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# Aberrant overexpression of 53BP2 mRNA in lung cancer cell lines

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**Abstract The p53-binding protein 2 (53BP2) was identified as a binding protein to a tumor suppressor p53. We examined the genetic aberrations of 53BP2 gene in various human cancer cell lines. Although no gross genomic alteration or mutation of 53BP2 gene was observed, 53BP2 mRNA levels were highly variable. There was no association between the 53BP2 mRNA level and the p53 status. When we examined sensitivities of these cell lines to DNA-damaging agents including UV irradiation, X-ray irradiation and *cis*-diamine-dichloroplatinum (CDDP), we found that higher 53BP2 mRNA expression was correlated with the sensitivity to these agents.**



*Key words:* p53-binding protein 2; p53; DNA-damaging agent

## 1. Introduction

The p53-binding protein 2 (53BP2) has been initially identified by its ability to bind the core domain of p53 tumor suppressor gene product [1]. The binding of 53BP2 to wild-type p53 but not some mutant forms of p53 suggested that some of the biological functions of p53 wild-type protein, such as induction of apoptosis and cell cycle arrest, might be mediated by its interaction with 53BP2 [2–5].

The 53BP2 protein consists of 1005 amino acids with an α-helical region, proline-rich (PXXP) region, ankyrin repeats and Src-homology 3 (SH3) domain [1]. These PXXP and SH3 structural motifs were found in various regulatory proteins and appeared to be involved in protein-protein interactions [2,6]. In fact, 53BP2 has been shown to bind a number of proteins other than p53, such as Bcl-2 [4], protein phosphatase 1 [7] and the p65 subunit of nuclear factor κB (NF-κB) [8] through the ankyrin repeats and SH3 domain of 53BP2. Recent crystallographic analysis has provided a structural basis for p53-53BP2 interaction and demonstrated that the 53BP2-binding site on the p53 core domain consists of evolutionarily conserved regions that are frequently mutated in cancer and that it overlaps the site of DNA-binding [3]. Moreover, we have recently demonstrated that overexpression of 53BP2 induced apoptosis and that NF-κB p65 subunit bound to 53BP2 following the inhibition of the 53BP2-induced apoptosis [8].

In cancer cells, mutations in p53 gene are the most common genetic change in human cancer. It has been reported that about half of human cancers have mutant p53 and the rest of cancers possess wild-type p53 [9–11]. In this paper, we first examined the possibility that those cancer cell lines with wild-type p53 may have a mutation in 53BP2. However, no gross genomic alteration or point mutation of the functional domains of 53BP2 was detected. Thus, we also examined the mRNA levels of 53BP2 in various cell lines and found the correlation between the 53BP2 mRNA level and the sensitivity to DNA-damaging agents.

## 2. Materials and methods

### 2.1. Cell lines

Cancer cell lines from various tissues including lung (NCI H460, A549, SBC3, SBC5 and Lu99), breast (MCF7), colon (LoVo, SW480, SW620, HT29 and DLD1) and leukocytes (MOLT4, CEM and THP1) were examined. NCI H460, LoVo, MCF7, HT29, SW620, MOLT4, CEM and THP1 were obtained from American Type Culture Collection (Rockville, MD, USA). A549, Lu99, SBC3 and SBC5 were obtained from Health Science Research Resources Bank (Osaka, Japan). DLD1 and SW480 were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). A549, SBC3, MCF7 and SBC5 were maintained in minimum essential medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum. NCI H460, Lu99, HT29 and DLD1 were maintained in RPMI 1620 (Iwaki, Tokyo, Japan) with supplements described above. SW620 and SW480 were maintained in Leibovitz's L-15 medium (Gibco BRL) and LoVo were maintained in Ham's F12 medium (Nissui, Tokyo, Japan) with above supplements. Cells were grown at 37°C in a humidified 5% $CO_2$ incubator.

