Expression of 53BP1 as a cisplatin-resistant marker in patients with lung adenocarcinomas

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Abstract. DNA repair is one of the major causes of spontaneous drug and radiation resistance in patients with lung adenocarcinomas (LADC). 53BP1 is a mediator that relays signals from DNA damage sensors and activates various effectors for the DNA repair and cell survival. In this study we investigated the clinical and biological significance of 53BP1. Expression of 53BP1 was detected by immunoblotting and immunohistochemistry. Our data showed that 53BP1 was detected in 166 (75.8%) of 219 LADC patients. Expression of 53BP1 correlated with tumor stage, cigarette smoking, lymphovascular invasion and poor clinical outcome. In vitro, increased 53BP1 expression elevated drug resistance, and silencing of 53BP1 expression reduced cisplatin resistance. Our results suggest that 53BP1 expression plays an important role in cisplatin resistance and predicts the prognosis for LADC.

Introduction

Lung cancer is the leading cause of cancer death worldwide. Among the four pathological subtypes, small cell lung cancer (SCLC), lung adenocarcinoma (LADC), squamous cell carcinoma and large cell carcinoma (1), LADC is featured by the rapid growth and high metastatic potential. Most importantly, the disease is resistant to therapy using anticancer drugs or radiation, the clinical performance that is reflecting in the poor prognosis (2,3). Drug and radiation resistance is correlated with cell cycle progression and capability of DNA repair (4). Both mechanisms are regulated by p53, a genome guardian, which induces cell cycle arrest whilst cells encounter genomic damage to allow DNA repair or induce apoptosis (5-7). Expression of p53 is thus important for determining drug and radiation resistance as well poor prognosis (8,9).

Using a yeast two-hybrid method, Iwabuchi et al identified two proteins, p53 binding protein 1 (53BP1) and 53BP2, which could interact with DNA binding domain of wild-type, but not mutant p53 (10). Both proteins are required for the induction of p53-dependent transcription activation (11). In addition, the 220-kDa 53BP1, in particular, acts as a mediator to relay signals from DNA damage sensors, ataxia-telangiectasia mutated (ATM) and ATM and Rad 3-related (ATR) kinases, to activate the various effectors, which are required for the DNA repair and cell survival (11-14). Therefore, it is reasonable to ask whether 53BP1 expression has any prognostic significance in patients with LADC.

In this study, we used an immunohistochemical method to determine 53BP1 expression in surgical specimens from patients with the LADC. Expression of 53BP1 was confirmed by immunoblotting. Correlation between clinicopathological parameters and 53BP1 expression and the prognostic significance of 53BP1 expression in patients with LADC were evaluated. The effect of 53BP1 expression on cisplatin resistance was measured in vitro.
Materials and methods

Non-small cell lung cancer (NSCLC) cell lines. One breast cancer (MCF-7) and seven NSCLC (H23, H226, H838, H1437, H2009, H2087 and A549) cell lines were used for the in vitro evaluation of gene expression. H23, H838, H1437, H2009, H2087 and A549 are LADC cells, and H226 is an epithelial type cell (2.3). Cells were grown at 37°C in a monolayer in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Patients and tissue specimens. From January 2001 to December 2002, tissue specimens were collected from 219 patients with newly diagnosed LADC. Samples from all patients, for whom at least one follow-up examination or death was documented, were pathologically confirmed LADC. The stage of the disease was classified according to the new international staging system for lung cancer (15). The Medical Ethics Committee approved the protocol, and written informed consent was obtained from every patient before surgery. All patients had undergone surgical resection and radical N2 lymph node dissection. Tumor size, lymph node number, differentiation, vascular invasion and mitotic number were also evaluated. Patients with lymph node involvement or loco-regional recurrence were irradiated at the affected areas. Those with distant metastasis were treated with chemotherapy. After treatment, patients were routinely followed every 3-6 months in the outpatient department. Results from blood examination, biochemical studies, chest radiography, abdominal sonography, whole body bone scan and computerized tomography scans of chest that indicated any evidence of disease were interpreted as tumor recurrence and metastasis. The average age of male patients (n=153) was 58.9±4.58 years and that of female patients (n=66) was 54.8±3.62 years (p<0.05). Immunohistochemical staining was carried out using a single-blind procedure.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from frozen tissues by a SNAP RNA column (Invitrogen, San Diego, CA). After measuring RNA yield, cDNA primed with oligo random primers was synthesized by AMV reverse transcriptase. Following total RNA extraction and synthesis of the first-strand cDNA, an aliquot of cDNA was subjected to 35 cycles of PCR to determine the integrity of β-actin mRNA (2.3). The cDNA used in the following RT-PCR was adjusted according to the quality and quantity of β-actin mRNA. The primer sequences were selected by Primer3 (http://frodo.wi.mit.edu/primer3). The primers for β-actin are: 5’-AGAGCTACGAGCTGCCTGTTGTA-3’ (antisense primer, nts 1477-1456). The DNA fragment is 943 base-pair (bp) for 53BP1 and 579 bp for β-actin.

