【原著】

# The methylation status of ASPP CpG island is changed in cancer cell lines retaining wild-type

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# 野生型癌細胞株におけるASPP CpGアイランドのメチル化状態の変化

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**ABSTRACT**

The p53 protein is one of the best-known tumor suppressors. Recently discovered ASPP1 and ASPP2 are specific activators of p53. To understand if ASPP inactivation offers a selective advantage to tumors that have wild-type p53, we measured the mRNA expression of ASPP1 and ASPP2 in tumor cell lines retaining wide-type p53. In addition, the CpG island methlyation status of ASPP1 gene and ASPP2 gene in the 5'-untranslated region was also investigated in order to understand the possible cause of abnormal expression of ASPP1 and ASPP2 in the tumor cell lines retaining wide-type p53. The data showed that mRNA expression of ASPP1 and ASPP2 is downregulated and CpG island tested is hypermethylated. These results indicated that ASPP CpG island aberrant methylation could be one molecular and genetic alteration in wild-type p53 tumors and one of the reasons that are responsible for the loss of ASPP activity.

Key words：ASPP; DNA methylation; Methylation-specific PCR; Reverse transcription PCR; p53

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## INTRODUCTION

The p53 protein is one of the best-known tumor suppressors. The p53 gene is also one of the most frequently mutated genes[1]. Mutations in p53 gene have been found in about 50% of human cancers[2-3]. The other 50% of human tumors retain wild-type p53. Why wild-type p53 is unable to perform its duty as a tumor suppressor in these tumors. This question remains unclear. Recently, a new family of protein, apoptosis-stimulating protein of p53 (ASPP) was discovered[4-5]. This discovery may suggest some new answers to this question. The ASPP family consists of three members: ASPP1, ASPP2 and iASPP. ASPP1 and ASPP2 act as potent activators of p53. iASPP acts as an inhibitor of p53. The apoptotic function of p53 is stimulated by ASPP1 and ASPP2 and inhibited by iASPP[6-8].

To understand if ASPP inactivation offers a selective advantage to tumors that have wild-type p53, we measured the mRNA expression of three p53 wild-type cancer cell lines, HEPG-2, MCF-7 and A549. We also detected the CpG island methlyation status of ASPP1 gene and ASPP2 gene in the 5'-untranslated region in order to understand the possible cause of abnormal expression of ASPP1 and ASPP2 in three cancer cell lines.

## MATERIALS AND METHODS

*Cell lines.* The human cancer cell lines used in this study were HEPG-2, MCF-7 and A549, obtained from the Southwest Hospital (Chongqing, PRC). The normal control used was fibroblast cell. All cell lines were grown in Dulbecco modified Eegle medium supplement with 10% fetal calf serum and 100 U/ml benzylpenicillin and 100 U/ml streptomycin sulfate in a humidified atmosphere containing 5% of $CO_2$ at 37℃

*DNA and RNA preparation.* Total DNA and RNA were extracted from various cancer cell lines and fibroblast cell using Tirpure-Isolation-Reagent (Roche, USA).

*RNA expression level determination by RT-PCR.* RT-PCR kits (TaKaRa, Japan) were used to synthesize cDNA from 2 $\mu$g of total RNA using random hexamer primers. cDNA synthesis was carried out as suggested by the kit protocol, using AMV reverse transcriptase. The reverse transcription was carried out for 45 min at 48℃ After an incubation at 99℃ or 5 min to inactivate the reverse transcriptase. 5 $\mu$l of cDNA were used to amplify the target regions of ASPP1 or ASPP2. $\beta$-actin cDNA fragments were also amplified as a positive control. The RT-PCR primers of ASPP1 and ASPP2 are designed using Primer Premier 5.0 software. The nucleotide sequences of these primers and their PCR conditions are summarized in Table 1. After PCR, 8 $\mu$l product was mixed with 1 $\mu$l of 10 x loading dye and then run on 1.5% agarose gel. Electrophoresis was carried out at 100V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining. Electrophoresis profiles were analyzed by the software, Gel-Pro3.1 (Media Cybernetics Inc., USA). The ratio of the integrated optical density (IOD) of ASPP and $\beta$-actin represented the mRNA level. The PCR products of ASPP1 and ASPP2 were 498 and 391 base pairs respectively.

