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Positive association of long telomeres with the invasive capacity of hepatocellular carcinoma cells

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ABSTRACT

Invasion, the representative feature of malignant tumors, leads to an increase in mortality. The malignant liver tumor – hepatocellular carcinoma (HCC) – has an enhanced invasive capacity that results in increased patient mortality. Moreover, this enhanced invasive capacity is due to the up-regulation of invasion promoters such as zinc finger protein SNAI1 (Snail) and matrix metalloproteinases (MMPs), and the down-regulation of invasion suppressor molecules such as E-cadherin. Telomerase reverse transcriptase (TERT), which encodes the catalytic subunit of telomerase, is highly expressed in a variety of invasive cancers, including HCC. Telomerase activation induces telomere elongation, thereby leading to cell immortalization during malignant tumor progression. However, the relationship between telomere length and invasion is yet to be experimentally corroborated. In this paper, we revealed that invasive HCC cells passing through the Matrigel display significantly longer telomeres than non-invasive HCC cells. Moreover, we established a method that can distinguish and sort cells containing long telomeres and short telomeres. Using this system, we observed that the HCC cells containing long telomeres had a high-level expression of invasion-promoting genes and a low-level expression of invasion-suppressing E-cadherin. Furthermore, HCC cells containing long telomeres exhibited a higher invasive capacity than HCC cells containing short telomeres. Taken together, our findings suggest that long telomeres are positively associated with the invasive capacity of HCC cells and may be a potent target for malignant liver cancer treatment.

1. Introduction

The acquisition of cellular immortality is a common occurrence in diverse cancers [1,2]. Telomere elongation is a prerequisite for the immortalization of cancer cells [1,3]. Telomerase facilitate the lengthening of telomeres, which are comprised of tandem repeats that contain a high number of T and G nucleotides at the chromosomal end [3,4]. Telomerase is composed of core components such as telomerase reverse transcriptase (TERT) and telomerase RNA (TERC) and accessory components such as DKC1, GAR1, NHP2, and NOP10 [5]. Among the telomerase components, TERT and TERC are necessary and required for the telomerase reconstitution [6,7].

Invasion is a primary cause of increased mortality in cancer patients [8,9]. Invasion-associated genes have been an active area of cancer research for the development and validation of essential drugs for cancer therapy [10–12]. Zinc finger protein SNAI1 (Snail), matrix metalloproteinases (MMPs), and E-cadherin are known to be important invasion-associated genes [13–15]. Notably, Snail accelerates tumor cell invasion via not only the down-regulation of E-cadherin but also up-regulation of the MMP family in HCC cell lines. The acceleration of tumor cell invasion is associated with poor prognosis for HCC patients [9,16].

Generally, an invasion assay using a Matrigel matrix is the assay of choice for detecting cell invasion in a cell culture system [13,14,17]. A Matrigel utilizes molecules such as laminin, collagen IV, and entactin to mimic the extracellular matrix and growth factors such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and fibroblast growth factor (FGF) to enhance invasive ability [18]. The invasive capacity of cells can be determined by the number of cells that pass through the Matrigel matrix [16,18].

Telomerase is activated in 80–90% of tumors [19]. The canonical role of telomerase is to add telomeric DNA repeats for telomere elongation [20]. Moreover, the effects of silencing or overexpressing TERT on tumor cell invasion have been reported [20,21]. However, there have been no studies on the association between telomere length and invasion using a cell culture system. In this paper, we established a method for separating HCC cells containing long telomeres from HCC cells containing short telomeres. Furthermore,

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we detected higher expression of invasion-promoting genes, lower expression of invasion-suppressing gene, and a higher invasive capacity in HCC cells with long telomeres than those with short telomeres. Thus, we expect that telomere elongation is essential for tumor cell invasion.

2. Materials and methods

2.1. Cell culture

HCC cells (Huh7 and Hep3B cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene, Korea) supplemented with 10% fetal bovine serum (FBS, GenDEPOT, USA) and 1% antibiotics (Gibco, USA) in a humidified atmosphere at 37 °C and 5% CO_2 (NuAire Incubator, USA) [14].

2.2. Invasion assay and the collection of groups including non-invasive and invasive cells

Invasion assays were performed as described previously [14,17]. For the invasion assay, 5×10^4 cells were suspended in 300 μL of DMEM containing 0.1% bovine serum albumin (BSA) and loaded onto the upper compartment of a Transwell chamber that contained a Matrigel-coated polycarbonate membrane with a pore size of 8 μm pore size (BD Biosciences, USA). The cells that invaded through the Matrigel to the lower surface of the membrane were fixed using methanol and stained using crystal violet. The invaded cells were counted in three randomly selected microscopic fields of the fixed cells. The experiments were performed in triplicate.

For the collection of invasive and non-invasive cells, 5×10^5 cells were seeded in the upper compartment of a Transwell chamber that contained a Matrigel-coated polycarbonate membrane. Non-invasive cells were obtained from the cells in the Matrigel. The Matrigel was depolymerized by the addition of the BD Cell Recovery Solution (BD Biosciences). The invasive cells present on the lower surface of the membrane were obtained by the addition of Trypsin (Gibco).

