Differential SIRT1 Expression in Hepatocellular Carcinomas and Cholangiocarcinoma of the Liver

Redha Al-Bahrani¹,*, Dominika Tuertcher²,*, Samar Zailaie¹,*, Yasser Abuetabh¹, Seishi Nagamori³, Nikolas Zetouni¹, Wesam Bahitham¹, and Consolato Sergi¹,²

¹Department of Lab. Medicine and Pathology, University of Alberta, Edmonton, Canada, 2 Institute of Pathology, Medical University of Innsbruck, Innsbruck, Austria

Abstract. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are two major liver malignancies. Although some phenotypic overlap is known, HCC and CCA are usually different with regard to etiology, histology, and prognosis. Gene expression and deacetylase activity of the class III histone deacetylase SIRT1 are up-regulated in cancer cells due to oncogene overexpression or loss of function of tumor suppressor genes. SIRT1 may play a critical role in tumor initiation, progression, and drug resistance by blocking senescence and apoptosis, and promoting cell growth and angiogenesis, but pleiotropic effects (synchronous or metachronous anti-proliferation and anti-apoptotic mechanisms) have been suggested in some cancers. Our aim was to investigate the expression of SIRT1 in liver epithelial malignancies. Thirty carcinomas of the liver, including 16 HCC and 14 CCA cases, were investigated by immunohistochemistry using monoclonal antibodies against SIRT1 and p53. Western blot analysis (WBA) was carried out for expression of SIRT1 in three CCA cell lines, one HCC cell line, and one cell line of Papova-immortalized normal hepatocytes. An expression of SIRT1 was found in 11 of 16 (68.75%) HCC and in 5 of 14 (35.71%) CCA. Moreover, we found an expression of p53 in 8 out of 16 (50%) HCC and 13 out of 14 (92.86%) CCA. WBA showed expression of SIRT1 in all cell lines studied, although a stronger signal was seen in the HCC cell line. Immunohistochemical data did not correlate to clinical stage or other clinical or histopathological parameters. Sirtuin 1 is a phylogenetically-conserved family of deacetylases and our data seem to indicate that (1) pleiotropic effects may be present in hepatic epithelial malignancies, and (2) there is no specificity of SIRT1 for either HCC or CCA.

Introduction

Hepatocellular carcinoma (HCC) is an aggressive primary tumor that accounts for 80-90% of primary liver cancer and is the 5th-most common cancer worldwide [1,2]. Due to its high mortality rate, HCC is the third most fatal malignant neoplasm and annually causes more than 500,000 deaths worldwide [3]. Several studies have reported risk factors that may play a central role in its development and progression, and cirrhosis is the major cause in more than 80% of cases [4]. Cholangiocarcinoma (CCA) is a malignant neoplasm that grows slowly from the epithelium of the intra- or extrahepatic biliary system. CCA is often clinically silent, making it difficult to diagnose until an advanced stage [5-7]. CCA is the second most serious primary liver cancer after HCC worldwide. Its etiology is still unclear, although several reports suggest that the highest incidence and mortality rate are in Korea, Thailand, and China, as well as in communities with people from those countries. CCA has been linked to underlying infection and inflammation following liver flukes (Clonorchis sinensis and Opisthorchis viverrini) infestation. Moreover, 7-40% of patients with primary sclerosis cholangitis (PSC) may develop CCA [8,9].

P53 is known as the guardian of the genome, or the gatekeeper of cell proliferation. It has been reported that about 50% of human cancers harbor a mutation of TP53 gene [11]. P53 becomes active in response to cellular stress factors including hypoxia, UV irradiation, DNA damage, and/or activated oncogene [12-14]. Activated p53 proteins aid in the repair of DNA errors, helping to eliminate the risk of abnormal cell proliferation. In fact, p53 induces a transient cell cycle arrest, allowing DNA to
be repaired or inducing apoptosis [14]. Mammalian sirtuin-1 (SIRT1) is a NAD (nicotinamide adenine dinucleotide)-dependent deacetylase and a homolog to Sir2 (silent information regulator 2), which is responsible for prolonged lifespan in yeasts, nematodes, and flies [17]. SIRT1 has been shown to play an essential role in anti-aging by probably keeping chromatin silent through the deacetylation of the histone groups as suggested from some authors [17]. SIRT1 regulates other cellular functions, like cell differentiation, proliferation, and apoptosis [18]. SIRT1 is capable of deacetylasing histone and non-histone proteins including p53, NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells), and PPAR (peroxisome proliferator-activated receptors) [19-21]. SIRT1 has a controversial role in cancer development: it has been shown to be both a tumor promoter and a tumor suppressor gene, indicating pleiotropic effects [22-23]. On the other hand, recent studies have revealed the significance of SIRT1 as a diagnostic biomarker in cancer. For instance, Zhang et al studied the expression of SIRT1 in Barrett’s esophagus (BE) and Barrett’s-related dysplasia. They reported that a high expression of SIRT1 is associated with high-grade BE dysplasia and invasive carcinoma [24].

