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Original Articles

Mycoplasma infection promotes tumor progression via interaction of the mycoplasmal protein p37 and epithelial cell adhesion molecule in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is currently the third leading cause of cancer death worldwide. To study how mycoplasma infection affects HCC progression, we investigated the characteristics of mycoplasma-infected tumor tissues and circulating tumor cells (CTCs) in HCC patients. The mycoplasmal membrane protein p37 showed significant correlations with higher histologic stages and vascular invasion and predicted poor disease-free survival of HCC patients. p37-positive CTCs were detected in 42 out of 47 HCC patients (89%). p37-positive circulating cells were also detected in 4 out of 10 healthy donors (40%), and all were epithelial cell adhesion molecule (EpCAM)-positive. In HCC patients, most of p37-negative CTCs (95%) showed intermediate phenotype with neither EpCAM nor vimentin expression, but p37-positive CTCs were EpCAM-positive (44%), vimentin-positive (32%), and both negative (24%), suggesting that EpCAM-positive CTCs are enriched with mycoplasma infection. Mycoplasma infection promoted migratory capacity of HCC cells with increased expression of EpCAM. Immunoprecipitation analysis revealed that p37 associates with EpCAM. The results suggest that mycoplasma infection promotes tumor progression in HCC patients via interaction of the mycoplasmal p37 and EpCAM.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive malignancy and ranks the third most common cancer-related death [1]. Although a variety of pathological, genetic, and molecular events that drive HCC formation and progression have been identified, the exact molecular mechanisms by which HCC is initiated and developed remain unclear [2]. As molecular indicators of biological and pathological status, biomarkers can serve as a useful tool to measure disease presence and progression and to develop a more targeted therapy. Many researchers have been trying to find and validate various prognostic and diagnostic markers in HCC for decades, but it has been very difficult to apply proper prognostic markers in this field.

Mycoplasma hyorhinis (*M. hyorhinis*) is a swine pathogen and is one of the common contaminants in animal and human cell cultures [3]. Mycoplasma infection is prevalent in many cancer tissues such as gastric, esophageal, lung, breast, glioma, renal, ovarian, cervical and prostate carcinomas [4–8], suggesting a close correlation between *M. hyorhinis* infection and tumorigenesis [4,9–16]. *M. hyorhinis*-encoded membrane protein p37 is responsible for the proliferation and metastases of cancer cells in various mycoplasma-infected cancer cells [7,15,17,18]. Furthermore, p37 expression predicts poor survival of

Abbreviations: HCC, Hepatocellular carcinoma; CTC, circulating tumor cells; EpCAM, epithelial cell adhesion molecule; EMT, epithelial-mesenchymal transition; EGFR, epithermal growth factor receptor; MAb, monoclonal antibody; TMA, tissue microarray; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; RT, room temperature; PFA, paraformaldehyde; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; csMVP, cell surface major vault protein

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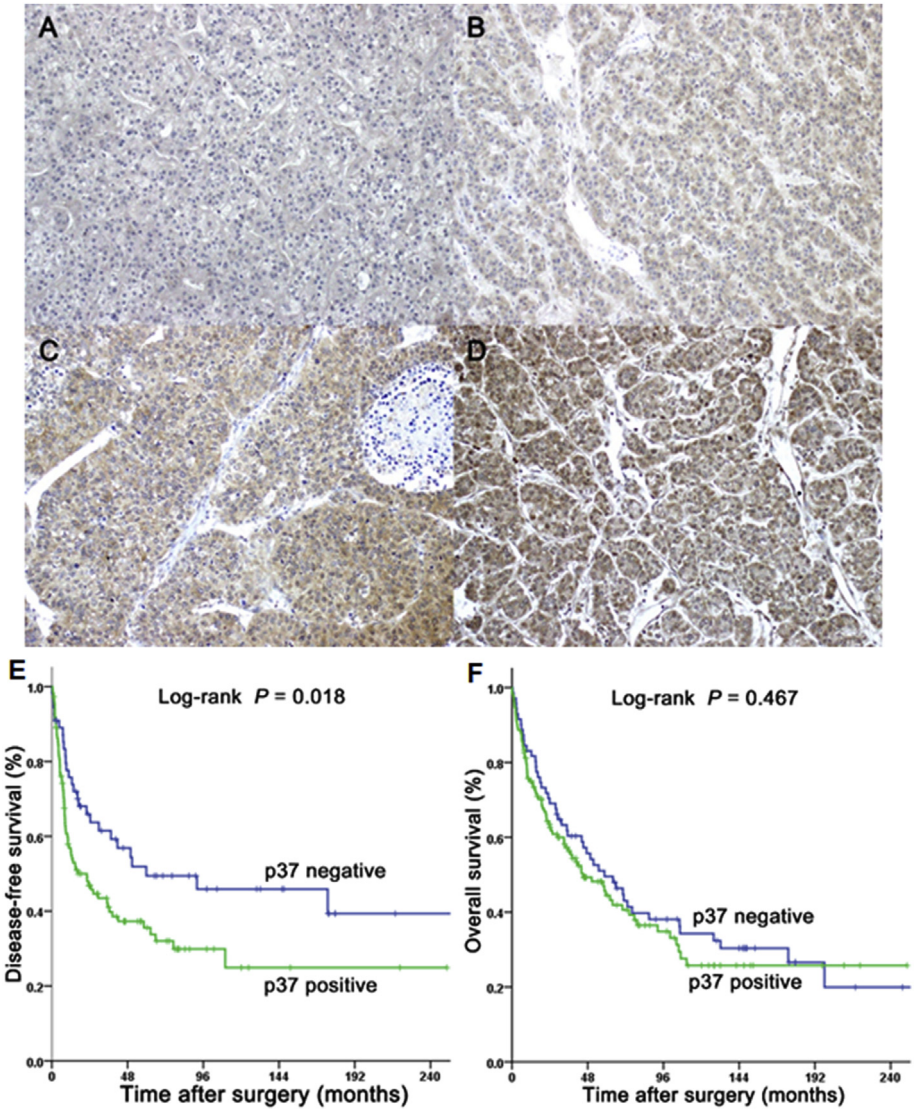

