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Diethyl phthalate enhances expression of SIRT1, DNMT3a and DNMT3b during apoptosis in PC12 Cells

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Running title: Diethyl phthalate enhances expression of SIRT1, DNMT3a and
DNMT3b

Abstract

Diethyl phthalate works as a phthalate plasticizer and is ubiquitously used in personal care products, cosmetics, medical equipment and pharmaceutical coating. Diethyl phthalate is considered a potential endocrine disruptor. Previously we found diethyl phthalate enhanced apoptosis induced by serum deprivation in PC12 cells. However, the relationship between diethyl phthalate and longevity related factors, sirtuins, and epigenetic factors (e.g., DNA methyltransferases) remains unclear, because genome modification caused by chemical toxicity, sirtuins and epigenetic factors have played key roles on abnormal metabolism and development. Here, we investigate whether diethyl phthalate affect sirtuins (SIRT1 and SIRT2) and methyltransferases (DNMT1 and DNMT3a) on the apoptosis of PC12 cells. We found that DNMT3a was significantly decreased by serum deprivation. However, DNMT3a, DNMT3b and SIRT1 were significantly increased under the enhancement of apoptosis induced by serum deprivation. These results suggest that SIRT1, DNMT3a and DNMT3b play multiple and complex roles in different apoptotic stages. Our results showed diethyl phthalate triggered epigenetic factors on PC12 cells apoptosis under nutrition stress. Finally, our results suggest that monitoring epigenetic factors such as DNMT3a, DNMT3b and SIRT1 could be a useful tool for chemical toxicity risk assessment.

Keywords: Apoptosis, DNA methylation, Endocrine disrupter, Epigenetic, Sir2

Abbreviations

DEP Diethyl phthalate

DMEM Dulbecco's modified Eagle's medium

DNMT DNA methyltransferase

FBS Fetal bovine serum

PAE Phthalates

Real-time RT-PCR Real-time reverse transcription-polymerase chain reaction

SAM S-adenosylmethionine

SIRT sirtuin

INTRODUCTION

Phthalates or phthalic acid esters (PAEs) are widely used in industrial production of plastics and other commodities. PAEs are used as a plasticizer to produce polymeric materials. Recently, many studies have reported that some PAEs were endocrine disrupting chemicals (Heudorf U et al., 2007; Alam et al., 2010), and PAEs showed toxicity and bioaccumulation (Janjua et al., 2007; Mose et al., 2007; Wu et al., 2010; Ferguson et al., 2011), especially affecting congenital abnormalities and cancers (Parks et al. 2000, Stroheker et al. 2005, 2006).

Diethyl phthalate (DEP) belongs to PAE plasticizers and is ubiquitously used in personal care products, cosmetics, soft toys, flooring, medical equipment and pharmaceutical coating. The general structure of phthalates is shown in Table 1. Usually, humans are exposed to DEP through inhalation and dermal exposure (Otake et al. 2001; Kao et al. 2011; Guo and Kannan, 2011). Recently, concerns of congenital and reproductive effects of DEP exposure are especially raised for infants, toddlers and pregnant women or capable of childbearing (Wormuth et al. 2006; Casas et al. 2011). However, there is little information about carcinogenicity by DEP.

Cancers involving multi-gene, multi-signal and multi-pathway unregulated cell growth are generated by an increase in cellular proliferation and/or a decrease in cell death, predominantly related to inhibition of apoptosis (Thompson, 1995). Apoptosis is the programmed cell death characterized by chromatin condensation, DNA fragmentation, cellular shrinkage and membrane blebbing (Kerr et al. 1972). Usually, cancer has been regarded to originate from genetic alterations such as mutations, deletions, rearrangements as well as gene amplifications, leading to abnormal expression of tumor suppressor genes and oncogenes. An increasing body of evidence indicates that, in addition to changes in DNA

sequence, epigenetic alterations contribute to cancer initiation and progression (Hagelkruys et al. 2011).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) in mammalian cells. There are currently five members of the DNMT family, including DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3l (Brooks et al. 2010; Rodriguez-Orsio et al. 2010). In organisms, DNA methylation status is altered by exposure to chemical substances (Pilsner JR, et al. 2010; Vandegehuchte et al. 2010). In addition, DNMT1 has been reported to work in embryonic period to maintain homeostasis (Chmurzynska, 2009), and DNMT3a and 3b was thought to play a role for *de novo* methylation (Li et al. 2003). Wang et al. (2005) reported that DNMT3a interacted with p53 to suppress p53 related gene expression. These methyltransferases have been considered to be key players in the regulation of mammalian tissues.

