Cancer Letters 375 (2016) 47–50

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Epithelial–mesenchymal transition-related genes are linked to aggressive local recurrence of hepatocellular carcinoma after radiofrequency ablation

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 18 February 2016
Accepted 19 February 2016

Keywords:
Hepatocellular carcinoma (HCC)
Radiofrequency ablation (RFA)
Epithelial–Mesenchymal Transition (EMT)
MicroRNA

ABSTRACT

We reported that poor prognoses of hepatocellular carcinoma (HCC) patients after radiofrequency ablation (RFA) are owing to up-regulation of expression of hypoxia-inducible factor-1 and epithelial cell adhesion molecule. We investigated aggressive progression in residual liver tumors (RLTs) after RFA to focus on expression of epithelial–mesenchymal transition (EMT)-related genes and miRNAs. Ten patients with recurrent HCC post-RFA who underwent hepatectomy (RFA group) and 78 patients with HCC without prior RFA (non-RFA group) were enrolled. We examined expression of transforming growth factor (TGF)-β, Twist, vimentin, and Snail-1 mRNAs in tumor tissues, and expression of miR-34a and miR-200c. Expression of TGF-β, Twist and Snail-1 in the RFA group was significantly higher than that in the non-RFA group ($P < 0.05$); vimentin expression in the RFA group was higher than that in the non-RFA group ($P = 0.07$). Expression of miR-200c and miR-34a in the RFA group was significantly lower than that in the non-RFA group (miR-200c: $P = 0.04$; miR-34a: $P < 0.01$). Increased expression of EMT markers through down-regulation of miRNA expression in RLTs after RFA may be related to poor prognoses of HCC patients with aggressive local recurrence after RFA.

Introduction

Radiofrequency ablation (RFA) is potential curative therapy for the early stages of primary hepatocellular carcinoma (HCC) [1]. RFA induces tumor necrosis with a low prevalence of complications, and is superior to percutaneous injection of ethanol in terms of tumor ablation [2]. However, several cases of rapid and aggressive recurrence of HCC after RFA have been reported [3–6]. Tajima et al. reported that, in patients with local recurrence after RFA, tumors showed greater growth and invasion of vascular structures, and less differentiation compared with tumors of patients who did not undergo RFA [6]. Recently, we reported that local recurrence of HCC after RFA showed an aggressive tumor phenotype and poor prognosis through enhanced expression of hypoxia-inducible factor (HIF)-1 and epithelial cell adhesion molecule (EpCAM) [7]. Hypoxia or HIF-1 overexpression-induced epithelial–mesenchymal transition (EMT) can increase migration of tumor cells [8].

EMT is a morphologic change of polarized epithelial cells from an epithelial phenotype to a mesenchymal fibroblastoid phenotype in a process characterized by dissolution of cell–cell junctions, cytoskeletal rearrangements, increased motility of cells, and synthesis of the extracellular matrix. These processes are accompanied by acquisition of mesenchymal properties (e.g., cell migration, invasive behavior), and EMT occurs during the invasion and metastasis of cancer cells [9]. Transforming growth factor (TGF)-β is, in general, accepted to be a major inducer of EMT [10]. TGF-β enhances the transcription factors of EMT (e.g., Snail, Twist) to activate expression of mesenchymal markers such as vimentin [11,12]. With regard to HCC cells after RFA, Yoshida et al. reported that insufficiently heat-treated HCC cells had enhanced malignant potential due to EMT changes [13].

MicroRNAs (miRNAs) are non-coding RNA molecules comprising approximately 21–23 nucleotides that regulate expression of target genes by interfering with their transcription or by inhibiting translation [14]. Several miRNAs regulate the genes associated with the development, proliferation, and apoptosis of cells, as well as stress responses and tumorigenesis [15,16]. The discovery that miRNA expression is often dysregulated in malignant tumors underpins their critical role, which is a matter of active investigation from a basic-science perspective and for its clinical usefulness [17]. Aberrant expression of several miRNAs is associated with multiple cancer types (including HCC) [18,19].