Fig. 1. p37 expression predicts poor disease-free survival in HCC patients. **(A–D)**: Immunohistochemical staining of p37 protein by CA27 MAb. p37 expression was scored according to the intensity of p37 staining (× 100). Score = 0 (**A**), score = 1 (**B**), score = 2 (**C**), and score = 3 (**D**). Score 1 and above were regarded as positive. **(E, F)**: Kaplan–Meier analysis of cumulative disease-free survival (**E**) and overall survival curves (**F**) according to p37 expression in 204 HCC patients. Statistical significance was determined using the Log-rank test.

gastric cancer patients and associates with metastasis of gastric cancer cells through the interaction of annexin A2 and epithermal growth factor receptor (EGFR) in host cells [15]. Previously, we generated monoclonal antibodies (MAb) against p37, and we observed for the first time mycoplasma infection in the circulating tumor cells (CTCs) of HCC patients [19,20]. In this study, we demonstrated that p37 expression associates with higher histologic stages and vascular invasion and predicts poor disease-free survival of HCC patients. To determine how mycoplasma infection affects HCC progression, we examined the characteristics of p37-positive CTCs in HCC patients and the migratory potential of mycoplasma-infected HCC cells. We further investigated the host-binding molecules of p37 in mycoplasma-infected HCC cells. Based on the results, we discuss and propose the biological role of mycoplasma infection in HCC progression.

2. Materials and methods

2.1. Tissue microarray and immunohistochemistry of HCC tissues

A total of 204 patients with HCC who underwent surgical resection in Hanyang University Hospital (Seoul, Korea) from 1991 to 2013 were collected. For tissue microarray (TMA) construction of HCC, we used a manual tissue microarray (Unitama, Korea). Immunohistochemical staining for p37 was performed in formalin-fixed, paraffin-embedded TMA blocks by using anti-p37 antibody CA27 (Youngin Frontier, Korea), and detailed protocols were described previously [21].

2.2. Patients and blood sample collection

From September 2015 to February 2017, 47 HCC patients were recruited. Peripheral blood samples (approximately 10 ml) were obtained in heparin-containing collection tubes with the informed consent from patients who would undergo treatment in Hanyang University Hospital (Seoul, Korea). Peripheral blood samples were also obtained from 10 healthy donors with the same informed consent. Cancer staging by the American Joint Committee on Cancer (AJCC, 2010) was used. For experiments involving human samples, approval was obtained from the institutional review board of Hanyang University Hospital (HUH IRB No.HY-16-053-1). All subsequent experiments were also performed in accordance with the relevant laws and institutional guidelines.

Table 1
Correlation between p37 expression and clinicopathologic factors in hepatocellular carcinoma (n = 204).