SIRT1 is a longevity related gene and encodes sirtuin1 (known as Nicotinamide Adenine Dinucleotide-dependent deacetylase sirtuin-1) and belongs to the SIRT gene family that includes SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 (Lavu et al. 2008). Among them, SIRT1 and SIRT2 are considered to play important roles in cell survival, differentiation, inflammation, metabolism and cell cycle, and have emerged as candidate therapeutic targets for many human diseases (Wakeling et al. 2009). Although classed as a histone deacetylase, Sirt1 deacetylates a broad range of substrates (Chung et al. 2010). Furthermore, SIRT1 is considered to play major functions in anti-apoptosis, anti-aging, anti-obesity, anti-inflammatory, stress response, caloric restriction and metabolism through deacetylation and modulation of protein functions (Boily et al. 2000; Kazantsev and Thompson, 2008; Csiszar et al. 2009; Nosho et al. 2009; Chuang et al. 2010; Holness et al. 2010; Zhao et al. 2011). Usually SIRT1 attenuated oxidative stress-induced apoptosis through p53 deacetylation (Kume et al. 2006). However, there is little information about

SIRT1 expression in apoptotic cells. In addition, the relationship between chemical substances such as endocrine disrupters and epigenetic factors such as SIRT and DNMTs is still unclear on the proliferation of cancer cells.

PC12 cells, a cell line of rat pheochromocytoma cells, is a mature neuroendocrine cell model for studying diseases of nervous and endocrine system (De Simone et al. 2003; Chestnut et al. 2011). PC12 cell is successful model to study apoptosis and environmental hormones (Yamanoshita et al. 2000). Apoptosis is induced when the PC12 cells are cultured in serum-free medium and DNA fragmentation appears as DNA ladder after agarose gel electrophoresis.

Epigenetics refers to changes in gene activation without altering the basic DNA structure (Rao-Bindal1 and Kleinerman, 2011). Epigenetic changes include CpG island methylation within gene promoter regions and acetylation, deacetylation, and methylation of histone proteins (Walkley et al. 2008; Ellis et al. 2009). Epigenetic regulation has been considered a mechanism for the inactivation of tumor suppressor pathways in several types of cancer (Boily 2009; Li et al. 2003; Qiu et al. 2002).

Altering gene expression and the signaling pathways that control the cell cycle and apoptosis can contribute to the tumorigenic process and cell transformation from a normal to a malignant phenotype (Peng et al. 2011). Recent advances in the study of epigenetics have shown that expression and signaling pathways may be regulated by methylation, histone modifications, and other epigenetic mechanisms. It means that cell cycle regulation and apoptosis may be closely related (Chung et al. 2010).

Many cancer studies have shown that epigenetic reactions are related to apoptotic reactions. However, the relationship between the chemical substances, such as DEP, which is involved in apoptosis, and epigenetic factors or SIRT gene family is still unclear.

Recently we reported that DEP enhances apoptosis induced by serum deprivation (Sun et al. 2012). Therefore, the aim of this study was firstly to investigate whether apoptosis affects expression of SIRT1 and SIRT2 genes and epigenetic modification enzymes such as DNMT1, 3a and 3b using PC12 cell system. Furthermore, we want to establish a novel method for health risk assessment of trace chemical substances in organisms, whether DEP affects longevity related genes (SIRT1 and SIRT2) and the epigenetic modification enzymes was investigated on the apoptosis stage using the same system.

MATERIALS AND METHODS

Materials

PC12 cells, a cell line of rat pheochromocytoma cells, were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), diethyl phthalate (DEP) were obtained from Sigma-Aldrich (St. Louis, MO USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD USA). High pure PCR template preparation kit and SV Total RNA Isolation System and Access RT-PCR Introductory System were purchased from Promega (USA). Agilent RNA 6000 Nano Reagents and Agilent DNA 7500 Reagents were from Agilent Technologies (Germany). The Rotor-Gene SYBR Green RT-PCR Kit was purchased from Qiagen (USA). Diethyl Phthalate was dissolved in ethanol. Other chemicals were of analytical reagent grade.