The transforming growth factor beta (TGF-β) superfamily regulates both human and animal embryonic development [25]. Failure or dysregulation of the TGF-β superfamily has been found to be involved in several diseases. Bone morphogenic proteins (BMPs) are one of the TGF-β isoforms that is essential in embryonic development events such as proliferation, differentiation, and morphogenesis of organs [26]. BMP4 is crucial in embryonic development and early development and differentiation of ectodermal tissue [27]. It has been reported that BMP4 is responsible for cellular senescence by regulating premature cellular senescence in vivo and in vitro [28]. Recently, it has been found that BMP4 acts as a negative regulator for the cellular senescence molecules (including p53 and p21) in retinal pigment epithelium (RPE) via both classical
(Smad1, 5 and 8) and non-classical (p35) pathways [28]. Indeed, in our previous study we found an overexpression of BMP4 in CCA, suggesting that BMP4 might regulate cellular senescence through p53 and p21 [29].

In this study, we investigated the differential expression of SIRT1 and the correlation between SIRT1 and p53 in both HCC and CCA.

Materials and Methods

Tissue Samples. Thirty tissue specimens of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) were obtained from archive files after approval by the Human Research Ethics Board (Pro00007657_ Molecular Pathology and Genetics of the Abnormalities of the Intrahepatic Biliary System and Pro00020274_ Twsg-1 Expression in Cancer). All of the samples were fixed in 4% buffered formaldehyde and routinely processed. Paraffin tissue blocks were sectioned at five μm and each section was stained with H&E. Consecutive sections were used for immunohistochemistry.

Cell culture. CCA cell lines OZ, Huh-28, and HuCCT1 were obtained from the cell culture bank of the Japan Health Sciences Foundation. All cell lines were grown as monolayer cultures in their appropriate media as previously described [30]. Furthermore, a human HCC cell line (HepG2) and a Papova virus-immortalised cell line of normal human hepatocytes (THLE-3) were purchased from American Type Cultural Collection (Manassas, VA, USA). All cell lines were cultured in their appropriate media according to the provider’s instructions.

Immunohistochemistry and Scoring. Formalin-fixed, paraffin-embedded samples were used in the study. Sections were cut at 5-6 μm. For immunohistochemistry, we used rabbit monoclonal antibody raised against SIRT1 (1:100 μl) according to the manufacturer’s recommendations (Abcam, Cambridge, MA, USA) and a rabbit monoclonal antibody raised against p53 (1:50 μl) according to the manufacturer’s recommendations (Dako, Vienna, Austria). Sections were deparaffinized in xylene and rehydrated through a series of graded alcohols, followed by incubation for 20 minutes in 3% hydrogen peroxidase to block endogenous peroxidase activity. Antigen retrieval was performed by heating in 10mM sodium citrate buffer (pH 6.0) for 15 minutes. Non-immunized goat serum was used to block non-specific protein binding for 60 minutes. Sections were incubated overnight at 4°C. SIRT1 and p53 primary antibodies were used separately, but in consecutive tissue sections. Tissue sections were given three washes of 5 minutes each with TTBS (Tris-buffered saline containing 0.1% Tween 20) and were incubated with the primary antibody, rabbit anti-goat immunoglobulin (IgG) for SIRT1, and p53 for 60 minutes before a 30-minute incubation with Avidin-Biotin Complex (ABC) (Vector Laboratories, Burlington, ON, Canada). The antibody complex was visualized with DAB Peroxidase Substrate (Dako, Carpinteria, CA, USA), and tissue sections were counterstained with Harris haematoxylin (Thermo Fisher Scientific Anatomical Pathology, Ottawa, ON, Canada). Negative controls (absence of primary antibody) and an internal positive control (a colorectal tumor section) were used.

Both semi-quantitative and quantitative methods were used. In particular, SIRT1-stained samples were scored by semi-quantitative methods, namely multiplying the percentage of positive tumor cells by the intensity rating according to a previous method validated for non-parametric evaluations [31]. Samples were scored by extent (0/none, 1/1-25%, 2/26-50%, 3/51-75%, 4/76-100% of the tumor cells being positive) and intensity of staining (0/negative, 1/weak, 2/moderate, 3/intense) [31]. A quantitative assessment was made for p53. Three observers (RAB, NZ, and CS) evaluated absolute p53, counting separately. Rates were averaged and supported by valid kappa scores. We used a clear ruler to measure the
diameter of a low-power field. The rate was calculated as the fraction of p53-positive tumor cells among all tumor cells in the magnification field.

