The Staining Patterns of 53BP1 Nuclear Foci and 53BP1 mRNA Level are Associated With Cervical Cancer Progression and Metastasis

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Summary: p53-binding protein 1 (53BP1) plays a key role in DNA damage response mechanism, which protects genome integrity and guards against cancer. Although abnormal DNA damage response type of 53BP1 nuclear foci (NF) have been indicated to be associated with many types of malignancies, how the staining pattern of 53BP1 NF and the mRNA level of 53BP1 correlate with the clinicopathologic characteristics of cervical cancer is still unclear. In this study, we examined the staining pattern and mRNA level of 53BP1 in cervical premalignant and malignant lesions and normal cervical tissue by immunofluorescence staining and quantitative real-time polymerase chain reaction. We found that the level of 53BP1 NF increased in the following order: normal cervical tissues, cervical intraepithelial neoplasia (CIN) 1, CIN2/3, and cervical cancers, indicating that the level of 53BP1 NF increases as cervical cancer initiates and progresses. In addition, we also found that abnormal DNA damage response type of 53BP1 NF and low mRNA level of 53BP1 was significantly correlated with high histologic grade of cervical cancer, and low mRNA level of 53BP1 was also significantly associated with positive lymph node metastasis of cervical cancer. Key Words: Cervical cancer—53BP1—Tumor suppressor—Immunofluorescence—Quantitative real-time PCR.
activation, DNA repair, and foci formation following DNA damage (14).

Some progress has been made recently in understanding the role of 53BP1 in DDR and maintaining genomic integrity. Lukas et al. (15) examined the assembly of 53BP1 bodies in tissue sections and cell lines and found that 53BP1 nuclear body assembly is induced by DNA or chromatin damage and largely confined to G1. They also proposed that the formation of 53BP1 nuclear bodies in G1 might sequester DNA or chromatin lesions that are transmitted into the daughter cells during mitosis, which then might trigger DNA repair mechanism (15).

Nakashima et al. (16) and Naruke et al. (17) showed that abnormal DDR type of 53BP1 NF is associated with high-grade thyroid cancer and invasive skin cancer, and suggested that the staining pattern of 53BP1 can be a useful tool to estimate the level of GIN and the malignant potential of human thyroid tumors and skin tumors. Matsuda et al. (18) investigated the correlation between 53BP1 NF and HPV infection in cervical lesions and found that the distribution of 53BP1 NF is associated with HPV infection and p16INK4a overexpression. Their results suggest the association of 53BP1 NF with viral infection and replication stress in cervical lesions (18). They also found that the number of 53BP1 NF in cervical tissue increases as cervical lesion progresses to malignancy (18). However, the correlation of the staining pattern of 53BP1 NF and the mRNA level of 53BP1 with clinicopathologic characteristics of cervical cancer is still unclear. Our study aimed to fill this knowledge gap.

MATERIALS AND METHODS

Tissue Specimen Collection

All the patients signed the informed consent form. The procedure for tissue specimen collection was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical College. The tissue samples of 60 cases of cervical cancer and 30 cases of cervical intraepithelial neoplasia (CIN) were obtained from patients treated at the Department of Obstetrics and Gynecology, First Affiliated Hospital of Wenzhou Medical College, between May of 2010 and August of 2011. Of the 30 cases of CIN, there were 7 cases of CIN 1 and 23 cases of CIN2/3. Normal cervical tissue samples were also collected from 20 patients undergoing myomectomy for uterine leiomyoma. A small piece of surgically removed tissue was sent for histopathologic diagnosis, and the remaining tissue was fresh-frozen for RNA extraction and immunofluorescence staining. All the hematoxylin-eosin slides from the tissue samples were reviewed independently by 2 pathologists who were kept blind to patients’ clinical data. The normal cervical tissues were confirmed for the absence of dysplasia or cancer cells. None of the patients received prior treatment.