Cell culture

PC12 cells in 25 cm² flasks were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO₂. Inactivation of FBS was carried out at 56°C for 30 min. The cells were preincubated overnight, and then the medium was replaced with serum/serum-free DMEM with or without DEP (final DEP concentration: 0, 1, 10, 100 and 1,000 ng/ml). Five µl of a 1000-fold concentration of DEP in ethanol was added to the 5 ml cell medium. Five µl of ethanol was added to the serum-free DMEM and DMEM supplemented with 10% FBS as positive and negative control of apoptosis, respectively. When the medium was changed to serum-deprived medium, cells in the flask were washed twice with serum-free DMEM. It is reported that 100mM ethanol induced apoptosis in cultured skin cells, though 40 mM ethanol rarely affected apoptosis in the cells (Neuman et al., 2002). Therefore, we assumed that using 1 µl/ml ethanol (approximately 18 mM) did not affect PC12 cells apoptosis in our study.

Electrophoresis of genomic DNA

PC12 cells were cultured in 5 ml DMEM with and without 10% FBS across a DEP concentration gradient (i.e., 0, 1, 10, 100 and 1,000 ng/ml), and 1 µl/ml ethanol as a control for 72 hr. After incubation with DEP, cells were harvested using a scraper. The obtained cells were centrifuged at 1,500 rpm for 5 min to remove the supernatant. Genomic DNA was isolated using high pure PCR template preparation kit according to the instruction manual. After RNAase incubation, ethanol precipitation was carried out. The ladder pattern of DNA was analyzed by agarose gel electrophoresis. From three to five µg of DNA was subjected to electrophoresis on 1.5% of agarose gel. After the electrophoresis, DNA was visualized by staining with ethidium bromide under UV illumination.

mRNA expression by real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR)

Total RNA of PC12 cells cultured in the serum/serum-free medium with 0 to 1,000 ng/ml of DEP for 72hr was prepared using the SV Total RNA Isolation kit. The PCR primers of DNMT1, DNMT3a, **DNMT3b**, SIRT1, SIRT2, **p53** and β -actin were synthesized according to the DNA sequence as described in Table 2. The real-time RT-PCR condition was executed according to the Rotor-Gene SYBR Green RT-PCR Kit instructions. Briefly, the reverse transcription reaction was carried out at 55°C for 10 min, and subsequently 40 PCR cycles were executed as shown next: initial activation step; 95°C for 5 min, denaturation; 95°C for 5 sec, annealing and extension step; 60°C for 10 sec. β -actin was chosen as an internal control. PCR products were real-time monitored using software of Rotor-Gene Q (USA). Real-time specificity and identity were verified by melting curve analysis of the real-time PCR products.

Statistical analyses

Relative mRNA expression values are presented as mean $\pm$ S.E.M and were compared with one-way analysis of variance (ANOVA), followed by the Fisher's test.

RESULTS

Detection of DNA fragmentation by agarose gel electrophoresis

To clarify whether DEP induced apoptosis in cultured PC12 cells, DNA fragmentation was observed in PC12 cells from serum enriched and serum deprived cultures with and without DEP (Figure 1).

Figure 1A shows a homogenous DNA band pattern, across all DEP concentrations. This band pattern does not fit the expectation of DNA degradation after apoptosis. By contrast, Fig. 1B shows DNA ladder patterns which have been observed in cultured cells following apoptosis. The ladder pattern in the control cells is the expression of smaller size DNA fragments that are produced by apoptosis induced nuclear cell DNA cleavage (Woodgate et al 1999). It means that serum deprivation induced apoptosis as described by Maroto and Perez-Polo (1997). The ladder pattern was enhanced by increasing DEP concentration (Figure 1B). These results indicate that DEP enhances apoptosis in nutrient deprived PC12 cells, as previously described (Sun et al. 2012).

mRNA Expression of epigenetic factors, DNMT1, DNMT3a, DNMT3b, SIRT1, SIRT2, and p53 in the cells cultured in the medium with and without FBS