*Sodium Bisulfite Treatment.* DNA bisulfite treatment was carried out using the reagents provided in the CpGenome DNA Modification Kit (Intergen, Purchase, NY, USA). Briefly, 1 $\mu$g of

DNA was denatured using NaOH and treated with sodium bisulfite for 18 h. Modified DNA was resuspended in 30 μl of TE (10 mM Tris / 0.1 mM EDTA, pH 7.5 ) and stored immediately at -20℃[9-10].

*Methylation-specific PCR Analysis Conditions.* PCR was performed using Taq polymerase (Promega) and the hot start procedure. The ASPP specific primer sets are designed to locate on the 5'untranslated region of ASPP gene using MethPrimer software[11-12] (Fig1). Primer set U will anneal to unmethylated DNA that has undergone a chemical modification. Primer set M will anneal to methylated DNA that has undergone a chemical modification. The unmethylated sequence of the ASPP gene was detected with ASPP-Uf and ASPP-Ur. The methylated sequence of ASPP gene was detected with ASPP-Mf and ASPP-Mr (Tab.1). The PCR reaction mixture (50 μl) contained 2.0 mM $MgCl_2$, 0.2mM each of 4 dNTPs, 1.0mM each of oligonucleotide primers and 5 units of Taq DNA polymerase. PCR conditions are listed in table 1. After PCR, 9 μl PCR product was mixed with 1.5 μl of 10 x loading dye and then run on 2% agarose gel. Electrophoresis was carried out at 100 V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining.

Table1　Summary of the primer sets and PCR conditions for ASPP1 and ASPP2

| Primer | Sequence | Pre-PCR incubation | Denature | Annealing | Extension | Cycle | Final incubation |
|---|---|---|---|---|---|---|---|
| **RT-PCR:** | | | | | | | |
| ASPP1-f | 5'-GCAGCACACAGCGCCTTAAATAAG-3' | 95℃,3min | 94℃,30s | 56℃,30s | 72℃,45s | 30 | 72℃,8min |
| ASPP1-r | 5'-TCCATTGTCCACATCGGCCAAGGT-3' | | | | | | |
| ASPP2-f | 5'- TATCTAATCCTTACCGAAACC -3' | 95℃,3min | 94℃,30s | 55℃,30s | 72℃,45s | 30 | 72℃,8min |
| ASPP2-r | 5'-CCCTCAGGCTCATAATCAA -3' | | | | | | |
| **Methylation:** | | | | | | | |
| ASPP1-Uf | 5'-TTTTGTATTTTGTTGTAGTTGTTGTT-3' | 94℃,5min | 94℃,30s | 51℃,30s | 72℃,30s | 40 | 72℃,8min |
| ASPP1-Ur | 5'-CACAAAAAAAATCCACAACACCC-3°Ø | | | | | | |
| ASPP1-Mf | 5'-CGTATTTCGTCGTAGTTGTCGTT3' | 94℃,5min | 94℃,30s | 51℃,30s | 72℃,30s | 40 | 72℃,8min |
| ASPP1-Mr | 5'-CGAAAAAAATCCGCGACGCCC-3' | | | | | | |
| ASPP2-Uf | 5'-GGTGTTTTAGTTTGTGTGGAGG-3' | 94℃,5min | 94℃,30s | 51℃,30s | 72℃,30s | 40 | 72℃,8min |
| ASPP2-Ur | 5'-CAAACTAAAATACCCCAAAAAATCA-3' | | | | | | |
| ASPP2-Mf | 5'-GTGTTTTAGTTCGCGCGGAGG-3' | 94℃,5min | 94℃,30s | 50.5℃,30s | 72℃,30s | 40 | 72℃,8min |
| ASPP2-Mr | 5'-ACCGAACTAAAATACCCCGAAAA-3 | | | | | | |