### 2.2. Southern and Northern blotting

Genomic DNA samples were prepared from each cell line by proteinase K digestion and phenol-chloroform extraction as described [12]. 10 μg of each DNA sample was digested with either *Eco*RI or *Hin*dIII, fractionated on a 0.7% agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N; Amersham, Arlington Heights, IL, USA). These membranes were cross-linked under ultraviolet (UV) light. They were incubated in Hybrizol I (Oncor, Perry Parkways, Gaithersburg, MD, USA) for 1 h at 42°C and hybridized at 42°C overnight with [α-$^{32}$P]dATP-labeled full-length 53BP2 cDNA as a probe.

In order to examine 53BP2 mRNA levels, total RNA samples from cell lines described above were prepared by using Trizol (Gibco BRL). 20 μg of total RNA was fractionated by electrophoresis on a 1.0% agarose gel containing 1×MOPS and 6.7% formaldehyde, transferred to Hybond N membrane (Amersham) in 10×saline sodium citrate (SSC), and were covalently linked by 1200 J/m$^2$ of UV light radiation. Hybridization was performed in Hybrizol I (Oncor) at 42°C overnight with the [α-$^{32}$P]dATP-labeled 53BP2 cDNA probe or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (internal control). The final wash was carried out in 0.1×SSC and 0.1% sodium dodecyl

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*Abbreviations:* 53BP2, p53-binding protein 2; SH3, Src-homology 3; UV, ultraviolet; CDDP, *cis*-diamine-dichloroplatinum; RT-PCR, reverse transcriptase-PCR


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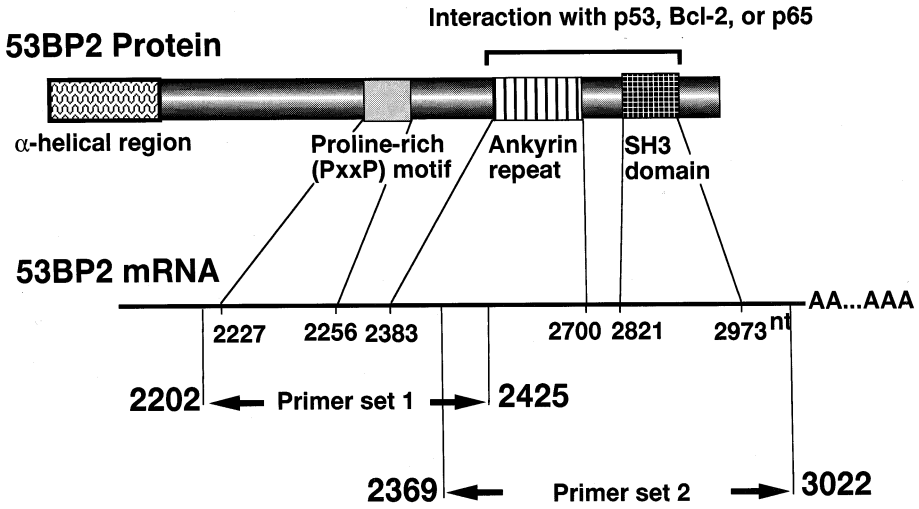


Fig. 1. Schematic diagram of 53BP2 protein and location of PCR primers used in this study. 53BP2 consists of 1005 amino acids with an α-helical region, proline-rich region, ankyrin repeats and SH3 domain. The primer sets 1 and 2 correspond to the PXXP region and to the ankyrin repeats and SH3 domain, respectively. The regions responsible for the interaction with other proteins are indicated.

sulfate at 65°C for 20 min. For quantitation of 53BP2 mRNA and that of GAPDH (as an internal control) in Northern blot, we used Fuji BAS 2500 image analyzer (Fujifilm, Tokyo, Japan) [13]. The relative ratio of 53BP2 mRNA level over GAPDH mRNA was calculated to compare the 53BP2 mRNA level of each cell line.