To examine whether expression of epigenetic factors was altered by apoptotic condition, SIRT1, SIRT2, DNMT1, DNMT3a, DNMT3b and p53 in the cells cultured in the medium with and without FBS were measured using real-time RT-PCR analysis. As shown in Figures 2B and F, only DNMT3a and p53 in PC12 cells were decreased by serum deprivation. Although DNMT3b is essential for *de novo* methylation as well as DNMT3a (Okano et al. 1999), DNMT3b was not changed by serum deprivation. The other three factors did not also significantly change ($P>0.05$) in the cells treated with and without FBS. These results indicate that DNMT3a and p53 might be related to apoptotic reaction.

mRNA Expression of epigenetic factors SIRT1, SIRT2, DNMT1 and DNMT3a in the cells cultured in the FBS-containing and FBS-free medium with and without DEP

To examine changes of epigenetic factors in the PC12 cells treated with DEP, mRNA expressions of SIRT1, SIRT2, DNMT1 and DNMT3a were measured by real-time RT-PCR methods (Figures 3-5).

As shown in Fig. 3, there was no significant difference between mRNA contents of DNMT1 (Figure 3A) and DNMT3a (Figure 3B) in the PC12 cells cultured in the medium containing FBS with 0 to 1,000 ng/ml DEP. When FBS enriched PC12 cells were exposed to 1 and 10 ng/ml DEP, the DNMT1 relative expression value had a wider variance than the control. With increasing DEP concentration in serum-free medium, DNMT1 expression in PC12 cells decreased, yet no significantly (Figure 3C). However, in the serum-free medium, when PC12 cells were exposed to 1 and 10 ng/ml DEP, the DNMT3a expression was significantly up regulated (Figure 3D). When DEP was 100 ng/ml, DNMT3a expression decreased comparing with that of 1 and 10 ng/ml DEP, but still higher than control. We did not detect significant changes for SIRT1 (Figure 4A) and SIRT2 (Figure 4B) mRNA expression in serum enriched PC12 cells with and without DEP. Figure 4C illustrates that SIRT1 mRNA expression was significantly up-regulated in PC12 cells cultured with different concentrations of DEP without serum, while mRNA contents of SIRT2 showed no change (Figure 4D). As same as DNMT3a (Figure 3D), DNMT3b was significantly increased when PC12 cells were exposed to low concentration of DEP under the serum deprivation condition (Figure 5A). It is indicated that both DNMT3a and 3b may relate to enhancement of apoptosis caused by DEP, different with changes of these two factors in PC12 cells cultured in the serum deprived medium. It is interestingly that p53 showed similar tendency to DNMT3b (Figure 5).

DISCUSSION

PC12 cells are useful to study neuroendocrine cancer and neuronal apoptotic death mechanism (Greene et al. 1976; Mesner et al. 1992). Our study showed that serum deprivation-induced apoptosis was enhanced by DEP. However, such enhancement was not observed in the serum-enriched treatments. Figure 1 clearly supports this affirmation, which is in concordance with our previous study (Sun et al, 2012). As shown in Figure 1B, the enhanced effects were observed when DEP concentration was above 1 ng/ml. Previous risk assessment studies estimated a mean value of DEP exposure of 0.76-3.48 $\mu\text{g/kg/day}$ in the general population, up to 20-42 $\mu\text{g/kg/day}$ in children and 78 $\mu\text{g/kg/day}$ in adult females (Wormuth et al. 2006; Guo et al. 2011; Koniecki et al. 2011). All of these values are below the reference dose of 800 $\mu\text{g/kg/day}$ (U.S. EPA, 1993). Our study clearly find out that reference value underestimates the potential toxicity of DEP, provided the enhanced apoptosis under nutritional stress for concentrations orders of magnitude below the tolerable intake of 5 mg/kg body weight per day from WHO standards (CICADs, 2003).

Yamanoshita et al. (2000; 2001) reported that tributyltin and 2,4,5-Trichlorophenoxyacetic acids inhibited apoptosis completely. On the other hand, in this study, DEP in serum-free cultured PC12 cells slightly enhanced apoptosis (Figure 1B). Similarly, nonylphenol, a phenolic endocrine disrupter chemically similar to DEP, has been shown to enhance apoptosis (Aoki et al. 2004).