| | | | | ASPP1 | | | |
|---|---|---|---|---|---|---|---|
| A | 1 | GAGCCCCGCA | TCCCGCCGCA | GCTGCCGCCT | CGCCGCGGCC | GGGCCGGAGA | GCACGGCGGC |
| B | 1 | GAGTTTTGTA | TTTTGTTGTA | GTTGTTGTTT | TGTTGTGGTT | GGGTTGGAGA | GTATGGTGGT |
| C | 1 | GAGTTTCGTA | TTTCGTCGTA | GTTGTCGTTT | CGTCGCGGTC | GGGTCGGAGA | GTACGGCGGC |
| A | 61 | GGGAGCGCGG | CCTTAGGAGG | CGGCCGGAGC | GGTGGGCACA | GCTCGGCGCG | GAGCGTCCTG |
| B | 61 | GGGAGTGTGG | TTTTAGGAGG | TGGTTGGAGT | GGTGGGTATA | GTTTGGTGTG | GAGTGTTTTG |
| C | 61 | GGGAGCGCGG | TTTTAGGAGG | CGGTCGGAGC | GGTGGGTATA | GTTCGGCGCG | GAGCGTTTTG |
| A | 121 | TCAGGCGGCG | GCCGAGGGCG | TCGCGGACTC | TCCCCGCGAT | GATGCCGATG | ATATTAACTG |
| B | 121 | TTAGGTGGTG | GTTGAGGGTG | TTGTGGATTT | TTTTTGTGAT | GATGTTGATG | ATATTAATTG |
| C | 121 | TTAGGCGGCG | GTCGAGGGCG | TCGCGGATTT | TTTTCGCGAT | GATGTCGATG | ATATTAATTG |
| | | | | ASPP2 | | | |
| A | 61 | GGGCTTGTTG | GTGCCCCAGC | CCGCGCGGAG | GGCCCTTCGG | ACCCGCGCGC | CGCCGCTGCC |
| B | 61 | GGGTTTGTTG | GTGTTTTAGT | TTGTGTGGAG | GGTTTTTTGG | ATTTGTGTGT | TGTTGTTGTT |
| C | 61 | GGGTTTGTTG | GTGTTTTAGT | TCGCGCGGAG | GGTTTTTCGG | ATTCGCGCGT | CGTCGTTGTC |
| A | 121 | GCCGCCGCCG | CCTCGCAACA | GGTCCGGGCG | GCCTCGCTCT | CCGCTCCCCT | CCCCCGCATC |
| B | 121 | GTTGTTGTTG | TTTTGTAATA | GGTTTGGGTG | GTTTTGTTTT | TTGTTTTTTT | TTTTTGTATT |
| C | 121 | GTCGTCGTCG | TTTCGTAATA | GGTTCGGGCG | GTTTCGTTTT | TCGTTTTTTT | TTTTCGTATT |
| A | 181 | CGCGACCCTC | CGGGGCACCT | CAGCTCGGCC | GGGGCCGCAG | TCTGGCCACC | CGCTTCCATG |
| B | 181 | TGTGATTTTT | TGGGGTATTT | TAGTTTGGTT | GGGGTTGTAG | TTTGGTTATT | TGTTTTTATG |
| C | 181 | CGCGATTTTT | CGGGGTATTT | TAGTTCGGTC | GGGGTCGTAG | TTTGGTTATT | CGTTTTTATG |
| A | 241 | CGGTTCGGGT | CCAAGATGAT | GCCGATGTTT | CTTACCGTGT | ATCTCAGTAA | CAATGAGCAG |
| B | 241 | TGGTTTGGGT | TTAAGATGAT | GTTGATGTTT | TTTATTGTGT | ATTTTAGTAA | TAATGAGTAG |
| C | 241 | CGGTTCGGGT | TTAAGATGAT | GTCGATGTTT | TTTATCGTGT | ATTTTAGTAA | TAATGAGTAG |

Fig.1. Design of MSP primers for ASPP1 and ASPP2. (A) Original sequence before bisulfite treatment.CpG dinucleotides are underlined. (B) Unmethylated sequence after bisulfite treatment. All cytosine were deaminated and converted to thymine. Unmethylated primers are double underlined. (C) Methylated sequence after bisulfite treatment. All cytosine residues at CpG sites remained unchanged although other cytosines were converted to thymine. Methylation primers are double underlined. The GenBank accession numbers for the ASPP1 and ASPP2 nucleotide sequences in this fig are AJ318887 and AJ318888, respectively. Shading indicates the ATG transitional initiation codon.

