Expression of Apoptosis Inhibitor Survivin: Common and Independent of p53 Aberration in Thai Cancer Patients

Ratchada Cressey\textsuperscript{1*}, Roongsiri Chotpadiwetkul\textsuperscript{1}, Nirush Lertprasertsuke\textsuperscript{2} and Watchara Kasinrerk\textsuperscript{3}

\textsuperscript{1}Division of Clinical Chemistry, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
\textsuperscript{2}Department of Pathology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
\textsuperscript{3}Division of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

*Corresponding author. E-mail: ratchada@chiangmai.ac.th

ABSTRACT

Survivin is an anti-apoptotic protein that is suppressed by wild-type p53 and overexpressed in various types of human tumours. Although wild-type p53 has been reported to repress expression of the survivin gene by binding to its promoter, whether loss of p53 function is responsible for the induction of survivin gene expression in tumour tissues is unknown. In this study, we examined the expression levels of survivin and p53 by Western blot analysis to determine the frequency and the relationship between their anomalies and with the clinicopathologic features of liver, lung and colorectal tumours in Thai patients.

The results suggested that in normal tissues, survivin and p53 could only be detected in 3 out of 36 (8.3%) and 5 out of 36 (13.9%), respectively. In contrast, these two proteins were significantly increased in tumour tissues. p53 protein was accumulated in 19 out of 36 tumour tissues (52.7%) and survivin was detectable in 32 out of 36 cases (88.9%). Survivin was overexpressed in 63.6% of the hepatocellular carcinomas, while all of the colorectal and non-small cell lung carcinomas overexpressed survivin. Survivin overexpression, but not p53 accumulation, was significantly associated with advanced stage of tumours (p=0.007) and metastasized tumours (p=0.025). No association between p53 and survivin positivity was observed.

Our results further indicate that the activation of survivin is required for tumourigenesis and is frequently found in Thai cancer patients, indicating that the approaches of down regulating survivin as a cellular target for cancer therapy should be very promising for Thai population. Although p53 has been previously reported to repress survivin gene transcription, overexpression of survivin frequently found in human tumours may not entirely depend on the loss of p53 function.

Key words: p53, Survivin, Thai cancer patients
INTRODUCTION

The balance between cell cycle and apoptosis is crucial for normal tissue homeostasis. Any disruption of this balance, which leads to inappropriate cell death and/or abnormal proliferation, can result in tumourigenesis. Dysregulation of apoptosis is involved in carcinogenesis by abnormally-prolonged cell survival, facilitating the accumulation of transforming mutations and promoting cell growth. One of the most important regulators of apoptosis is a recently-discovered member of the inhibitor of apoptosis proteins (IAP) called “survivin”. This protein blocks apoptosis by inhibiting activation of caspase-3 and caspase-7 and regulates the G2/M phase transition of the cell cycle (Altieri, 2003). One of the most significant characteristics of survivin is its differential expression in cancer versus normal tissues. Survivin is expressed during embryonic and fetal development but is undetectable in the majority of adult tissues. Interestingly, in several types of tumours, i.e., lung, breast, prostate, pancreas and colon, this protein is reexpressed (Andersen and Thor, 2002). However, the mechanism regarding the up-regulation of survivin gene expression in tumourigenesis is poorly understood.

The tumour-suppressor protein p53 plays a pivotal role, switching between cell cycle arrest and apoptosis induction. Functional p53 is a transcription factor with the capabilities of transactivation and transrepression of hundreds of its target genes. Although wild-type p53 has been reported to repress expression of the survivin gene by binding to its promoter (Hoffman et al., 2002; Mirza et al., 2002; Beltrami et al., 2004), whether loss of p53 function is responsible for the induction of survivin gene expression in tumour tissues is unknown.

Damage to p53 plays a major role in human carcinogenesis. Point mutations or deletions of the p53 gene are observed in approximately 50% of malignant diseases, although the frequencies of damage depend on the tumour type (Soussi, 2000). A common feature shared by mutant p53 proteins is impaired specific DNA binding and sequence-specific transactivation, along with reduced ability to suppress cell growth and induce apoptosis (Wallace-Brodeur and Lowe, 1999). Mutant p53 is frequently found to be metabolically-stable protein with a long half-life (Anker et al., 1993; Lepelley et al., 1994). Wild type p53 has a short half-life, 6-60 minutes, and thus does not generally accumulated to levels high enough to be detected by standard immunohistochemistry or Western blot analysis. In contrast, due to its longer half-life, mutated p53 can accumulate to levels which can be detected by such techniques. Therefore, an increase in the p53 protein level is usable as an indication for p53 mutations, although there are still some discrepancies (Top et al., 1995; Coggi et al., 1997).