Western Blot Analysis. Western blotting was performed on proteins electrophoretically transferred from SDS-PAGE (9%) onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare Inc., Baie d’Urfe, Quebec, Canada). After blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with monoclonal primary antibodies against SIRT1 (1:500μl) (Abcam, Cambridge, MA, USA) and β-actin (1:5000μl) (Cell Signaling Technology Inc., Danvers, MA, USA). The membranes were then incubated with horseradish peroxidase-labeled (HRP) rabbit anti-goat IgG for 60 minutes at room temperature. Detection was performed with an enhanced chemiluminescent substrate according to manufacturer’s instructions (Perkin-Elmer Inc., Waltham, MA, USA) and then the blots were exposed to Kodak X-ray film (Kodak Graphic Communications Company, Burnaby, BC, Canada). All experiments were performed in triplicate.

Statistical Analysis. The tumor cells were randomly selected and counted. The total number of counted cells in a determinate structure and the percentages of positive cells were presented as mean ±SD and SE. The Mann-Whitney test was used because of the use of two groups with paired data and a non-parametric platform. All p values were two-sided, and p values <0.05 indicated statistical significance. The statistical software used was SPSS Version 20 (IBM, Armonk, NY, USA).

Results

Immunohistochemistry. First we investigated SIRT1 expression in human liver cancer tissues using immunohistochemical staining. We found faint to moderate expression of SIRT1 in normal or non-tumorous hepatocytes surrounding the liver carcinoma in the tissue sections and absent to strong expression in the tissue sections of both HCC and CCA. Epithelial expression of SIRT1 was detected in 11 HCC cases (Figure 1). Epithelial SIRT1 expression was significantly higher in liver carcinoma than in normal hepatocytes. High expression was found in 11 of 16 cases (68.75%, SD±0.98), while negative expression of SIRT1 was noticed in five of 16 cases of HCC (31.25%). A score of 1 of SIRT1 expression was noticed in 4 of 16 cases (25%), a score of 2 in 6 (37.5%), and a score of 3 in one case (6.25%) (Figure 2). In contrast, immunohistochemical staining of CCA showed a moderate to strong expression of SIRT1 in the epithelial bile duct tumor. An epithelial expression was found in 5 of 14 cases (35.7%, SD±3.34) (Figure 1). Negative expression of SIRT1 was detected in 9 of 14 cases (64.28%). Moreover, when we scored SIRT1 in CCA, we found two cases with scores of 2 (14.28%), one case with score of 3 (7.1%), and only one case (7.1%) with a score of 12 (6.25%) (Figure 2). SIRT1 was expressed in both HCC and CCA. However, statistically there was no significance comparing both tumors (p=0.473).

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>SIRT1 Score</th>
<th>P53 Variation per cent Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>21.27%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2.1%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>27.37%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>34.48%</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>34%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>14.28%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>12.2%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>CCA</td>
<td>0</td>
<td>5.9%</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>29%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>15.9%</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>12.37%</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>1.28%</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>1.3%</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>13.59%</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>24.18%</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>HCC</td>
<td>0</td>
<td>3.4%</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>16.66%</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>0</td>
<td>66.66%</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>2</td>
<td>8.19%</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
We also investigated the expression of p53 for a possible correlation with SIRT1 in consecutive tissue sections. Immunohistochemical staining of HCC cases showed 62.5% p53 expression, while CCA showed 85.7% p53 expression. We also found that the expression of p53 was significantly related to the absence or low expression of SIRT1 in both tumors ($p=0.028$) (Table 1).

**Western Blot Analysis.** We detected SIRT1 in all three CCA cell lines, although the expression of SIRT1 was faint in all CCA cell lines (Figure 3). Normal hepatocytes cell line (THLE-3) also showed an expression of SIRT1. HepG2 had a band of strong intensity compared to CCA and normal hepatocytes cell lines. The β-actin signal was present at comparable levels in all cell lines examined.

**Discussion**

Sirtuin enzymes are a conserved family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-ribosyltransferases that regulate lifespan in lower organisms and mediate numerous processes in mammals [32-36]. HCC and CCA are the most common primary liver malignancies with poor prognoses. Recent studies have shown that the NAD-dependent deacetylase sirtuin-1 (SIRT1) plays an essential role in cell differentiation, proliferation, and apoptosis [37]. However, the role of SIRT1 in liver tumors remains poorly understood. In this study, we investigated the expression pattern of SIRT1 in the primary liver tumors CCA and HCC using immunohistochemistry and western blotting technique. Our results indicate that overexpression of SIRT1 is correlated with cell proliferation and progression of both tumors [38,39]. By immunohistochemistry, we found epithelial overexpression of SIRT1 in both tumors.