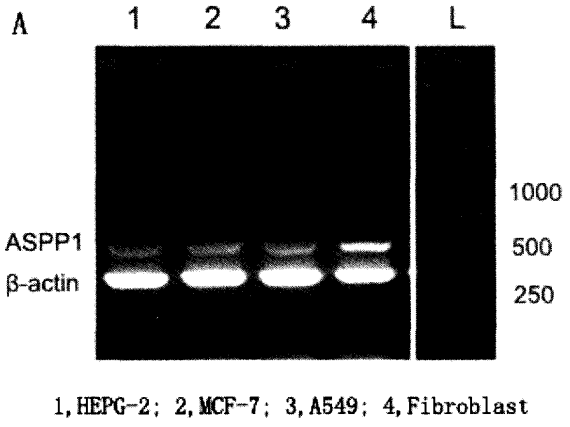
## RESULTS AND DISCUSSION

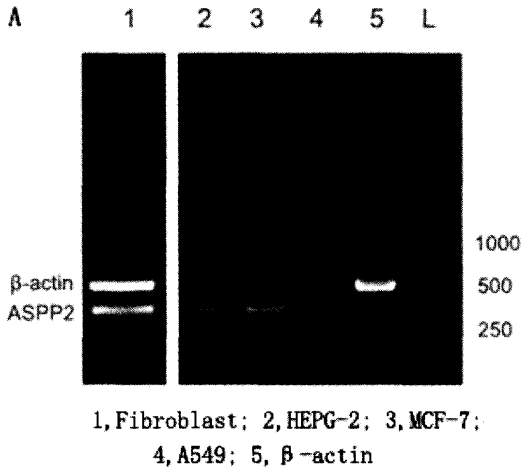
Recently, the ASPP family was identified as potent activators of p53[4]. The apoptotic function of p53 is stimulated by two members of the ASPP family, ASPP1 and ASPP2. Binding to the DNA binding domains of p53, ASPP1 and ASPP2 specifically stimulates the transactivation function of p53 on promoters of proapoptotic genes[4]. Therefore, the ASPP proteins appeared to be acting to specifically enhance the ability of p53 to cause apoptosis.

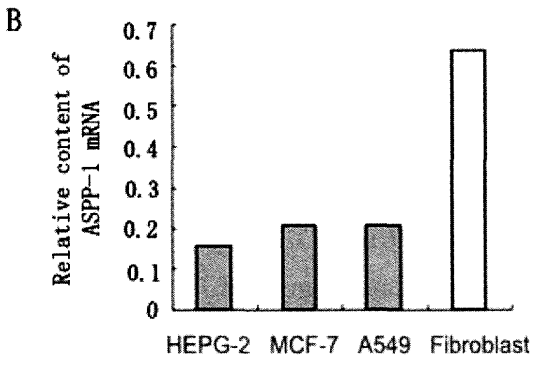
Human tumors can be divided into two kinds. One is tumors with wide-type p53. The other is tumors with mutated p53. In tumors where p53 has been mutated, p53 is no longer able to interact with the ASPP proteins, then a cell which would otherwise have died is now able to survive, allowing the mutation to persist[13]. In addition, in tumors where p53 has not been mutated, a selective advantage may have occurred as a consequence of the down-regulation of ASPP1 and ASPP2, which would then prevent p53 from causing cell death[13]. Our study in three tumor cell lines with wide-type p53 shows that the mRNA expression of ASPP1 and ASPP2 is lower than that of ASPP in the normal control (Fig 2 and Fig 3). These results, together with the research of Lu and coworkers in breast cancer[4], suggest that the down-regulation ASPP may be one of characters in tumors with wild-type p53.

What is the cause of ASPP down-regulation in tumors retaining wild-type p53. Some of the

possible reasons may be gene mutation or epigenetic modification. But, up to now, ASPP gene mutation has not been found[13]. Whether the ASPP gene themselves are subject to mutation during tumorigenesis remains to be seen. However, our study here shows that epigenetic modification may happen to ASPP gene in tumor cell lines retaining wild-type p53.