We hypothesized that expression of EMT-related genes is up-regulated in RFA patients, and that miRNAs are regulated by HCC

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http://dx.doi.org/10.1016/j.canlet.2016.02.041
0304-3835/

after RFA. We investigated whether EMT-associated genes are more prevalent in HCC when recurring after RFA compared with patients undergoing liver resection without previous RFA.

Materials and methods

Patients

Between January 2005 and December 2012, 88 HCC patients underwent hepatic resection at the Department of Surgery within Tokushima University Hospital (Tokushima, Japan). None of these patients had extra-hepatic metastatic tumors. Hepatectomy was indicated for local recurrence of HCC after RFA (n = 10, "RFA group") and for HCC without prior RFA (n = 78, "non-RFA group"). All 10 patients in the RFA group underwent RFA once. "Local recurrence after RFA" was defined as any findings of progression in the treated tumor on follow-up computed tomography or magnetic resonance imaging [7]. Experienced hepatologists carried out RFA for all patients in the RFA group. If local recurrence was diagnosed, they undertook additional RFA sessions whenever possible. Consequently, patients with recurrent tumors in whom additional RFA sessions were difficult to carry out were referred to the surgical department of our center [7].

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Expression of TGF-β, Twist, Snail-1, and vimentin in specimens of tumor tissue were determined using quantitative RT-PCR. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was done using a 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA). The following assays (assay identification number) were used: TGF-β (Hs00998133_m1), Twist (Hs00361186_m1), Snail-1 (Hs00195591_m1), and vimentin (Hs00185584_m1). Triplicate PCR reactions were obtained for each sample containing 10 μL of AB TaqMan Universal PCR Master Mix, 1 μL of the relevant 20x assay, 1 μL target cDNA and dH_2O under the following conditions: one cycle of 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. TaqMan Human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Endogenous Control (4326317E) was used as the control gene. Expression of mRNA described above was calculated as a ratio to that of GAPDH. For miRNA quantitative RT-PCR, total RNA was reverse-transcribed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems), and quantitative RT-PCR was undertaken using the Applied Biosystems 7500 real-time PCR system. TaqMan probes for human patients were used to assess expression of miRNA (has-miR-34a: ID: 000425; has-miR-200c: ID: 002300), and the endogenous control was RNU6B (ID: 001093).

Immunohistochemical staining

We used formalin-fixed, paraffin-embedded samples. Serial sections were cut at a thickness of 5 μm. Sections were dewaxed, deparaffinized in xylene, and rehydrated through a graded series of ethanol. For better retrieval of antigen, samples were boiled for 20 min in a microwave oven in citrate buffer (pH = 6.0). Endogenous peroxidases were blocked by treatment with 0.3% hydrogen peroxidase for 30 min. Samples were incubated in 5% goat serum for 60 min to prevent nonspecific binding to antigen. Slides were incubated with primary antibodies overnight at 4 °C. The following primary antibodies and dilutions were used: rabbit monoclonal antibody against Twist (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal antibody against vimentin (1:50; Dako, Santa Clara, CA, USA); rabbit monoclonal antibody against TGF-β (1:200; Abcam, Cambridge, UK); rabbit monoclonal antibody against Snail-1 (1:250; Abcam). A secondary peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins was applied for 60 min. Sections were developed in 3,3-diaminobenzidine and counterstained with Mayer's hematoxylin. Slides were dehydrated through a graded series of alcohol and coverslips were applied. Two investigators decided independently which cells had stained positive on each slide.

Statistical analyses

Statistical analyses were carried out using JMP v8.0.1 (SAS, Cary, NC, USA). Groups were compared in terms of perioperative parameters using the Mann–Whitney U-test. A P value of <0.05 was considered statistically significant.

Results

TGF-β expression in the RFA group (median value: 13.7) was significantly higher than that in the non-RFA group (median value: 4.7) (P < 0.001, Fig. 1a). Twist expression in the RFA group (median value: 116.7) was significantly higher than that in the non-RFA group (median value: 10.2) (P = 0.02, Fig. 1b). Snail-1 expression in the RFA group (median value: 12.1) was significantly higher than that in the non-RFA group (median value: 2.9) (P = 0.006, Fig. 1c). Vimentin expression in the RFA group (median value: 7.7) tended to be higher than that in the non-RFA group (median value: 4.0) (P = 0.07, Fig. 1d).