In this study, we used Western blotting techniques to examine the expression levels of survivin and p53 in order to determine the frequency and the relationship between their anomalies in Thai cancer patients. The Western blot results were further correlated with clinicopathologic features of the tumours to investigate a possible influence of survivin and p53 on tumour progression in Thai patients.
MATERIALS AND METHODS

Selection of patients and sample

All tissues in this study were obtained from Thai cancer patients who underwent curative resection at Maharaj Nakorn Chiang Mai Hospital during April 2002 to June 2004. In each case, adjacent normal mucosa was collected for comparison. These specimens were immediately placed in vials, frozen in embedded medium for the preservation of cell integrity and stored at -70°C until analysis. All tissues were classified by a pathologist according to pathological features of the tumours, which included tumour size in maximal diameter, histological grading, and depth of invasion, lymph-node metastasis and distant metastasis. The study was approved by the Ethical Committee of the Faculty of Medicine, Chiang Mai University, according to document number 56/2545.

Western blotting

Western blotting was performed to evaluate the expression of survivin and p53. Frozen tissues were thawed, cut into small pieces and homogenized in SDS lysis buffer (0.5M Tris-HCl pH 6.8, 2% SDS (w/v) and 10% glycerol (v/v)) containing a protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2.2 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64; Sigma, U.S.A). The tissue homogenate was then centrifuged at 10,000 g for 15 minutes at 4°C, after which the supernatant was removed and the protein concentration of the supernatant was estimated, using the BCA protein assay kit (PIERCE, U.S.A).

Twenty-five micrograms of protein from the tumour tissue and normal tissue from each patient was resolved on a 10% SDS polyacrylamide gels under reducing conditions and electrotransferred onto a PVDF membrane (PALL Gelman Laboratory, U.S.A). The membrane was blocked with 5% non-fat milk in TBS, containing 0.05% Tween-20 (TBS-Tween) for 1 hour before being incubated with monoclonal antibodies specific for survivin (D-8, dilution 1:500, Santa Cruz Biotechnology, USA) and p53 (DO7, dilution 1:1000, Novocastra, UK) for 1 hour at room temperature (RT), and with horseradish peroxidase-conjugated goat anti-mouse IgG (Dako, U.S.A) for 1 hour at RT, respectively.

After extensive washing with TBS-Tween, immunoreactive protein was visualized by a chemiluminescence-based procedure, using the ECL Plus detection kit according to the manufacturerís protocol (Amersham, U.S.A).

Examination of total proteins loaded on polyacrylamide gels by coomassie blue staining

As an internal loading control, several studies generally use GAPDH (Glyceroldehyde-3-phosphate dehydrogenase) or β-actin protein which are classified as housekeeping gene (Molina et al., 1999; Konturek et al., 2001). However, accumulated evidence have suggested that the use of GAPDH should be avoided in experimental hypoxia, cell proliferation and carcinogenesis (Sturzenbaum and Kille, 2001) and the total actin content can vary with development, cell culture condition, pathologically and potentially between cells within tissue (Gately, 2000). Therefore, in this...
study the amount of total protein loaded onto polyacrylamide gel was examined by staining with coommassie blue to ensure equality of protein loaded into each lane.

This method can provide an estimate of total amount of protein in each sample loaded onto a gel by quantifying total protein present but not one of protein in a mixture of several. The polyacrylamide gel carrying separated protein was submerged into generous amount of coomassie Blue staining solution (0.025% Coomassie Brilliant Blue R250, 40% (v/v) methanol, 7% (v/v) acetic acid) and incubate at RT, overnight. Next morning, the stained gel was de-stained by replacing coomassie blue staining solution with the de-stained solution I (40% (v/v) methanol, 7% (v/v) acetic acid), and shaked slowly for 30 min. This removed the buck of the excess stain. The de-stained solution I was replaced with de-stained solution II (7% (v/v) acetic acid, 5% (v/v) methanol) and the solution was periodically until the gel background was clear.

Statistical analysis

The data were analyzed using SPSS for Window version 10 (SPSS, Inc., Chicago, IL, USA). The relationships between expression of survivin and p53 and clinicopathologic features were analyzed using chi-square test.