Cytotoxicity assay. Cells were seeded at 5000 cells/well 18 h prior to challenge with various doses of cisplatin. The control group was treated with RPMI-1640. Following cell challenge for 48 h, 10 μl of WST-1 (BioVision, Mountain View, CA) was added and continued incubation for 2 h. Percent survival of cells was quantified by comparing the number of viable cells in the treatment group with that in the control group. All procedures were performed in triplicate. This assay measures both replicating and static cells (2.3).

Colony forming assay. Cells were seeded at 100, 500, 2000 and 10000 cells/6 cm culture plate. The cells were treated with various concentrations of cisplatin for 12 h, and then the drug was washed off with PBS. Following drug challenge, the cells were incubated at 37°C for 10 days until cell colony (≥50 cells/colony) appeared (16). Percent survival of cells was quantified by comparing the number of viable cell colonies in the treatment group with that in the control group. All procedures were performed in triplicate. This assay measures only replicating cells.

Preparation of mouse antibodies. DNA sequence corresponding to N-terminal fragment (amino acid residues 119-422) of human 53BP1 (h53BP1-N) was amplified by primer sequences containing EcoRI (sense) and HindIII (antisense) restriction sites respectively. The primer sequences were 53BP1s: 5’-TCCGGAATTCTTCTGGAATGTCACTGGT-3’ (EcoRI site is underlined) and 53BP1a: 5’-GCAGAATTCTGAGTCTCGTGTGGTATA-3’ (HindIII site is underlined).

The DNA fragment of h53BP1 was cloned into an expression vector pET-32a (Promega KK, Tokyo, Japan). Bacterial colony containing pET32-h53BP1-N was selected and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to mass-produce h53BP1-N. The recombinant protein was purified by a nickel-affinity column. Affinity-purified h53BP1-N was used to immunize BALB/c mice, and sensitivity of antisera (OD405 >0.3 at 1:6000 dilutions) was measured by enzyme-linked immunosorbent assay (ELISA). Specificity of antibodies was determined by the appearance of a 220-kDa band in immunoblotting of lung cancer cell extract (10). Monoclonal antibodies were produced by a hybridoma technique, and 53BP1-specific antibodies were screened by the above-mentioned methods.

Immunoprecipitation. gel electrophoresis and protein analysis by MALDI-TOF. Total cell lysate was prepared by mixing 5x10^7 cells/100 μl phosphate-buffered saline with equal volume of 2X NP-40 lysis buffer [40 mM Tris-HCl, pH 7.6, 2 mM EDTA, 300 mM NaCl, 2% NP-40 and 2 mM phenylmethylsulfonylfluoride (PMSF)], Protein G sepharose™ (Amersham Biosciences AB, Uppsala, Sweden) was pre-washed before mixing with 500 μg of total cell lysate. The reaction mixture was induced at 4°C for 60 min, and then centrifuged at 800 x g for 1 min. The supernatant was reacted with 5 μg of purified monoclonal antibodies and 20 μl of fresh protein G sepharose at 4°C for 18 h. The reaction mixture was centrifuged at 800 x g for 1 min. Following removal of the supernatant, the precipitate was washed with 1X PBS, and dissolved in loading buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM disodium EDTA, 1 mM PMSF, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and 1%
Signals were classified as 53BP1-.

was inactivated with 3% H2O2 and 0.1% sodium azide at room temperature (RT) for 10 min. Endogenous myeloperoxidase and absolute alcohol, antigen was retrieved by treating samples in boiling water for 10 min. Eletrophoresis was carried out in two 10% polyacrylamide gels with 4.5% stacking. One gel was processed for immunoblotting, and the other gel was stained with Coomassie blue (2,3). Protein on the Coomassie-stained gel, which corresponded to the immunoblotting-positive band, was extracted from the gel for identification by MALDI-TOF on a Voyager-DE™ pro biospectrometry workstation (Applied Biosystems, Milpitas, CA, USA). Fragments of peptide fingerprints were matched with those on the SwissProt database by MS-fit (ProteinProspector 4.0.5., The Regents of the University of California).