Epigenetic changes in DNMTs and SIRT6 gene expression in the PC12 cells under the apoptotic condition are shown in Figure. 2. As shown in Figure 1, the ladder pattern was appeared by serum deprivation due to induction of apoptosis in PC12 cells. Following apoptosis DNMT3a mRNA expression was significantly decreased. DNMT3a and 3b are

well-known enzymes responsible for *de novo* methylation. As observed in other cancer cells, the expression of DNMT3a consistently increased (Lin et al. 2001; Majumder et al. 2002), decreased expression of the enzyme might contribute to the hypomethylation in the apoptotic condition. From the results, this methyltransferase may play an anti-tumor regulative role. On the other hand, DNMT3b was not changed by serum deprivation (Figure 2C). Bai et al. (2005) reported that DNMT3b was increased in growth factor-mediated differentiation in PC12 cells cultured in the medium containing nerve growth factor and 1% horse serum, although DNMT3a was decreased. From these results, it was suggested that DNMT3b was not responsible for serum deprivation-induced apoptosis. In addition, p53 was significantly decreased 72 hr after serum deprivation (Figure 2F). It may be due to the apoptosis was completely accomplished.

As shown in a previous report (Sun et al. 2012) and here (Figure 1), DEP enhanced apoptosis induced by serum deprivation. However, DNMT3a and 3b were also increased with increasing of DEP concentration dose (Figures 3D and 5A). The reason why such discrepancy occurred is still unclear. Likewise, DNMT3a was increased by treatment with camptothecin, an antitumor drug, in NSC34 cells (Chestnut et al. 2011). In contrast, with serum deprivation, it was suggested that silencing of anti-apoptotic related genes was enhanced by up regulation of *de novo* methylations by DEP treatment. We, thus, think that DNMT3a and 3b expression was increased by adding DEP to PC12 cells. In addition, as expected, DNMT1 and DNMT3a expression in serum-enriched PC12 cells did not show any significant changes following DEP exposure. Furthermore, DNMT1 expression did not change between the DEP exposed serum-free PC12 cell cultures. Our results indicate that methylation status of global genome was stable under the DEP treatment. In summary, DEP treatment is promising in light of developing a potential cancer treatment, since limited and localized exposure to DEP could induce apoptosis of cancer cells.

SIRT1 expression was also significantly increased under the apoptotic condition with serum deprivation in PC12 cells (Figure 4C). SIRT1 is the closest homolog of yeast Sir2 protein. A deacetylating histone, SIRT1 also deacetylates other protein such as Forkhead transcription factors FoxO, MyoD, and the tumor suppressor p53, p200, Ku70, PPAR γ , and the PPAR γ coactivator-1 α (PGC-1 α) (Chung et al. 2010). SIRT1 activity may be regulated by phosphorylation, since it is phosphorylated on Ser27 and Ser47 in vivo (Nasrin et al. 2009). Thus, SIRT1 can modulate cellular metabolism and exert corresponding effects on gene expression. SIRT2 also has NAD-dependent deacetylase activity. It was verified that resveratrol protects cardiomyocytes from H₂O₂-induced apoptosis or hypoxia-induced apoptosis by activating SIRT1, 3, 4 and 7 (Yu et al. 2009; Chen et al. 2009). Usually SIRT1 attenuated oxidative stress-induced apoptosis through p53 deacetylation (Kume et al. 2006). However, as shown in Figure 1, DEP enhanced apoptosis induced by serum deprivation. We suggested that DEP mediated apoptosis enhancement on increasing of Bax expression (Sun et al. 2012). These contradictory results suggest that SIRT1 role in apoptosis is complex and may depend on cell types. Similar phenomena were also observed in SIRT1 activation by resveratrol induced apoptosis in BRCA-1 deficient cancer cells (Wang et al. 2008). Alternatively, the enhanced SIRT1 expression in conditions of nutritional stress could sensitize cells to the action of FOXO3/4 (Marfè et al. 2011). Nevertheless, further investigation on whether p53, FOXO3 and NF- κ B change under activated SIRT1 or apoptotic factors such as Bcl2 families is granted.