RESULTS

Expression of survivin and p53 in normal and tumour tissues

Western blot was performed on tissues obtained from patients with cancer of lung, liver and colorectal. Representative immunoblots of tissue samples, using a specific anti-survivin antibody and anti-p53 antibodies are shown in Figure 1. In normal tissues, survivin and p53 could only be detected in 3 out of 36 (8.3%) and 5 out of 36 (13.9%), respectively. In contrast, these two proteins were significantly increased in tumour tissues. p53 protein was accumulated in 19 out of 36 tumour tissues (52.7%) and survivin was detectable in 32 out of 36 (88.9%) cases. All 4 out of 36 cases which did not express a detectable level of survivin were hepatocellular carcinomas (Table 1).

Expression of survivin is significantly associated with tumour progression

Representative sections from each paraffin-embedded block of tumour tissue were stained with hematoxylin and eosin for morphological examination by a pathologist who was blinded to the results of the Western blot analysis. Association of survivin and p53 expression with clinicopathologic features of tumour tissues is shown in Table 2. The results showed that survivin overexpression, but not p53 accumulation, was significantly associated with the advanced stage (p=0.007) and metastasized tumours (p=0.025). No association between p53 accumulation and survivin overexpression (p=0.345) was observed.
Figure 1. Representative Western blots showing protein level of survivin and p53 in tumour tissues and their corresponding adjacent normal tissues. Tumour tissues significantly possess higher amount of p53 and survivin protein, despite about the same amount of total proteins was loaded into each lane as examined by coomassie blue staining. (T, tumour tissues; N, normal tissues).

DISCUSSION

Survivin is believed to play important roles in tumourigenesis due to the observation that it is highly expressed in cancers, but is undetectable in non-proliferating adult tissues. In this study, we investigated the expression level of the survivin in colorectal, lung and liver tumour tissues and compared the expression level with normal tissues from each organ, respectively. By using Western blot analysis, almost none of the normal tissues possessed a detectable level of survivin (8.3%). Survivin was found to be overexpressed in 63.6% of the hepatocellular carcinoma investigated in this study. Interestingly, all (100%) of the colorectal and non-small cell lung carcinoma from Thai cancer patients overexpressed survivin. The role of survivin in colorectal (Zhang et al., 2001; Lin et al., 2003; Chen et al., 2004; Ponnelle et al., 2005), non-small-cell lung (Monzo et al., 1999; Hofmann et al., 2002; Karczmarek-Borowska et al., 2005) and liver (Duan et al., 2005; Kannangai et al., 2005; Zhu et al., 2005) tumourigenesis is well documented at both protein and mRNA levels. Survivin has been reported to be overexpressed in around 50-70% of colorectal adenocarcinomas, 40-60% of hepatocellular carcinomas and around 65-100% of non-small cell lung carcinomas. Taken together, these results indicate an important role of survivin in colorectal, lung and liver tumourigenesis, which was further supported by our study with a higher percentage in colorectal and lung tumours (100%).
Table 1. Expression of survivin in relation to p53 accumulation.

<table>
<thead>
<tr>
<th>Survivin expression</th>
<th>p53 accumulation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>negative</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>positive</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 2. Expression of survivin and p53 in relation to the clinicopathologic features of tumour tissues (p<0.05 was considered significant).

<table>
<thead>
<tr>
<th>Pathological features</th>
<th>Survivin overexpression</th>
<th>p53 accumulation</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
<td>p value*</td>
<td>No. of cases</td>
</tr>
<tr>
<td>Type of cancer (total cases)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung (5)</td>
<td>5 (100%)</td>
<td>0.006</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Liver (11)</td>
<td>7 (63.6%)</td>
<td></td>
<td>7 (63.6%)</td>
</tr>
<tr>
<td>Colorectal (20)</td>
<td>20 (100%)</td>
<td></td>
<td>8 (40%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (15)</td>
<td>15 (100%)</td>
<td>0.073</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Male (21)</td>
<td>17 (80.9%)</td>
<td></td>
<td>13 (61.9%)</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤ 5 cm (22)</td>
<td>18 (81.8%)</td>
<td>0.091</td>
<td>12 (54.5%)</td>
</tr>
<tr>
<td>&gt; 5 cm (14)</td>
<td>14 (100%)</td>
<td></td>
<td>7 (50%)</td>
</tr>
<tr>
<td>Tumour stage grouping</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Stage I (5)</td>
<td>4 (80%)</td>
<td>0.007</td>
<td>2 (40%)</td>
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<tr>
<td>Stage II (9)</td>
<td>8 (88.8%)</td>
<td></td>
<td>6 (66.6%)</td>
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<tr>
<td>Stage III (3)</td>
<td>1 (33.3%)</td>
<td></td>
<td>2 (66.6%)</td>
</tr>
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<td>Stage IV (19)</td>
<td>19 (100%)</td>
<td></td>
<td>9 (47.3%)</td>
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<td>Histological grading</td>
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<tr>
<td>Well (20)</td>
<td>16 (80%)</td>
<td>0.165</td>
<td>12 (60%)</td>
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<td>Moderately (11)</td>
<td>11 (100%)</td>
<td></td>
<td>3 (27.2%)</td>
</tr>
<tr>
<td>Poorly (5)</td>
<td>5 (100%)</td>
<td></td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
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<tr>
<td>No (17)</td>
<td>13 (76.4%)</td>
<td>0.025</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>Yes (19)</td>
<td>19 (100%)</td>
<td></td>
<td>9 (47.3%)</td>
</tr>
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*p-chi-square test