Immunoblot analysis. After gel electrophoresis, proteins were transferred to a nitrocellulose membrane for immunoblotting (2,3,16). Nitrocellulose membrane was probed with specific antibodies. The signal was amplified by biotin-labeled goat anti-mouse IgG and peroxidase-conjugated streptavidin. The protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY) with chemiluminescent reagent (NEN, Boston, MA) (2,3).

Immunocytochemistry and immunohistochemistry. Both immunocytochemical and immunohistological stainings were performed by an immunoperoxidase method using labeled streptavidin-biotin complex (Dako, LSAB2 System, Carpenteria, CA) (2,3). For confocal immunofluorescence microscopy, the second antibodies contained fluorescence labeled streptavidin. The protein was visualized by exposing the specimen to an X-Omat film (Eastman Kodak, Rochester, NY) with chemiluminescent reagent (NEN, Boston, MA) (2,3).

Immunofluorescence, both the antibodies contained fluorescence labeled streptavidin. The protein was visualized by exposing the specimen to an X-Omat film (Eastman Kodak, Rochester, NY) with chemiluminescent reagent (NEN, Boston, MA) (2,3). Following sequence analysis (by Mission Biotech, Taipei, Taiwan; www.missionbio.com.tw), nucleotide sequence of cDNA fragments from the seven lung cancer cell lines matched that of 53BP1: Homo sapiens p53 tumor suppressor-binding protein 1, Homo sapiens, AF078776.1, mRNA, identities = 936/943 (99.26%). Expression of 53BP1 mRNA, however, was not correlated with that of either wild-type or mutant p53 gene in these cells.

Slide evaluation. In each pathological section, NTLT served as internal negative control. Slides were evaluated by two pathologists blinded to the clinicopathological knowledge. The ImmunoReactive Scoring system was adapted for this study (17). Briefly, a specimen was considered having a strong signal when >50% of cancer cells were positively stained; intermediate, if 25-50% of the cells stained positive; weak, if <25% or >10% of the cells were positively stained; and negative, if <10% of the cells were positively stained. Cases with strong and intermediate 53BP1 signals were classified as 53BP1+, and those with weak or negative 53BP1 signals were classified as 53BP1-.

Statistical analysis. The relationship between 53BP1 expression and clinicopathological parameters was analyzed by χ² test. The χ² test for trend was used when corresponding variables exceeded two categories. Survival curves were plotted using the Kaplan-Meier estimator (18). Statistical difference in survival between different groups was compared by the log-rank test (between 53BP1+ and 53BP1- groups) (19). Analysis of covariance and the Mantel-Haenszel χ² procedure were used to evaluate the difference of cytotoxicity curves among the various groups. Statistical analysis was performed using GraphPad Prism 5 statistical software (San Diego, CA). Statistical significance was set at p-value <0.05.

Results

Expression of 53BP1 in LADC cells determined by RT-PCR. Expression of 53BP1 mRNA was determined by RT-PCR in seven lung cancer cell lines, and 53BP1 mRNA was detected in all the cancer cell lines (Fig. 1). Following sequence analysis (by Mission Biotech, Taipei, Taiwan; www.missionbio.com.tw), nucleotide sequence of cDNA fragments from the seven lung cancer cell lines matched that of 53BP1: Homo sapiens p53 tumor suppressor-binding protein 1, Homo sapiens, AF078776.1, mRNA, identities = 936/943 (99.26%). Expression of 53BP1 mRNA, however, was not correlated with that of either wild-type or mutant p53 gene in these cells (Fig. 1).

Characterization of monoclonal antibodies to 53BP1 and expression of 53BP1 in LADC cell lines. Specificity of monoclonal antibodies was determined by immunoblotting of the H226 and MCF-7 cell lysate. The predicted molecular mass of 53BP1 is 220-kDa. However, our antibodies recognized an extra 250-kDa protein band, which was about 30-kDa larger than the anticipated 53BP1, and we labeled this protein band as 53BP1* (Fig. 2A). The unique 250-kDa protein band was also detected in H1437, the 250-kDa 53BP1* band was also detected. In H1437, the 250-kDa 53BP1* band was also detected (Fig. 2C). However, the 250-kDa 53BP1* signal was not recognized in the lung cancer specimens (Fig. 2D).
The higher molecular weights in MCF-7, H226 and h1437 suggested that the 250-kDa 53BP1* was modified post-translationally. Treatment of cell lysate with calf intestinal alkaline phosphatase (CIP) before immunoblotting showed a reduction of 250-kDa protein, and an increase of 220-kDa protein (Fig. 2E), indicating that the 250-kDa 53BP1* could be a phosphorylated form (20). We also probed 53BP1 specific antibody-precipitated proteins with antibodies to ubiquitin or to sumoyl residues. The result was negative (data not shown).

