Expression of the serum response factor in hepatocellular carcinoma: Implications for epithelial-mesenchymal transition

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Received July 9, 2007; Accepted September 4, 2007

Abstract. The acquisition of a migratory and invasive phenotype by cells of epithelial origin is associated with a gain of mesenchymal characteristics concomitant with a loss of the epithelial phenotype, a phenomenon referred to as epithelial-mesenchymal transition (EMT). Vimentin, a cytoplasmic intermediate filament, is characteristic of mesenchymal cells and is usually not expressed in epithelial cells. Increased expression of vimentin in carcinomas correlates with parameters of malignant potential such as tumor grade and tumor invasion. Serum response factor (SRF) regulates transcription of immediate early genes and triggers proliferation, migration and differentiation in several types of cells. However, the role of SRF in hepatocellular carcinoma (HCC) has not been explored. The aims of this study were to evaluate the expression of SRF and to assess a functional role of SRF in HCC. First, we examined the expression of SRF in 55 human specimens of HCC and four different HCC cell lines, including a sarcomatoid HCC cell line. We also examined the role of SRF in HCC by transfection of an SRF expression plasmid into a HCC cell line. SRF was expressed in 13 of 55 cases of HCC. SRF was predominantly expressed in HCC cells, with intense labeling in the nucleus. No staining was observed in hepatocytes of normal and cirrhotic liver outside the tumor. SRF was significantly up-regulated in high grade HCC, especially in sarcomatoid HCC. Overexpression of SRF in hepatocellular carcinoma cells accelerates migration and invasion with subsequent acquisition of mesenchymal phenotype by expression of a mesenchymal marker (vimentin) and activation of immediate early genes. We propose for the first time that the expression of SRF in HCC cells associated with EMT may play an important role in HCC progression. Thus, functional antagonism of SRF will provide an additional therapeutic approach by controlling tumor cell invasion and metastasis.

Introduction

Epithelial cells represent the most important cell type in malignant tumor formation and carcinomas (malignant epithelial tumors) comprise >90% of human malignancies. Tumor progression toward the malignant phenotype requires the loss of the epithelial phenotype and the acquisition of a mesenchymal one, a re-programming known as an epithelial-mesenchymal transition (EMT) (1,2). The EMT is hallmarkd by an expression of vimentin in epithelial cells and is accompanied by loss of tight cell-cell adhesion and acquisition of a fibroblastic morphology (3). Vimentin, a cytoplasmic intermediate filament, is characteristic of mesenchymal cells and it is not usually expressed in epithelial cells. The atypical expression of vimentin in epithelial cancer cells might be associated with local invasiveness and metastasis potential. The aberrant overexpression of vimentin and its relation to tumor metastasis have been reported for hepatocellular carcinoma (HCC) (4), breast carcinoma (5), cervical carcinoma (6), and prostate carcinoma (7).

Serum response factor (SRF) is a transcription factor of the MADS box family (8,9). DNA binding sites for SRF [serum response elements (SREs)] have been found in the promoters of ~50 different genes so far, including immediate early genes like c-fos and Egr-1 and muscle-specific genes (10-12). SRF has been shown to be implicated in cellular processes such as immediate early and tissue-specific gene expression, cell proliferation, differentiation, and the apoptotic pathway (13-17).

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Key words: hepatocellular carcinoma, serum response factor, epithelial-mesenchymal transition, vimentin

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy and is responsible for more than one million deaths annually worldwide. The poor prognosis of HCC is commonly associated with recurrence and metastasis. Cell motility is an important factor in the progression and metastasis of HCC. At the molecular level, a number of different proteins, including adhesion molecules and growth factors have been shown to be involved in the regulation of cell migration (18-20). However, the molecular mechanisms of HCC metastasis remain unclear. A recent study reported that SRF plays a role in tumor progression, specifically in
the mesenchymal transition of epithelial tumor cells (21). However, the expression of SRF in HCC and its role have not been investigated.

In the present study, we examined: i) SRF expression and its relationship to tumor differentiation, tumor size, and the proliferating cell nuclear antigen labeling index (PCNA-LI) in surgical specimen of human HCCs; ii) whether over-expression of SRF by transfection of an SRF expression plasmid into HCC cell line accelerates the EMT and vimentin expression; and iii) the role of SRF in migration and invasion of HCC cells.

Materials and methods

Patients and specimens. This study was approved by the Human Ethics Committee of Chonbuk National University Medical School. We retrospectively studied HCC specimens that were obtained from 55 patients who underwent surgical resection between 1999 and 2002 at the Chonbuk National University Hospital. Of the 55 patients with HCC, 45 were men and 10 were women. The mean age of patients was 55 years (age range: 13-79 years). HBV and HCV serologies were negative in 15 and 10 patients, respectively.