*2.3. Nucleotide sequencing*

The 53BP2 cDNA fragment, containing the proline-rich region, ankyrin repeats and SH3 domain, was amplified by reverse transcriptase-PCR (RT-PCR) with Titan One Tube RT-PCR system kit (Boehringer Mannheim, Mannheim, Germany). The following primer pairs were used for RT-PCR reactions: primer pair 1 (spanning 2202–2425 nucleotides), 5′-TGTGCTTGATGTGTACCTGGAGG-3′ and 5′-CTAGCAGTAAAGCAAGGGGGTT-3′; primer pair 2 (spanning 2369–3022 nucleotides), 5′-CAGAGCGTATCGCTCAT-3′ and 5′-AGTTTCAGGCCAAGCTC-3′ (Fig. 1). RT-PCR reactions were performed with approximately 1 μg of total RNA prepared from each cell line in 50 μl reactions under the following conditions: reverse transcription at 50°C for 30 min; followed by 10 amplification cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 68°C for 1 min and additional 25 amplification cycles consisting of denaturation at 98°C for 1 min, annealing at 60°C for 1 min, extension at 68°C for 1 min with increment for 5 s to each cycle at 68°C and final extension at 68°C for 7 min. The amplified products were purified with phenol-chloroform extraction and ethanol precipitation. The amplified DNA fragment was cloned into pCR Blunt vector plasmid (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommendations. Dideoxynucleotide sequencing reactions using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA) were performed for sequence analysis on ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) according to the manufacturer's recommendations.

*2.4. UV irradiation, X-ray irradiation and cis-diamine-dichloroplatinum (CDDP) treatment, and sensitivity or viability analysis*

Cancer cell lines were cultured in six-well plates and incubated for 24 h until they reached half-confluent. These cells were exposed to UV-C (254 nm) or X-ray irradiation (M80-WE; Softex, Tokyo Japan) at various doses, and incubated for an additional 46 h. In order to determine the sensitivity to CDDP (Sigma, St. Louis, MO, USA), cells were similarly cultured, incubated with CDDP at various concentrations and incubated for an additional 48 h. The cell viability was

Table 1
Results of 53BP2 cDNA sequencing in various tumor cell lines

| Cell line | Tissue derived | Pathology[a] | p53 status[b] | 53BP2 sequences[c] | |
|---|---|---|---|---|---|
| | | | | Primer pair 1 | Primer pair 2 |
| A549 | Lung | ACC | wt | N | N |
| NCI H460 | Lung | LCC | wt | N | N |
| Lu99 | Lung | LCC | wt | N | N |
| SBC3 | Lung | SCLC | wt | N | N |
| SBC5 | Lung | SCLC | R248L | N | N |
| MCF7 | Breast | ACC | wt | N | N |
| LoVo | Colon | ACC | wt | N | N |
| SW620 | Colon | ACC | R273H | N | N |
| SW480 | Colon | ACC | R273H/P309S | N | N |
| DLD1 | Colon | ACC | S241F | N | N |
| HT29 | Colon | ACC | R273H | N | N |
| MOLT4 | Blood | T cell ALL | wt | N | N |
| CEM | Blood | T cell ALL | R175H/R248H | N | N |
| THP1 | Blood | AMoL | 174del 26 | N | N |

[a]Abbreviations for pathological classification. LCC, large cell carcinoma; GCC, giant cell carcinoma; SCLC, small cell lung carcinoma; ACC, adenocarcinoma.
[b]The p53 status of each cell line was according to Jia et al. [19]. Mutant types including substitutions and deletions are described. 'wt', wild-type.
[c]The nucleotide sequence was determined with at least five independent clones for each preparation. N, no mutation.