2.3. Quantitative RT-PCR

Total RNA was isolated using the Ribozol RNA extraction reagent (Amresco) according to the manufacturer's protocol. Complementary DNA was synthesized from a total RNA of non-invasive and invasive cells using an AMV-Reverse Transcriptase kit (Promega). PCRs were performed using the QuantiTect SYBR Green PCR kit (Qiagen) and an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). The primer sequences for each gene are indicated below: for TERT, forward (5′-GGAGCAAGTTGCAAAGCATTG-3′) and reverse (5′-TCCCACGACGTAGTCCATGTT-3′); for TERC, forward (5′-GGTGGTGGCCATTTTTTGTC-3′) and reverse (5′-CTAGAATGAACGGTGGAAGGC-3′); for DKC1, forward (5′-TGAAAAGGACCACATGGTGA-3′) and reverse (5′-TAATCTTGGCCCCATAGCAG-3′); for NHP2, forward (5′-GGTCAACCAGAACCCCATC-3′) and reverse (5′-GTGTCTCCTGCCAAAACCAT-3′); for NOP10, forward (5′-TACCTCAACGAGCAGGGAGA-3′) and reverse (5′- GGTCATGAGCACCTTGAAGC-3′); for GAR1, forward (5′-CAAGGACCTCCAGAACGTGT-3′) and reverse (5′-CCACTTTTCCAATTTGTTCTTTG-3′); for MMP7, forward (5′-TGCTGACATCATGATTGGCTTT-3′) and reverse (5′-TCCTCA TCGAAGTGAGCATCTC-3′); for MMP9, forward (5′-ATGCGTGGAGAGTCGAAATCTC-3′) and reverse (5′-GGTTCGCATGGCCTTCAG-3′); for Snail, forward (5′-TTCAACTGCAAATACTGCAACAAG-3′) and reverse (5′-CGTGTGGCTTCGGATGTG-3′); for E-cadherin, forward (5′-GTCATCCAACGGGAATGCA-3′) and reverse (5′-TGATCGGTTACCGTGATCAA AA-3′); for β-Actin, forward (5′-GCAAAGAC CTGTACGCCAACA-3′) and reverse (5′-TGCATCCTGTCGGCAATG-3′).

2.4. Telomere measurements

To measure telomere lengths, flow-FISH and immuno-FISH were performed as previously described with modifications [22,23]. For flow-FISH, cells were washed twice with PBS containing 0.1% w/v BSA. Subsequently, the cells were re-suspended in a hybridization buffer (70% deionized formamide [Amresco], 20 mM Tris–HCl [pH 6.8], 1% BSA, and 1 nM FAM-labeled 5′-$[TTAGGG]_3$-3′ peptide nucleic acid [PNA] probe [Panagene, Korea]). Next, the samples were incubated in an 85 °C water-bath for 10 min. After hybridization with the telomere probe in the dark at room temperature for 3 h, the samples were washed in each of washing solutions (washing solution I: 70% deionized formamide [Amresco], 10 mM Tris–HCl [pH 6.8], 0.1% BSA, and 0.1% Tween 20; washing solution II: 0.1% BSA and 0.1% Tween 20 in PBS) and incubated in a separate solution (0.1% BSA, 10 μg/mL RNase A, and 0.06 μg/mL 7-Aminoactinomycin D [7-AAD] in PBS) at 37 °C for 1 h. A total of 20,000 nuclei from each experimental group were analyzed. The telomere fluorescence intensity of the nuclei gated at the G1-G0 cell cycle stage was measured using a BD FACSCalibur flow cytometer (BD Biosciences) running on the CELLQUEST software.

For immuno-FISH, cells were fixed using 4% paraformaldehyde and subsequently dehydrated for 2 min each using 70%, 90%, and 100% ethanol. After drying in air, the cells were denatured using a hybridization solution (70% formamide in 2 × SSC, 5% $MgCl_2$, 0.25% blocking reagent [Roche], 12 nM Cy3-labeled 5′-$[CCCTAA]_3$-3′ PNA probe [Panagene], and 12.4 nM FAM-labeled centromere PNA probe [Panagene]) for 5 min at 85 °C. Subsequently, the cells were incubated in a humidified chamber for at least 2 h at room temperature. After hybridization, the cells were washed thrice for 15 min each in 70% formamide in 2 × SSC, washed in 2 × SSC twice for 10 min each, and finally washed in 0.1% Triton X-100 in PBS for 10 min. The cells were then mounted using mounting medium containing DAPI (Vector Laboratories). TFI/CFI refers to the ratio of telomere fluorescence intensity to centromere fluorescence intensity. A centromeric probe was used as an internal control. Images were obtained using a confocal microscope. Image analysis was performed using Image-Pro plus 6.0 software (Media Cybernetics).

2.5. Cell sorting according to telomere fluorescence intensity via flow cytometry

A Huh7 cell line stably expressing TPP1-GFP was established using 1 mg/mL of G418 selection antibiotic (Duchefa). The cells were dissociated at 37 °C in 0.2% w/v trypsin–EDTA (Gibco) for 10 min. After gentle pipetting to induce dissociation, the cells were washed twice with cold PBS containing 0.6% w/v BSA and filtered using a cell strainer (BD Bioscience). P3 cells displaying strong GFP expression and P4 cells displaying weak GFP expression were isolated from the stable Huh7 cell line expressing TPP1-GFP using a FACS Aria II (BD Bioscience).

2.6. Cell viability assay

The cell viability of Huh7 cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Ten thousand cells were seeded in 96-well plates. After 24 h incubation, cells were washed with PBS. And then, 180 μL of phenol red-free medium and 20 μL of MTT solution (5 mg of MTT/mL PBS) were added to each well. After 4 h incubation,