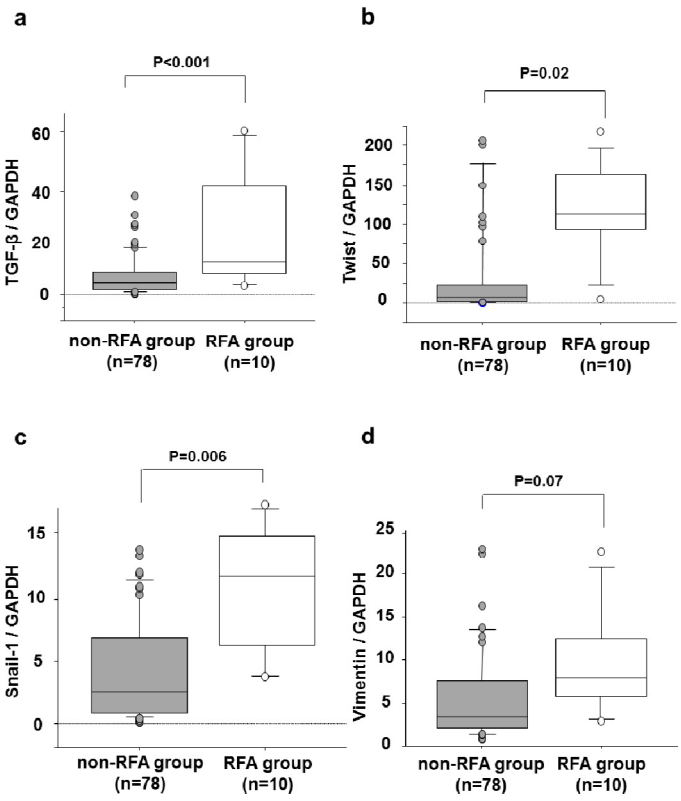

Fig. 1. mRNA expression of (a) TGF-β, (b) Twist, (c) Snail-1, and (d) vimentin in the RFA group and the non-RFA group.

TGF-β, Twist, Snail-1and vimentin exhibited positive immunohistochemical staining in RFA-group patients. Stains of Twist, Snail-1and vimentin were localized in tumor cells near necrotic areas (Fig. 2a–d).

miR-200c expression in the RFA group (median value: 1.1×10^{-2}) was significantly lower than that in the non-RFA group (median value: 1.9×10^{-2}) (P = 0.04, Fig. 3a). miR-34a expression in the RFA group (median value: 0.5×10^{-1}) was significantly lower than that in the non-RFA group (median value: 1.3×10^{-1}) (P = 0.009, Fig. 3b).

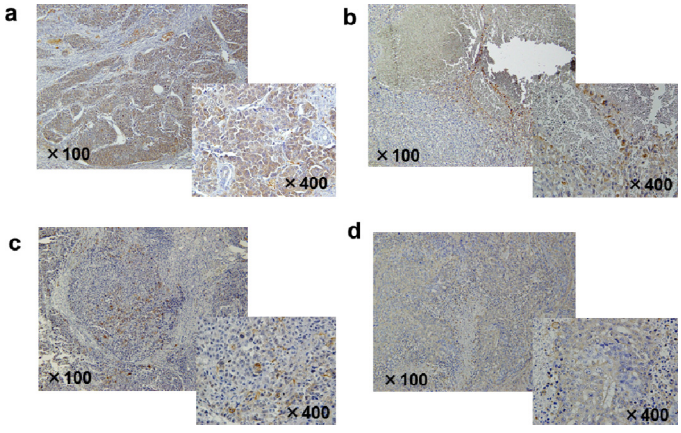

Fig. 2. Immunohistochemical expression of EMT markers in the RFA group ((a) TGF-β, (b) Twist, (c) Snail-1, and (d) vimentin).