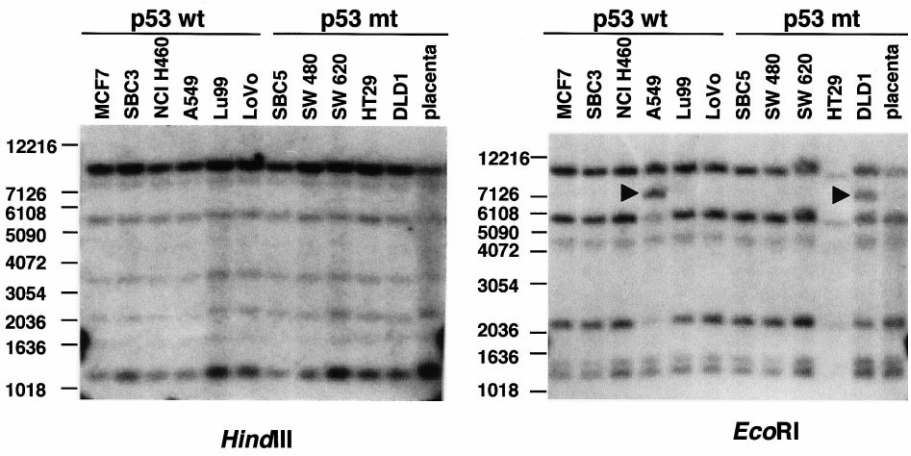


Fig. 2. Southern blot analysis of the 53BP2 gene in human tumor cell lines. Approximately 10 μg of the genomic DNA was restricted with *Eco*RI or *Hin*dIII, electrophoresed in a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the [α-$^{32}$P]ATP-labeled human 53BP2 cDNA probe. A549 and DLD1 revealed an aberrant polymorphic band upon *Eco*RI digestion (arrow heads). The low densities of 53BP2 bands in HT29 (*Eco*RI) are due to the loss of DNA during the experimental procedure.

determined by sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. The cell viability was quantified by adding XTT dye to the cultures and allowing it to be reacted upon for 3 h by the mitochondrial dehydrogenases generated by viable cells. Absorbance was measured at 450/690 nm. All the measurements were performed in more than triplicates.

## 3. Results

### *3.1. Analysis of 53BP2 genome in human tumor cell lines*

The genomic DNA was isolated from various tumor cell lines and digested with restriction enzymes, *Hin*dIII or *Eco*RI for Southern blotting hybridization. These cell lines included five from lung cancer (two from small cell lung cancer, two from large cell carcinoma and one from adenocarcinoma), five from colon adenocarcinoma and one from breast adenocarcinoma. As demonstrated in Fig. 2, no gross alteration of 53BP2 gene was observed in these cell lines irrespective of the status of p53 gene (either wild-type or mutant). Upon *Eco*RI digestion, A549 and DLD1 revealed an aberrant band of 8.1 kb, which is considered to be a polymorphism since no alteration was detected with other digestions. We also examined three human leukemia cell lines including MOLT4, CEM and THP1, and detected no gross mutation in 53BP2 gene (data not shown).

### *3.2. 53BP2 cDNA sequencing*

We have then determined the nucleotide sequences of the 53BP2 cDNA regions which are known to be involved in the interaction with other proteins. Thus the 53BP2 cDNA fragments corresponding to the PXXP motif (using primer set 1; Fig. 1) and to the ankyrin repeats and SH3 domain (primer set 2) were amplified from various tumor cell lines, cloned into a vector, and subjected to the sequencing analysis. Although we analyzed at least five independent clones from each set of sample, no substitution, deletion or insertion was detected among 14 cell lines tested (more than 140 cDNA clones were examined) (Table 1). It was noted that even in seven cell lines with wild-type p53 alleles, there was no mutation in these regions of 53BP2 cDNA.