Elevated expression of 220- and 250-kDa 53BP1 increases cisplatin resistance. Because nicotine, a major ingredient in cigarette, induces DNA damage (21), we tested the effect of nicotine on expression of 53BP1. Addition of nicotine to H23 cells increased levels of both 220-kDa 53BP1 and 250-kDa 53BP1* (Fig. 3A) as well as cell resistance to cisplatin (Fig. 3B). Using siRNA to knock down 53BP1 (53BP1<sup>−/−</sup>) expression in H1437 cells (Fig. 3C), however, reduced cisplatin resistance (Fig. 3D). These results supported our anticipation that 53BP1 expression could be associated with cisplatin resistance of the lung cancer cells.

Expression of 53BP1 in LADC. Using immunohistochemistry, 53BP1 was detected in 166 (75.8 %) of LADC pathological samples (Fig. 4A-C). Among 148 of the 166 (89.1%) pathological specimens the 53BP1 could be identified in the nuclei of cancer cells (Fig. 4A and B). 53BP1 expression was also detected in 84.9% (79/93) of metastatic lymph nodes (data not shown). Statistical analysis showed that expression of 53BP1 correlated with tumor stage, cigarette smoking, and lymphovascular involvement (Table I), suggesting that 53BP1 expression was associated with tumor cell growth, and possibly metastasis of LADC cells. Moreover, among the 166 patients who had high levels of 53BP1, 98 (59.0%) had tumor recurrence during follow-up examination. Among the 53 patients who had low levels of 53BP1, 15 had tumor recurrence (28.3%). All 103 patients who had recurrence developed tumors within 24 months after operation. The risk of recurrence for patients with high levels of 53BP1 was 3.651-fold higher than that for patients with low levels of 53BP1 (p<0.01). Survival of patients with low 53BP1 levels was significantly better than that of patients with high 53BP1 levels (Fig. 4E). The hazard ratio between these two groups was 1.987, and the difference in cumulative survival was significant (p=0.0016).
Multivariate analysis also showed a significant statistical difference in 53BP1 expression between the two groups (p=0.0276).

Discussion

The results showed that antibodies generated in our study recognized the 53BP1. In LADC sections 53BP1 was frequently detected in the nuclei of cancer cells. Expression of 53BP1 correlated with the advanced tumor stage, lympho-vascular invasion and patients' cigarette smoking habits, suggesting that 53BP1 expression was associated with cigarette smoking and disease progression of LADC.

As shown by immunoblotting, in addition to the 220-kDa protein which was frequently detected in the pathological specimens, a 250-kDa 53BP1* was detected in H226, H1437 and MCF-7 cells. Intensity of the 250-kDa protein reduced to 220-kDa following CIP treatment, supporting the fact that 53BP1 was phosphorylated (20). Since increase of 53BP1...
expression facilitates DNA repair, it is thus interesting to note that addition of nicotine elevates 53BP1 expression and increases cisplatin resistance (21). Silencing of 53BP1 by siRNA, on the other hand, reduces cisplatin resistance. These data suggest that expression of 53BP1 is important for drug resistance of LADC. Patients who have higher 53BP1 levels are more resistant to cisplatin-based chemotherapy and have worse prognosis. Patients who have lower levels of 53BP1, on the other hand, are more sensitive to cisplatin-based chemotherapy and have better prognosis. By showing that cancer cells with dominant-negative 53BP1 phenotype have higher frequency of camptothecin-induced cell death, results by Yoo et al support our observations and indicate that 53BP1 expression is associated with drug resistance (12).

Using immunohistochemistry Gorgoulis et al detected 53BP1 expression in the non-small cell lung cancer (NSCLC) (22). Detection rate was 93.2% (69/74), and 53BP1 expression was not dependent on the status of p53, wild-type or mutant (22). Moreover, 53BP1 expression in NSCLC cells correlated with cell proliferation, as determined by Ki67 expression, as well as protein and protein phosphorylation levels of CHK2, a DNA damage-related signaling kinase (22,23). Interestingly, CHK2 was not phosphorylated in the absence of 53BP1 (22,24), and CHK2 expression was essential for cisplatin resistance (25).