Immunohistologic Staining for 53BP1 and Evaluation Criteria for the Staining Pattern of 53BP1

Tissues were cut into 4- to 10-μm sections and mounted on slides. The tissue sections were then fixed in cold acetone for 10 min and washed with PBS for 3 times. Ten percent normal blocking serum in PBS was used to block the tissue section for 20 min. The tissue sections were then incubated with anti-53BP1 rabbit polyclonal antibody (Santa Cruse, Montgomery, TX) at 1:200 dilution. Alexa-fluor 488-conjugated goat anti-rabbit antibody was then used (Invitrogen, Carlsbad, CA). The tissue specimens were counterstained with DAPI-I (Vysis, Downers Grove, IL) and observed under a fluorescence microscope (Olympus BX51, Japan). Staining images were captured and analyzed at 1000 x magnification.

Caski and HeLa cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Invitrogen, Groud Island) in humidified 37°C incubator with 5% CO2. After fixing with methanol and blocking with 10% normal goat serum, the cultured cells were stained for 53BP1 in the same manner as described for tissue specimens. The pattern of 53BP1 staining was classified into 5 types according to the following criteria (16,18): (1) stable type—faint and diffuse nuclear staining; (2) low DDR type—1 or 2 discrete NF; (3) high DDR type—3 or more discrete NF; (4) abnormal DDR type—discrete NF that are larger than 1.0 μm; and (5) lost type—no nuclear staining.

Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from frozen tissues using the Trizol LS Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instruction. RNA concentration and quality were determined by a spectrophotometer (Beckman, Brea, CA) and gel electrophoresis, respectively. First-strand cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) in a total volume of 20 μL. qRT-PCR analysis for 53BP1 gene expression was performed in triplicate using the SYBR Green
PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. The fold-change of 53BP1 expression in cervical lesion compared with that in the normal cervical tissue was calculated using ΔCT and $2^{-\Delta\Delta CT}$. GAPDH was used as the reference gene. The relative expression of 53BP1 to GAPDH was calculated. The following primers were used: 53BP1 forward primer: 5'-CCTCAGGCTCTGGTGACTTC-3'; reverse primer: 5'-TGACAGCACAGCCCAGTAAG-3'; GAPDH forward primer: 5'-GGTCGGAGTCAACTTG-3'; and reverse primer: 5'-ATGAGCCCCAGCCTTCCAT-3'.

The amplified DNA fragment is 292 bp for 53BP1 and 314 bp for GAPDH.

Statistical Analysis
Comparisons between the 2 groups were carried out by Student t test. Correlations between the types of the staining pattern of 53BP1 NF (stable, low DDR, high DDR, abnormal DDR, and lost type) and clinicopathologic characteristics of cervical cancer were analyzed by Fisher exact test or χ² test. P <0.05 was considered significantly different. All statistical analyses were performed using the SPSS software (version 15.0). Data were presented as mean ± SD.

RESULTS
The Level of 53BP1 NF is Significantly Increased in Cervical Cancer
Carcinoma cell line Hela and Caski expressed high DDR type and abnormal DDR type of 53BP1 NF, respectively (Fig. 1A, a and b). The staining pattern of 53BP1 in the normal cervical tissue from the 20 uterine leiomyoma patients was stable type (Fig. 1B, a). All the 7 cases of CIN1 expressed low DDR type of 53BP1 NF (Fig. 1B, e). Of the 23 cases of CIN2/3, 14 (60.9%) expressed low DDR type, whereas the remaining 9 cases (39.1%) showed high DDR type (Fig. 1B, f). In contrast to normal cervical tissue and CIN, no stable type or low DDR type was observed in the 60 cases of cervical cancer, of which 2 cases (3.3%) showed lost type (Fig. 1B, b), 24 cases (40.0%) showed high DDR (Fig. 1B, c), and 34 cases (56.7%) exhibited abnormal DDR (Fig. 1B, d). These results highlighted that the level of 53BP1 NF was significantly increased (Table 1) and the staining pattern of 53BP1 NF changed during cervical cancer initiation and progression.