### *3.3. Expression of 53BP2 mRNA in tumor cell lines*

Northern blotting analysis was performed with these cell lines using full-length 53BP2 cDNA as a probe. The expected 4.5 kb band corresponding to 53BP2 mRNA was detected from all the samples examined (Fig. 3). However, the amounts of 53BP2 mRNA were variable among these cell lines. Thus, we compared the level of 53BP2 mRNA with regard to the GAPDH mRNA level in each cell line. There was no association between the p53 status and the 53BP2 mRNA level among these cell lines. However, regardless of the p53 status (either wild-type or mutant), two cell lines (SBC3 and SBC5) from small cell lung cancer showed a relatively high expression level. Another cell line (Lu99) from lung giant cell carcinoma also had a relatively high level of 53BP2 mRNA expression. Since it is known that small cell lung cancer tends to be highly sensitive to anti-cancer agents and irradiation [14–16], we then examined the sensitivity of these cell lines to various DNA-damaging agents.

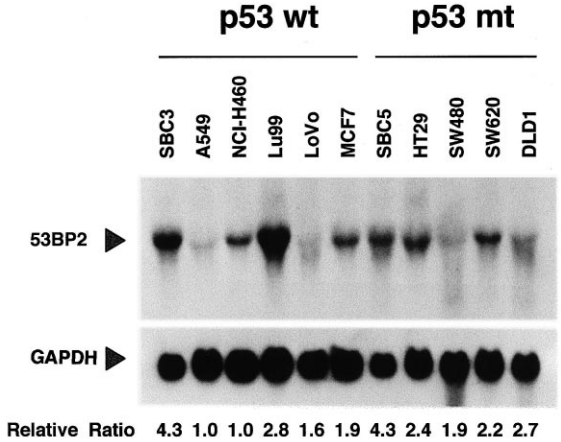


Fig. 3. Northern blot analysis of total RNA probed with 53BP2 cDNA (upper panel) and GAPDH (lower panel). Total RNA (20 μg) was loaded onto each lane. 53BP2 mRNA levels represent the relative amount of the 53BP2 mRNA normalized to that of GAPDH mRNA. The mRNA levels were quantified by a Fuji BAS 2500 image analyzer.

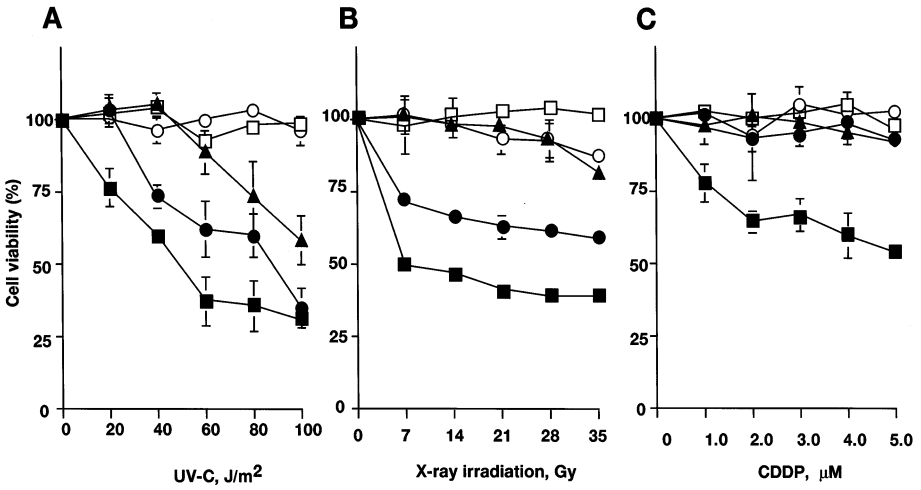


Fig. 4. Cell viability of various tumor cell lines upon treatment with (A) UV, (B) X-ray irradiation and (C) CDDP. The cell viability was measured by XTT assay. Data represent the mean ± S.D. of each cell line compared with the untreated control culture determined in triplicates. □, A549; ○, NCI H460; ●, Lu99; ▲, SBC5; ■, SBC3.