Pleiotropy occurs when multiple effects are associated with a single gene. Pleiotropic effects are not unusual in carcinogenesis. Cathepsin D (CD) is a part of the lysosomal degradation program and has been associated with some gastrointestinal tumors. In fact, CD is able to both activate and inactivate cryptic anti-angiogenic factors including angiostatin, 16k prolactin, and endostatin [40]. Activating Transcription Factor-3 (ATF3) is another interesting example of pleiotropy that has been demonstrated to have either oncogenic or tumor-suppressive effects on different types of cancer [41-46]. ATF3 is a member of a large family known as the ATF/cyclic AMP response element binding family. Ishiquro T et al. (2001) have demonstrated that overexpression of ATF3 has an oncogenic effect in colon cancer by promoting its invasion [44], but Bottone FG Jr. et al. (2005) have reported that overexpression of ATF3 in colorectal cancer results in an anti-invasion activity [45]. Recently, Hackl C. et al (2010) have argued that down-regulation of ATF3 promoted both colon-cancer cell migration and cancer growth [46]. Similarly, it has been suggested that SIRT1 has pleiotropic effects (anti-proliferation and anti-apoptotic mechanisms) in different types of malignant tumors from the pancreas, prostatic gland, and lower gastrointestinal tract [38,39,47]. It has been reported that SIRT1 downregulates the tumor suppressor p53 via deacetylation [22]. Cancer cells have increased rates of glycolysis due to irreversible defects in mitochondrial
oxidative phosphorylation (OXPHOS). This phenomenon known as the Warburg effect, in which glucose is converted to pyruvate, and pyruvate is then converted to lactate by NADH rather than transported into mitochondria [48]. It has been recently indicated that SIRT1 and PGC-1α, a transcription coactivator, are involved in mitochondrial biogenesis and regulate the expression of mitochondrial genes in the nuclear and mitochondrial compartments [49]. A recent study by Bernier M. et al. (2011) indicated that SIRT1 might suppress mitochondrial activity via modulation of STAT3, an important transcription factor regulated by SIRT1, deacetylation, and deactivation of PGC-1α transcription using SIRT1 null cell lines [50]. It has been recently demonstrated that SIRT1 selectively stimulates activity of the transcription factor hypoxia-inducible factor 2 alpha (HIF-2α) during hypoxia. HIF-2α is able to suppress the supply of fatty acids and pyruvate for mitochondrial metabolism [51]. Thus, SIRT1, PGC-1α, and HIF-2α may act together to reprogram the metabolism of cancer cells. P53 is an important regulator of metabolic pathways and contributes to the regulation of glycolysis, oxidative phosphorylation, glutaminolysis, insulin sensitivity, nucleotide biosynthesis, mitochondrial integrity, fatty-acid oxidation, and antioxidant response [52], p53 was the first nonhistone target identified for SIRT1 [53]. The effect of SIRT1 on p53-mediated metabolic alterations has yet to be investigated. Thus, our investigations are intended to elucidate SIRT1 role in liver cancer. Interestingly, we had an almost 2 in 3 rate of nuclear detection of p53 in all cases with an absence or low expression of SIRT1. Our data are consistent with Chen J et al’s data suggesting that SIRT1 deacetylates p53 in liver tumors, which inactivates the cell cycle arrest mechanism [54]. Furthermore, it has been proposed that BMP4 regulates cellular senescence in the tumor suppressors p53, p21CIP1 and p27KIP1 in retinoblastoma, although cell proliferation was not related to BMP4 induction [54]. Up-regulation of BMP4 expression is linked to inhibition of NFkB [55], and deacetylated NFkB via SIRT1 might activate and increase the expression of BMP4, which down-regulates p53 and inhibits the cell cycle arrest. In fact, in our previous study, we demonstrated that BMP4 is upregulated in CCA [29]. Our findings suggest that there might be a correlation between NAD-dependent deacetylating SIRT1, BMP4, and p53 in liver cancer development, warranting further investigation.

In conclusion, sirtuin enzymes are a phylogenetically-conserved family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-riboseyltransferases. Our data emphasized the absence of mono-directional expression in a single epithelial malignancy and the pleiotropic effects of SIRT1 in both liver epithelial malignancies. Pleiotropic effects during cancer development in the liver may be at the root of mitochondrial dysregulation in the cellular metabolism of malignant hepatocytes and cholangiocytes.

Acknowledgements

The authors are very grateful to the Saudi Cultural Bureau, Ottawa, ON, Canada, for the financial support of RA, SZ, YA, and WB, Laboratory Medicine and Pathology at the University of Alberta, Edmonton, AB, Canada.

References