Table I. Correlation of 53BP1 expression with clinicopathological parameters of LADC.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Expression of 53BP1</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>High (n=166)</td>
<td>Low (n=53)</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male (n=153)</td>
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<td>Female (n=66)</td>
<td>47</td>
<td>19</td>
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<tr>
<td>Cigarette smokingb</td>
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<tr>
<td>Smoker (n=149)</td>
<td>134</td>
<td>15</td>
</tr>
<tr>
<td>Non-smoker (n=70)</td>
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<td>38</td>
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<tr>
<td>Stage</td>
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<tr>
<td>I (n=76)</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>II (n=81)</td>
<td>67</td>
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</tr>
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<td>III (n=62)</td>
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<td>8</td>
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<tr>
<td>Cell differentiation</td>
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<tr>
<td>Well (n=52)</td>
<td>38</td>
<td>14</td>
</tr>
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<td>Moderate (n=102)</td>
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<td>27</td>
</tr>
<tr>
<td>Poor (n=65)</td>
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<td>12</td>
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<tr>
<td>Lymphovascular invasion</td>
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<td>Positive (n=93)</td>
<td>79</td>
<td>14</td>
</tr>
<tr>
<td>Negative (n=126)</td>
<td>87</td>
<td>39</td>
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</table>

*a Two-sided p-value determined by χ² test. **In this study, smoker is defined as a person who is constantly exposed to cigarette smoking, including non-smoker patients living in the same household where the spouse or a relative smoked more than one pack per week (~50 packs/year).

DNA repair, in particular nucleotide excision repair (NER), has long been associated with drug resistance (26-28). An elegant in vitro study by Niedernhofer et al showed that excision repair cross-complementation group 1 (ERCC1) was required for repairing cisplatin-induced DNA damage, confirming that DNA repair was indeed involved in drug resistance (29). Clinically, Olaussen et al found that NSCLC patients with ERCC1-negative tumors responded better to cisplatin-based adjuvant chemotherapy and had better prognosis than those with ERCC1-positive tumors, supporting the facts that ERCC1 was associated with increased drug resistance (30). These results corresponded well with those reported by Yu et al that both ERCC1 and CHK2 were essential for cisplatin resistance (25). Xeroderma pigmentosum complementation group (XP) C is important for NER as well. In association with human homolog of yeast Rad23 protein B (HHR23B), XPC binds DNA damage sites, XPC-hHR23B complex then recruits XPA, RNA polymerase II basal transcription factor b (TFIIB), transcription factor B5 (TFB5) and human replication protein A (RPA) to initiate DNA repair (31,32). 53BP1 also binds RPA, and it is required for the phosphorylation of RPA2 and CHK2 (12,22,24). Interestingly, studies of the DNA damage mediators’ functions in ATM activation indicate that 53BP1 not only acts as a mediator for relaying DNA damage signals, but also as a modulator,
which directly interacts with the Mre11/Rad50/NBS1 (MRN) complex and promotes kinase activity of ATM (33). The MRN complexes are essential for the repair of DNA double-strand breaks, e.g., homologous recombination, non-homologous end-joining (NHEJ) and preservation of telomeres (34-36).

It is worth noting that cigarette smoke introduces both intrastranded and interstranded DNA damage as well as shortening of telomeres (37-39). It is possible that cigarette smoke concurrently increases expression of p53 and DNA repair-related proteins, including 53BP1, XPC and ERCC1. However, for such pathophysiological responses, other explanations are feasible, for instance, cigarette smoke increases expression of hepatocyte growth factor (HGF) in type II pneumocytes and lung cancer cells, which not only elevates metastatic potential, but also down-regulates apoptosis-inducing factor (AIF) expression and reduces cisplatin toxicity in LADC cells (2,3).

In conclusion, immunoblotting and immunohistochemistry revealed abundant expression of 53BP1 in lung adenocarcinoma cells. Pathological results suggest that 53BP1 expression is associated with tumor stage, lymphovascular invasion and cigarette smoking habits of the patients, which reflects the increased tumor cell growth, metastasis and poor prognosis of patients with LADC. In vitro, nicotine increased 53BP1 expression and cisplatin resistance. Silencing of 53BP1 expression, however, reduced cisplatin resistance. These data suggest that 53BP1 plays an important role in drug resistance of lung adenocarcinoma cells.

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