### 3.4. Sensitivity to UV, X-ray irradiation and CDDP

We examined five cell lines, including three (SBC3, SBC5 and Lu99) expressing high levels of 53BP2 mRNA and two (A549 and NCI H460) expressing low levels of 53BP2 mRNA. These cells were exposed to UV-C, X-ray irradiation, or treatment with CDDP. The cell viability was measured by XTT assay. As demonstrated in Fig. 4A, the cells expressing high levels of 53BP2 mRNA showed higher sensitivity to UV-C. Similarly, these cell lines are relatively sensitive to X-ray irradiation although to a lesser extent (Fig. 4B). In contrast, A549 and NCI H460 were relatively resistant to UV-C and X-ray irradiation. Upon treatment with CDDP, only SBC3 appeared to be sensitive to the DNA damage-induced cell death. Although we do not currently know the reason why other cell lines expressing high levels of 53BP2 mRNA were resistant to CDDP, additional mechanisms such as acquisition of multiple drug resistance phenotype may have been involved.

## 4. Discussion

In this study, we have demonstrated the lack of genetic abnormality of 53BP2 in various tumor cell lines irrespective of the genetic status of p53. Thus, it is indicated that the genetic abnormality of 53BP2 per se is not implicated in carcinogenesis, which is consistent with our previous results of the chromosomal mapping of 53BP2 gene that was mapped at 1q42.1 and did not correlate with any genetic marker for the susceptibility of hereditary cancers [17]. However, we also found that 53BP2 mRNA is abundantly expressed in some cell lines, which are relatively sensitive to various DNA-damaging reagents.

Biological activities of 53BP2 have been implicated in previous studies. Iwabuchi et al. [1] initially reported that 53BP2 bound to the wild-type p53 but not to its mutant form and recently that 53BP2 might be involved in augmentation of the transcriptional activity of p53 thus reducing the oncogene-mediated transformation [5]. Additionally, Naumovski and Cleary et al. [4] demonstrated that Bcl-2 interacted with p53 with higher affinity and that overexpression of 53BP2 induced cell growth arrest and suggested that one of the actions of Bcl-2 might be through interfering the 53BP2-binding to p53. More importantly, we have recently demonstrated that 53BP2 induced apoptosis, when overexpressed, and that the p65 subunit of NF-κB could block the 53BP2-induced apoptosis by directly interacting with 53BP2. These observations that 53BP2 could interact with p53 as well as Bcl-2 and p65 may indicate that various biological actions of these proteins may be regulated by 53BP2. Since p53, Bcl-2 and p65 are important regulatory factors involved in stress response, cell growth and apoptosis, functional interactions among these proteins may determine the direction of cellular responses.

We found that 53BP2 mRNA levels were diverse among various cell lines and there was no association between the 53BP2 mRNA level and the p53 status. The mRNA levels of 53BP2 of SBC3, SBC5 and Lu99 were relatively higher than other lung cancer cell lines. SBC3 and SBC5 were derived from small cell lung cancer, which is known highly sensitive to anti-cancer agents and irradiation [14–16]. We thus examined the sensitivity of these cell lines to various DNA-damaging agents and found that these cell lines were sensitive to UV and X-ray irradiation. In contrast, cell lines with lower 53BP2 mRNA levels such as A549 and NCI H460 were resistant to the cell death induced by these reagents. The lack of sensitivity of SBC5 and Lu99 to CDDP may possibly be ascribed to other factors involved in the cellular sensitivity to anti-cancer reagents such as P-glycoprotein, topoisomerase II and *O*-6-alkylguanine-DNA-alkyltransferase, although this possibility should be further substantiated [18]. In addition, p53 mutation (in case of SBC5) might be involved in sensitivity to anti-cancer agents [9,19]. These observations suggested a possibility that expression of 53BP2 might be correlated with the sensitivity to DNA damage agents. Further studies are needed to confirm this possibility by, for example, downregulating the 53BP2 mRNA by somatic gene targeting or transduction of anti-sense 53BP2 mRNA. It is also interesting to explore whether 53BP2 interacts with other members of the p53 family such as p73 [20] and p51/p73L/p63 [21–23] since these proteins share the similar DNA-binding motif that is known to be involved in the interaction with 53BP2 as well.

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