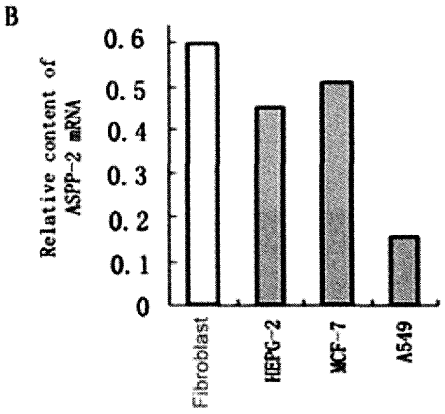




Fig 2. mRNA expression of ASPP-1 in tumor cell lines (HEPG-2, MCF-7 and A549) and normal control (Fibroblast). (A) Agarose gel electrophoresis of RT-PCR amplified products; (B) Relative content of ASPP-1 mRNA. The ratio of the integrated optical density (IOD) of ASPP-1 and $\beta$-actin represented the relative mRNA level.

Fig 3. mRNA expression of ASPP-2 in tumor cell lines (HEPG-2, MCF-7 and A549) and normal control (Fibroblast). (A) Agarose gel electrophoresis of RT-PCR amplified products; (B) Relative content of ASPP-2 mRNA. The ratio of the integrated optical density (IOD) of ASPP-2 and $\beta$-actin represented the relative mRNA level.

We observed that ASPP1 and ASPP2 gene CpG islands in the 5'-untranslated region were hypermethylated in three tumor cell lines retaining p53 wild-type and were unmethylated in normal fibroblast cell (Fig 4). These results indicated that the changes of CpG island methylation status of ASPP1 and ASPP2 gene might be an important factor in tumors that possess wild-type p53.

DNA methylation is the main epigenetic modification in humans[14], and changes in methylation patterns play an important role in tumorigenesis. Hypermehylation of normally unmethylated CpG islands in the promoter regions often occurs in important tumor suppressor genes such as VHL, hMLH1 and p16[15-17]. Our study shows that hypermethylation is also happened in the 5'-untranslated region of ASPP1 and ASPP2 gene. This observation suggested that ASPP down-regulated expression in tumor cells retaining wild-type p53 might be relative to the abnormal methylation status of ASPP gene. Therefore, the ASPP aberrant methylation could be one molecular and genetic alteration in tumors retaining wild-type p53 and one of the reasons that are responsible for the loss of ASPP activity.

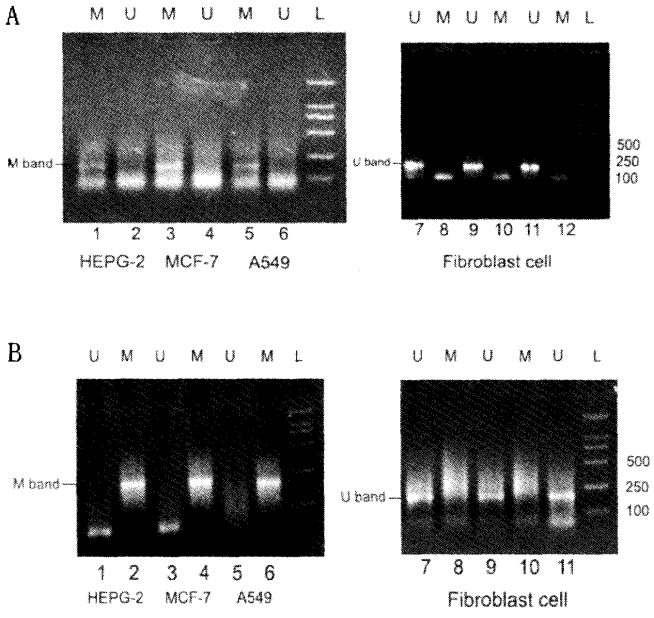



Fig 4. Methylation status of ASPP1(A) and ASPP2(B) in cancer cell lines and normal fibroblast cell. 1-2, HEPG-2; 3-4, MCF-7; 5-6, A549; 7-12, fibroblast cell. M band, methylation band; U band, unmethylation band; M, methylation primer sets amplified products; U, unmethylation primer sets amplified products; L, DNA Marker.

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