To determine the correlation between the staining pattern of 53BP1 NF and the clinicopathologic characteristics of cervical cancer, we performed Spearman’s analysis on the 60 cases of cervical cancer. The 2 cases of cervical cancer with lost type were excluded from the analysis. Our analysis revealed that high histologic grade of cervical cancer significantly correlated with abnormal DDR type of 53BP1 NF ($P<0.0001$), whereas no significant correlation was detected between the staining pattern of 53BP1 NF and age, histology type, clinical stage, and lymph node metastasis status of the cervical cancer patients ($P>0.05$, Table 2).

53BP1 mRNA Level in Cervical Cancer is Significantly Decreased
We analyzed 53BP1 mRNA level by qRT-PCR. The level of 53BP1 mRNA in cervical cancer tissue and CIN tissue relative to that of normal cervical tissue was 0.5415 ± 0.194 and 1.424 ± 0.402, respectively (Fig. 2), suggesting that the level of 53BP1 gene expression in cervical cancer is significantly lower than that in CIN tissues ($P<0.05$). No significant difference in 53BP1 mRNA level was detected between CIN and normal cervical tissues ($P=0.097$). We divided 60 cases of cervical cancer into 2 groups according to their mRNA level of 53BP1. We used 0.5415 as the dividing criteria. Cervical cancer with a relative 53BP1 mRNA level >0.5415 was classified into the high expression group, whereas cervical cancer with a relative 53BP1 mRNA level <0.5415 was classified into the low expression group. We then analyzed whether the relative 53BP1 mRNA level was correlated with any of the clinicopathologic characteristics of cervical cancer. Surprisingly, we found that low-relative 53BP1 mRNA level was significantly correlated with high histologic grade ($P=0.006$) and positive lymph node metastasis ($P=0.011$, Table 3). In contrast, low-grade cervical cancer and negative lymph node metastasis were associated with high-relative 53BP1 mRNA level.

DISCUSSION
In this study, we found abnormal patterns of 53BP1 NF immunofluorescence in preneoplastic and neoplastic cervical lesions. No discrete NF of 53BP1 was observed in normal cervical tissue, 70.0% (21/30) of CIN showed low DDR and 30.0% (9/30) showed high DDR type of 53BP1 NF, and 96.7% (58/60) of
cervical cancer displayed either high DDR or abnormal DDR of 53BP1 NF. Similar staining pattern of 53BP1 NF, which shows that the level of 53BP1 NF is increased in the order of benign precancerous lesion—cancer in situ—invasive cancer, has been observed in skin, thyroid, and cervical tumors as well (16–18). We also found that abnormal DDR type of 53BP1 NF was significantly correlated with high histologic grade of cervical cancer. Similarly, Lai et al. (19) showed that 53BP1 NF pattern

<table>
<thead>
<tr>
<th>TABLE 1. The staining pattern of 53BP1 NF changed during cervical cancer initiation and progression</th>
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<tbody>
<tr>
<td>Stable</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>UL (n = 20)</td>
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<tr>
<td>CIN1 (n = 7)</td>
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<tr>
<td>CIN2/3 (n = 23)</td>
</tr>
<tr>
<td>Cervical cancer (n = 60)</td>
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53BP1 indicates p53-binding protein 1; DDR, DNA damage response; CIN, cervical intraepithelial neoplasia; NF, nuclear foci; UL, uterine leiomyoma.

FIG. 1. Immunofluorescence staining for p53-binding protein 1 (53BP1) expression in the cervical cancer cell lines and cervical tissues. (A) HeLa cells (a) and Caski cells (b) expressed high DNA damage response (DDR) type and abnormal DDR type of 53BP1 nuclear foci (NF), respectively. (B) Immunofluorescence staining for 53BP1 expression in the cervical tissue. Stable type in the normal cervical epithelium (a). Lost type in the cervical cancer tissue (b). High DDR type of 53BP1 NF in cervical cancer tissue (c). Abnormal DDR type of 53BP1 NF in the cervical cancer tissue (d). Low DDR type in cervical intraepithelial neoplasia (CIN)1 tissue (e). High DDR type in CIN2/3 tissue (f). Tissue section was stained as described in the Material and methods section. The illustrated images were the representative images.
correlated with tumor stage, cigarette smoking, lymphovascular invasion, and poor clinical outcome in lung cancer. These results suggest that DSBs and GIN occur in precancerous cervical lesions and continuously accumulate as the cervical cancer progresses, and the staining pattern of 53BP1 evolves to reflect such DSB and GIN accumulation. Thus, we propose that the staining pattern of 53BP1 NF might be a potential biomarker of DSB in premalignant cervical lesions and cervical cancer.