HCC cell lines. The human HCC cell lines HLE and HuH-7 were purchased from the Health Science Research Resources Bank (Osaka, Japan) and HepG2 was obtained from the American Type Culture Collection (Manassas, VA). In addition, we used the sarcomatoid HCC cell line, designated as SH-J1, which was established in our laboratory (22). The HepG2, HLE and HuH-7 cell lines were cultured according to the recommendations of the cell banks.

Preparation of nuclear protein. HCC cells were lysed in a lysis buffer (0.5% NP-40, 20 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 2 mM MgCl2) containing 0.1% SDS, 25 μg/ml aprotinin, and 25 μg/ml leupeptin for 20 min at 4°C. After centrifugation at 500 x g for 5 min, supernatants were collected and used as cytoplasmic proteins. The nuclear pellets were washed in lysis buffer lacking NP-40, and then resuspended. Nuclear pellets were resuspended by vortexing in 1 pellet volume of a nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, and 2.3 μg/ml aprotinin, leupeptin, and pepstatin). Protein concentrations were determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of denatured nuclear protein (30 μg) were resolved on SDS-PAGE gels and used for Western blotting.

Western blotting. Total protein was isolated by homogenization of cultured cells in a lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet p-40) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM sodium fluoride and 1 mM sodium orthovanadate. Western blotting was performed as described previously (23). SRF and vimentin levels were first examined in the 4 different human HCC cell lines (HepG2, HLE, HuH-7 and SH-J1). Since SRF is known to regulate expression of genes such as c-Fos and Egr-1, the expression levels of c-Fos, Egr-1 and SRF were evaluated in an SRF transfected HLE cell line. To examine the effect of SRF over-expression on vimentin expression in HCC cells, we probed with an antibody against vimentin in SRF transfected cells. The signal of the bound antibodies was visualized by chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). The membranes were stripped and reprobed with a monoclonal anti-ß-actin antibody (Sigma, St. Louis, MO, USA) as a control for the protein loading and transfer. The U2OS, osteosarcoma cell, was used as a positive control for the expression of vimentin. Quantification of the data was performed using ImageQuant software (Molecular Dynamics, Piscataway, NJ, USA). Each signal was normalized against the corresponding ß-actin signal.

In vitro assays of cell migration and invasion. The cell migration assay was performed using Transwell chambers as described previously (24). The HLE cells transfected with either the SRF expression plasmid or the vector control plasmid were serum starved for 48 h. Transwell chambers equipped with 8 μm Matrigel-coated filters (24-well format) (Becton-Dickinson, Franklin Lakes, NJ, USA) were rehydrated, and 4x10^4 cells in 400 μl of serum free medium were
seeded in the upper chamber. DMEM containing 2% or 5% FBS was used in the lower chamber. After incubation for 12-22 h at 37˚C, cells on the upper surface of the filter were mechanically removed with a cotton swab. The filters were fixed and stained using a Diff-Quick staining kit (Dade Behring, Newark, DE, USA). Cells on the lower surface were counted under a microscope. The cells were counted under a light microscope at x100 magnification in five randomly selected fields per well. The migration assay was also performed with Transwell chambers, in which the filters were not coated with Matrigel using the same procedure.

Statistical analysis. Correlations between the expressions of SRF in the cancer cells and the tumor differentiation were tested by use of the \( \chi^2 \) test. The comparisons between the expressions of SRF and PCNA-LI and tumor size were performed using the Student's t-test. The results are presented as means ± SE. P-values <0.05 were considered as significant.

Results

Relationship between the SRF expression and tumor differentiation. We first evaluated the expression of the SRF in 55 pairs of HCC tissue and the corresponding non-tumorous liver tissue by immunohistochemistry. A representative immunostained specimen is shown in Fig. 1. Positive staining for SRF was found in 13 of 55 (24%) HCC specimens, but not in the specimens of hepatocytes of both normal and cirrhotic liver outside the tumors. In addition to the tumor cells, SRF expression was also observed in the smooth muscle cells of blood vessels and in stromal cells as well as in some of endothelial cells of both HCC and non-malignant liver tissue (Fig. 1). Nuclear localization of SRF in cancer cells was significantly increased in poorly differentiated HCC specimens (P<0.001). Nuclear localization of SRF in cancer cells was observed exclusively in Edmonson grade III or IV HCC specimens including 2 sarcomatoid HCCs (Table I). The PCNA-LI of the HCC cells ranged from 9 to 79% with an average of 40.9%. The mean size of the tumor was 4.3 cm (size range: 1.0-15 cm). The SRF positive tumors showed a higher PCNA-LI and larger tumor size than the SRF negative tumors; however, there was no significant difference between the PCNA-LI of tumor cells and the tumor size (Table II).