In addition, as shown in Figure 5B p53 was significantly increased in the PC12 cells cultured in the serum deprived medium exposed to DEP as expected, because DEP enhanced apoptosis. Sun et al. (2012) reported that caspase 3 activity and bax increased also in PC12 cells in the serum deprived medium with DEP. DNMT3a and 3b, SIRT1 accompanying with

p53 might contribute to enhance apoptosis induced by serum deprivation in PC12 cells exposed to DEP.

In this study, no significant difference was observed between SIRT1 and SIRT2 expression in DEP exposed and serum-enriched PC12 cells. In serum-deprived PC12 cell culture, SIRT2 did not change. From these results, we summarized the estimated roles of DNMT3a and 3b, SIRT1, and p53 in PC12 cells treated with serum deprivation medium and/or DEP as shown in Figure 6. Peng et al. (2011) proposed cooperation of SIRT1 and DNMT1 in MDA-MB-231 breast cancer cells. Therefore, we believe a similar cooperation might happen among these factors in PC12 cells. Furthermore, up regulated DNMT3a and 3b that induced *de novo* methylation in CpG islands may silence anti-apoptotic genes.

In conclusion, when apoptosis was induced by serum deprivation, DNMT3a was significantly decreased. DEP enhanced serum-deprivation induced apoptosis. However, DNMT3a, DNMT3b and SIRT1 were significantly increased by treatment with DEP in cells cultured in serum-deprived medium as same as p53. These contradictory results suggest that role of SIRT1 and DNMT3a or 3b are complex, and these genes may play multiple roles in different apoptotic stages and/or epigenetically affect apoptosis as shown in Figure 6. This study is the first to elucidate that serum-deprivation induced apoptosis has epigenetic expression (DNMT3a, DNMT3b and SIRT1) enhanced by DEP. We showed that DEP, one of the endocrine disrupters, showed epigenetic related apoptosis in PC12 cells under nutrition stress condition. Although further research is required to clarify the detailed mechanism of epigenetic changes by DEP, measurement of epigenetic factors might be useful tool for risk assessment of chemical toxicity.

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Table 1 General chemical structure of phthalates and diethyl phthalate

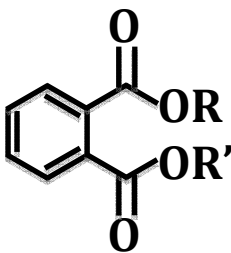
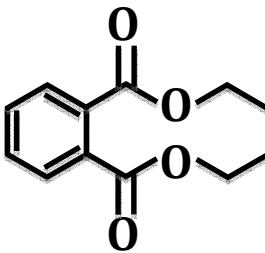
	Phthalates	Diethyl phthalate
General chemical structure	 <chem>O=C(O[R])c1ccccc1C(=O)O[R']</chem>	 <chem>CCOC(=O)c1ccccc1C(=O)OCC</chem>

Table 2 Primers used in Real-Time RT-PCR analyses of β -actin, DNMT1, DNMT3a, DNMT3b, SIRT1, SIRT2, and p53.

Primer's Name	Sequence (5'→3')	Length (bp)	Tm (°C)
β -actin F	AAGTCCCTCACCTCCCAAAAG	22	68.3
β -actin R	AAGCAATGCTGTCACCTTCCC	21	67.4
Dnmt1 F	AGGACCCAGACAGAGAAGCA	20	64.0
Dnmt1 R	GTACGGGAATGCTGAGTGGT	20	63.9
Dnmt3a F	ACTTGGAGAAGCGGAGTGAA	20	63.9
Dnmt3a R	GGATTCGATGTTGGTCTGCT	20	64.0
Dnmt3b F	ACTCGAGGAGGGAGCTTAGG	20	60.0
Dnmt3b R	TTTGTCATTTCCTCAACCAT	20	60.0
Sirt1 F	CGCCTTATCCTCTAGTTCCTGTG	23	64.8
Sirt1 R	CGGTCTGTCAGCATCATCTTCC	22	68.1
Sirt2 F	CTCCCACCAAACAGATGACC	20	64.4
Sirt2 R	ATTCAGACTCGGACACTGAGG	21	63.2
p53 F	GTCGGCTCCGACTATACCACTATC	24	62.3
p53 R	CTCTCTTTGCACTCCCTGGGGG	22	68.5