Interestingly, we observed 2 cases of cervical cancer losing 53BP1 protein expression. Our qRT-PCR analysis also showed that the level of 53BP1 mRNA in cervical cancer was significantly lower than that in CIN, the precancerous cervical lesions. We also found that low 53BP1 expression was significantly correlated with high histologic grade and positive lymph node metastasis of cervical cancer. These results suggest that 53BP1 might be a tumor suppressor. In fact, a number of studies have demonstrated that 53BP1 carries the characteristics of a tumor suppressor. Gorgoulis et al. (20) observed aberrant absence of 53BP1 in subsets of the human lung tumors and melanomas, and Bartkova et al. (21) found aberrant reduction or lack of 53BP1 in breast and lung cancer. Nuciforo et al. (22) also detected a progressive and marked loss of 53BP1 during tumor progression at the early transition from normal to dysplastic change in lung cancer. In addition, Morales et al. (23) found that the transgenic mice carrying 53BP1 deficiency (53BP1−/−) are predisposed to tumors. However, lost 53BP1 expression is not observed in thyroid cancer and skin cancer (16,17). The inconsistent results from different types of cancer might be because of various mechanisms underlying the tumorigenesis of the different types of cancer. In our study, because of a small sample size, only 2 cases of lost type were detected in the cervical cancer tissues. The effect of 53BP1 deficiency on cervical cancer initiation and progression and the mechanism underlying 53BP1 deficiency in cervical cancer are still unclear and need further investigation.

The association between 53BP1 function and cancer initiation and progression has recently been investigated intensively. 53BP1 is believed to act as a mediator or adapter protein to facilitate the

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**TABLE 2. Correlation between the staining pattern of 53BP1 NF and the clinicopathologic characteristics of cervical cancer**

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>No. cases</th>
<th>DDR high</th>
<th>DDR abnormal</th>
<th>$\chi^2$</th>
<th>$P^*$</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$\leq 40$</td>
<td>38</td>
<td>14</td>
<td>24</td>
<td>0.935</td>
<td>0.344</td>
</tr>
<tr>
<td>$&gt; 40$</td>
<td>20</td>
<td>10</td>
<td>10</td>
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<td></td>
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<tr>
<td>FIGO clinical stage</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>31</td>
<td>10</td>
<td>21</td>
<td>2.3</td>
<td>0.317</td>
</tr>
<tr>
<td>II</td>
<td>23</td>
<td>12</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>4</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Histology type</td>
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<td></td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td>52</td>
<td>20</td>
<td>32</td>
<td>0.299</td>
<td>0.673</td>
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<tr>
<td>Adenocarcinoma</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>Histologic grade [n (%)]</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G1</td>
<td>20</td>
<td>16</td>
<td>4 (20)</td>
<td>19.94</td>
<td>$&lt;0.0001^{**}$</td>
</tr>
<tr>
<td>G2</td>
<td>25</td>
<td>6</td>
<td>19 (76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>13</td>
<td>2</td>
<td>11 (85)</td>
<td></td>
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<tr>
<td>Lymph node invasion</td>
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<tr>
<td>Positive</td>
<td>11</td>
<td>6</td>
<td>5</td>
<td>0.416</td>
<td>0.519</td>
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<tr>
<td>Negative</td>
<td>47</td>
<td>18</td>
<td>29</td>
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*$\chi^2$ test.**
**$P<0.05$, represents significant difference.
53BP1 indicates p53-binding protein 1; DDR, DNA damage response; NF, nuclear foci.