Table I. Correlation between the expression of SRF and tumor differentiation in HCC.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 1 (n=6)</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
</tr>
<tr>
<td>SRF positive</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SRF negative</td>
<td>6 (100)</td>
</tr>
</tbody>
</table>

SRF, serum response factor, \( \chi^2 \) test (P=0.000869).

Figure 1. Immunohistochemical staining for SRF in HCC. (A) Nuclear expression of SRF in smooth muscle cells and endothelial cells of blood vessel. Internal positive control (x10). (B) Low grade HCC (Grade II) showed negative staining. Note the high expression of SRF in the smooth muscle cells of unpaired arteries. (C) High grade HCC (Grade III) and (D) sarcomatoid HCC (Grade IV) showed diffuse strong nuclear staining for SRF on the nucleus of HCC cells (x20).
HCC and mesenchymal cells, we examined the SRF and vimentin levels in four different human HCC cell lines and a U2OS osteosarcoma cell line. Consistent with the immunohistochemistry analyses, the expression levels of SRF in all epithelial cell lines (HepG2, HLE and HuH-7) were very low whereas the expression in SHJ1 cell line having undergone EMT was substantially high (Fig. 2A). Furthermore, vimentin expression was observed only in the SRF expressing sarcomatoid HCC cell line (Fig. 2B).

Transfection of SRF cDNA promotes expression of immediate early genes and vimentin. Next, we analyzed the expression levels of SRF itself and the SRF target genes, c-Fos and Egr-1, in the SRF-transfected HCC cells by Western blotting. In the SRF-transfected HLE cells, SRF, c-Fos and Egr-1 were increased by 3-, 4- and 31-fold, respectively (Fig. 3A). Furthermore, the representative mesenchymal marker, vimentin, was highly expressed in the SRF-transfected HLE cell line (Fig. 3B). Together, these findings suggest that vimentin expression is closely associated with SRF expression in HCC cells. Since it has been well established that the E-cadherin/catenin complex plays a crucial role in tumor progression and metastasis, we also examined the expression level of E-cadherin and β-catenin in the SRF transfected HCC cells. Overexpression of SRF in HCC cells decreased the expression level of E-cadherin, however it did not alter the expression level of β-catenin (Fig. 3C).

**Table II. Correlation between the expression of SRF and the PCNA-LI and the tumor size in HCC.**

<table>
<thead>
<tr>
<th></th>
<th>No. of cases</th>
<th>PCNA-LI (%)</th>
<th>Tumor size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> SRF positive</td>
<td>13</td>
<td>41.46±4.52</td>
<td>4.45±0.67</td>
</tr>
<tr>
<td>SRF negative</td>
<td>42</td>
<td>40.79±2.36</td>
<td>4.25±0.46</td>
</tr>
</tbody>
</table>

Student's t-test, P=0.891.

**B.**

<table>
<thead>
<tr>
<th></th>
<th>No. of cases</th>
<th>Tumor size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRF positive</td>
<td>13</td>
<td>4.45±0.67</td>
</tr>
<tr>
<td>SRF negative</td>
<td>42</td>
<td>4.25±0.46</td>
</tr>
</tbody>
</table>

Student's t-test, P=0.552.

**Figure 2.** Western blot analysis of SRF and vimentin in hepatocellular carcinoma cell lines. (A) HepG2, HLE and HuH-7 cell lines showed minimal expression of SRF while the sarcomatoid, SHJ1 cell line showed strong expression of SRF. (B) Vimentin expression was only observed in the SRF expressing, sarcomatoid HCC cell line (B).

**Figure 3.** (A) Enhanced expression of SRF, c-Fos and Egr-1 proteins in HLE cells transfected with the SRF gene. (B) Western blot analysis showed a marked increase in the protein level of vimentin in the SRF-transfected HLE cells. (C) Western blot analysis showed that overexpression of SRF in HLE cells decreased the expression level of E-cadherin.
expression of SRF in HLE cells increased cell migration by 2.2-fold (Fig. 4A) compared to that of the control. Overexpression of SRF also increased cell invasion by 2.5 to 2.7-fold when compared to that of the control (Fig. 4B).

Discussion

This study demonstrated for the first time the following: i) expression of SRF in HCC cells is correlated with cell de-differentiation; ii) epithelial HCC cell lines show minimal expression of SRF, while the SHJ1 cell line having undergone EMT exhibits high expression of SRF and vimentin; iii) overexpression of SRF in HCC cells promotes the expression of immediate early genes (c-Fos and Egr-1) and vimentin; iv) overexpression of SRF in HCC cells decreases the expression of E-cadherin; and v) overexpression of SRF in HCC cells promotes cell migration and invasion. These findings clearly indicate the important role of SRF in HCC progression, specifically at the EMT of carcinoma cells. In addition, overexpression of SRF is associated with enhanced cell motility and invasiveness of HCC cells.