The underlying mechanisms of survivin expression in association with prognosis may partly be related to the processes involved in the regulation of apoptosis via survivin (Suzuki and Shiraki, 2001). In addition, it has been demonstrated that expression of survivin was decreased in cells in the G0/G1 phase and increased in the S and G2/M phase, indicating that this protein also has an important role, directly or indirectly, in cell proliferation (Beardmore et al., 2004). Therefore, survivin can potentially control both spindle checkpoint and apoptotic checkpoint. The overexpression of survivin in neoplasm may dismiss these checkpoints, thus allowing aberrant progression of transformed cells to mitosis.
Tumourigenesis is an extremely complicated course. Though it is known that genes have direct interaction \textit{in vitro}, whether they actually have interaction \textit{in vivo} is unknown. For that reason, although survivin overexpression has been linked to the loss of wild-type p53 function \textit{in vitro} (Hoffman et al., 2002; Mirza et al., 2002), it is not known whether this occurs in tumourigenesis. In addition, there are still some discrepancies about the underlying mechanisms of how p53 represses survivin gene transcription. While Hoffman et al., (2002) demonstrated that p53 suppressed the transcription of the survivin gene by binding to its promoter, Mirza et al., (2002) showed that in spite of the presence of two putative p53-binding sites in the survivin promoter, deletion and mutation analysis suggested that neither site was required for transcriptional repression of survivin expression. The authors also suggested that modification of chromatin structure within the promoter could be the molecular explanation for silencing of the survivin gene by wild-type p53.

In our study, survivin was overexpressed in almost all tumour tissues, whereas p53 was accumulated in approximately 50% of the tumours tested. In addition, there was no association between p53 and survivin positivity suggesting that they have no direct interaction \textit{in vivo}. Therefore, we propose that overexpression of survivin is an early event before the loss of p53 function during the transformation of normal cells into cancer cells and the overexpression of survivin is not a consequent of p53 dysfunction. In agreement with this hypothesis, it has been indicated in a colorectal tumour model, the most studied tumour model, that immunoreactivity of survivin significantly increased in the transition from adenoma with low dysplasia to high dysplasia (Kawasaki et al., 2001) whereas dysfunction of p53 has been proposed to take place during the conversion of late adenoma to carcinoma (Kinzler and Vogelstein, 1996). Therefore, we further propose that although p53 can directly and/or indirectly repress survivin gene transcription, overexpression of survivin, frequently seen in human tumours, may not entirely depend on the loss of p53 function.

Survivin is one of the few proteins which is differently expressed in tumour cells as compared to most normal cells. This characteristic enables it to be a potential marker of cancer as well as a potential target for cancer therapy. Many approaches have been made in order to down-regulate survivin, both \textit{in vivo} and \textit{in vitro}. One of the approaches is to block survivin expression by anti-sense techniques. Many research groups have synthesized anti-sense drugs targeting survivin and successfully demonstrated their growth-inhibition effect (Carter et al., 2003; Li et al., 2006). Survivin expression can also be inhibited by ribozyme. Survivin-specific mRNA undergoes cleavage at 3’end due to ribozyme treatment, resulting in inhibition of translation and consequent retardation of tumour growth (Pennati et al., 2002; 2003; 2004; Choi et al., 2003). The observations that overexpression of survivin which occurred at early stage and was frequent in Thai tumour tissues as shown in our study indicated that these cancer therapy approaches, in combination with proper chemotherapy and radiotherapy, should be very promising for Thai cancer patients.
ACKNOWLEDGEMENTS

This work was financially sponsored by the National Center for Genetic Engineering and Biotechnology (BIOTEC) which is part of the National Science and Technology Development Agency (NSTDA) of Thailand.

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