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**FIG. 2.** The relative p53-binding protein 1 (53BP1) mRNA level in the cervical intraepithelial neoplasia (CIN) and cervical cancer tissue. Total RNA extraction and quantitative real-time polymerase chain reaction were performed according to the description in the Materials and methods section. The mRNA level of 53BP1 in the normal, cervical intraepithelial neoplasia (CIN), and cervical cancer tissue was first normalized to the reference gene GAPDH. Then, the relative mRNA level of 53BP1 in the CIN tissue and cervical cancer tissue was calculated as fold of the mRNA level of 53BP1 in the normal cervical tissue according to $2^{\Delta \Delta Ct}$. *$P<0.05$ cervical cancer tissue versus CIN tissue.
transmission of DNA damage signal to transducer kinases such as checkpoint kinase 1 and 2, which play roles in signal transduction pathways targeting downstream DDR components (24). Thus, malfunction of 53BP1 might very likely lead to DNA repair disorder, resulting in carcinogenesis. Several recent studies demonstrated evidence supporting that 53BP1 loss might be associated with the progression of ovarian and breast cancer. Hong et al. (25) found that the overexpression of 53BP1 induces G2/M arrest and apoptosis of the ovarian cancer cells; in contrast, 53BP1 knockdown significantly reduces apoptosis. Li et al. (26) found that 53BP1 level shows a gradual decrease during the progression from precancerous to cancer lesion in breast cancer, and knockdown of 53BP1 by RNA interference significantly increases the proliferation and invasion of the breast cancer cells. A recent study investigated the mechanism underlying 53BP1 loss in breast cancer and demonstrated that, in triple-negative breast cancer, the loss of the tumor-suppressor gene, BRCA1, can activate cathepsin L (CTSL)-mediated degradation of 53BP1, which then rescues homologous recombination repair and allows BRCA1-deficient cells to escape cell-cycle arrest (27). Our results suggest that 53BP1 might be overexpressed and recruited to DSB sites because of the accumulation of DSB in premalignant lesions. The progressive accumulation of DSB increases GIN, and a high level of GIN might allow the occurrence of genetic alterations associated with carcinogenesis such as loss or mutation of tumor-suppressor genes.

The loss of tumor-suppressor genes might subsequently initiate the mechanism to induce 53BP1 loss. 53BP1 loss then facilitates the escape of growth arrest, ultimately resulting in carcinogenesis. Thus, 53BP1 might not directly contribute to cancer initiation and progression; instead, it might indirectly facilitate tumor development because of its functional association with other molecules that are critical in carcinogenesis.

In cervical cancer, the distribution of 53BP1 NF has been found to be associated with HPV infection. Matsuda et al. (18) showed that the punctate HPV signals from in situ hybridization resemble the distribution pattern of 53BP1 NF in CIN, suggesting an association of 53BP1 NF with viral infection and replication. Gudjonsson et al. (28) recently also found that 50% of HPV-associated cervical tumors show expanded 53BP1 bodies, whereas none of the HPV-negative carcinomas showed these phenotypes. These findings indicate that HPV infection can cause DSB and GIN, and the level of GIN might be reflected by 53BP1 expression and distribution. Lukas et al. (15) investigated the molecular mechanism by which 53BP1 regulates the cell cycle and found that 53BP1 bodies form in G1 phase and are resolved by early S-phase. We will further investigate the involvement of 53BP1 in regulating the cell cycle in cervical cancer cells in our future study.

In summary, our study showed that the level of 53BP1 NF was significantly increased during cervical cancer initiation and progression, whereas the relative level of 53BP1 mRNA was significantly decreased in the cervical cancer compared with that in the precancerous cervical lesion tissue. The abnormal DDR type of 53BP1 NF and low-relative 53BP1 mRNA level were significantly correlated with high-grade cervical cancer. Low-relative 53BP1 mRNA level was also significantly correlated with positive lymph node metastasis of cervical cancer. Thus, the staining pattern and mRNA level of 53BP1 might be indicators for the magnitude of DSB reflecting GIN level, and the level of GIN might indicate the progressiveness and metastasis of cervical cancer.

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REFERENCES