Most cancer deaths are due to the spread of tumor cells resistant to conventional therapies (25). Metastatic cells acquire genetic and epigenetic changes that cause their aggressive phenotypes. The acquisition of increased motility and invasiveness is essential for metastasis (25). Epithelial-to-mesenchymal transition (EMT), a switch of polarized epithelial cells to a migratory fibroblastoid phenotype, is increasingly considered as an important event during malignant epithelial tumor progression and metastasis (1,2). Classes of molecules that change in expression, distribution, and/or function during EMT and that are causally involved include growth factors, molecules of the cell-to-cell adhesion axis, cytoskeletal modulators, and extracellular proteases (26).

The classic EMT is hallmarked by a change from cytokeratin expression to vimentin expression and is accompanied by a loss of tight cell-cell adhesion and the acquisition of spindle cell morphology (3-7). Clinically, vimentin-expressing spindle shaped tumor cells sometimes exist in advanced HCC nodules, and this phenomenon is regarded as the 'sarcomatous change'of HCC (27). Sarcomatoid HCC has a worse prognosis than ordinary HCC and is associated with extensive lymph node metastasis (28,29). In this study, the human epithelial HCC cell lines, HLE, HuH-7, and HepG2 cells did not express vimentin and showed minimal expression of SRF. However, the sarcomatoid HCC cell line, designated as SH-J1, highly expressed vimentin and SRF. Furthermore, expression of SRF in HCC cancer cells was significantly associated with tumor dedifferentiation in resected HCC specimens as seen in our study. Together with the fact that vimentin-positive carcinomas are mostly high grade carcinomas (5-7), our findings suggest that aberrant SRF expression in HCC cancer cells leads to tumor progression through expression of vimentin, a mesenchymal marker. In agreement with our findings, a previous study has demonstrated that expression of SRF is up-regulated and highly active in tumor cells that have undergone EMT and have acquired a mesenchymal phenotype (21).

The immediate early gene c-fos is one of the SRF targets that can be activated by a variety of growth factors and mitogens through several different signaling pathways (30). In this study, we showed that expression of SRF in HCC cells is closely associated with vimentin expression. Our results also demonstrated that transfection of the SRF gene in HCC cells induces expression of c-Fos, one of the target genes of the SRF. Mejlvang and colleagues have reported that expression of c-Fos in mammary epithelioid adenocarcinoma cells (MT1TC1) leads to prominent alterations in cell morphology, increased expression of mesenchymal
markers, vimentin and S100A4 (31). Although it is not clear whether the overexpression of SRF itself or the regulation of one or more of its target genes is responsible for progression to the mesenchymal phenotype, the above findings indicate a critical role of SRF in EMT and the progression of HCC.

In pathologic processes such as oncogenesis, EMT may endow cancer cells with reduced cell-cell adhesion and enhanced motility. Vimentin expression was interpreted by others as a sign of the EMT, reflecting the final step of tumor dedifferentiation, which is generally associated with a high potential of tumor invasion (2). Numerous studies have demonstrated that unexpected expression of vimentin in carcinomas correlates with parameters of malignant potential such as grade and survival incidence (1-7,32). It has also been shown that the aberrant expression of vimentin is significantly associated with metastasis in HCC (4,33). Here we showed that overexpression of the SRF in HCC cell stimulates motility and promotes invasiveness with concurrent overexpression of vimentin. Taken together, these findings suggest that overexpression of SRF increases cell motility and invasiveness through the expression of vimentin.

During EMT, the epithelial cell-specific adhesion molecule E-cadherin is known to be down-regulated. E-cadherin, which is considered to act as part of an invasion suppressor system, can also contribute individually to the invasive property and dedifferentiation of tumor cells when down-regulated (1,2,7,33-35). A recent study has shown that c-Fos, one of the target genes of the SRF, induces down-regulation of E-cadherin with abrogation of cell-cell adhesion, a strong β-catenin-independent proliferative response in MT1TC1 cells, and stimulation of cell motility, invasion and adhesion to different extracellular matrix proteins (31). Similarly, we found dramatic decrease in the expression level of E-cadherin when SRF was overexpressed in human HCC cells, suggesting that overexpression SRF can alter the E-cadherin/β-catenin complex. Further studies are needed to elucidate the SRF action mechanisms that regulate vimentin expression and the E-cadherin/β-catenin complex in HCC cells.

In conclusion, our results indicate that SRF may play an important role in tumor progression, specifically at the transition to an invasive metastatic stage of HCC. This study may also provide a rationale for a novel therapeutic approach for HCC through controlling tumor cell invasion and metastasis.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A06-0066-AA1018-06N1-00010A).

References