Factors	n	p37 expression		p-value
		Negative (%) (n = 71)	Positive (%) (n = 133)	
Tumor size				0.235
< mean	129	41 (31.8%)	88 (68.2%)	
≥mean	75	30 (40.0%)	45 (60.0%)	
Histologic grade				**< 0.001**
Low grade (G1, G2)	80	41 (51.2%)	39 (48.8%)	
High grade (G3, G4)	124	30 (24.2%)	94 (75.8%)	
Tumor focality				0.474
Single	167	60 (35.9%)	107 (64.1%)	
Multiple	37	11 (29.7%)	26 (70.3%)	
Small vessel invasion				**0.010**
Absent	116	49 (42.2%)	67 (57.8%)	
Present	88	22 (25.0%)	66 (75.0%)	
Large vessel invasion				**0.047**
Absent	180	67 (37.2%)	113 (62.8%)	
Present	24	4 (16.7%)	20 (83.3%)	
Vascular invasion				**0.006**
Absent	114	49 (43.0%)	65 (57.0%)	
Present	90	22 (24.4%)	68 (75.6%)	
Perineural invasion				0.098
Absent	199	71 (35.7%)	128 (64.3%)	
Present	5	0 (0.0%)	5 (100.0%)	
AJCC stage				**0.027**
I	99	42 (42.4%)	57 (57.6%)	
II, III, IV	105	29 (27.6%)	76 (72.4%)	

2.3. Enrichment of CTCs by depletion of CD45-positive cells

Peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood of healthy donors or HCC patients by Ficoll-Paque Plus (GE healthcare, Korea) gradient centrifugation. PBMCs were washed with phosphate buffered saline (PBS, pH 7.4) containing 0.5% bovine serum albumin and 2 mM EDTA, and resuspended in the same buffer at a concentration of 1×10^8 cells/ml. The enrichment of CTCs by CD45 depletion of the leukocyte fraction was described previously [22]. The recovered cells were centrifuged at 3560 ×g for 5 min, resuspended in 400 μl of RPMI1640 medium, and seeded onto glass slides coated with 0.1 μg/ml of poly-L-lysine. To induce the spontaneous binding of the live cells to the glass slides, the cells were incubated for 2–4 h at room temperature (RT). Unbound cells were washed with PBS before fixation. Bound cells were fixed in 3.7% paraformaldehyde (PFA) and stored in the refrigerator for further studies.

2.4. Immunocytochemistry of CTCs

For immunostaining of CTCs from blood samples, CD45-depleted cells were used as described previously [22]. For triple immunofluorescence staining for CA27/vimentin/EpCAM, cells were fixed in 3.7% PFA, blocked with 10% normal horse serum, and then incubated with Dylight 488-conjugated CA27. The cells were further incubated with rabbit polyclonal anti-vimentin or EpCAM (Santa Cruz Biotechnology, USA) and then incubated with Dylight 650-conjugated anti-rabbit IgG (Thermos, Korea). The cells were further incubated with Alexa 555-conjugated anti-EpCAM or vimentin (Cell Signaling Technology, USA). For triple immunofluorescence staining for CA27/EpCAM/E-cadherin, cells were incubated with Dylight 488-conjugated CA27. The cells were further incubated with rabbit polyclonal anti-EpCAM or E-cadherin (Santa Cruz Biotechnology) and then incubated with Dylight 650-conjugated anti-rabbit IgG (Thermos). The cells were further incubated with Alexa 555-conjugated anti-EpCAM or E-cadherin (Cell Signaling Technology). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and fluorescence signals were detected with a Leica TCS SP5 confocal microscope.

Table 2
Expression profile of EMT markers in nucleated cells from HCC patients and healthy donors by immunofluorescent staining.

Markers in HCC patients	Patients with marker -positive CTCs/patients examined	Marker-positive CTCs/ cells examined
$DAPI^+$	45/47 (95.7%)	1803
$DAPI^+$ $CD45^+$		0/17 (0%)
$DAPI^+$ $csMVP^+$		15/35 (42.9%)
$DAPI^+$ $p37^+$	42/45 (93.3%)	1091/1803 (60.5%)
$DAPI^+$ $p37^-$	3/45 (6.7%)	712/1803 (39.5%)
$p37^+$ $EpCAM^+$ $E\text{-}cadherin^+$	6/38 (15.8%)	61/468 (13.0%)
$p37^+$ $EpCAM^+$ $E\text{-}cadherin^-$	20/38 (52.6%)	138/468 (29.5%)
$p37^+$ $EpCAM^-$ $E\text{-}cadherin^+$	10/38 (26.3%)	25/468 (5.3%)
$p37^+$ $EpCAM^-$ $E\text{-}cadherin^-$	23/38 (60.5%)	244/468 (52.1%)
$p37^-$ $EpCAM^+$ $E\text{-}cadherin^+$	0/38 (0%)	0/321 (0%)
$p37^-$ $EpCAM^+$ $E\text{-}cadherin^-$	2/38 (5.3%)	4/321 (1.2%)
$p37^-$ $EpCAM^-$ $E\text{-}cadherin^+$	2/38 (5.3%)	2/321 (0.6%)
$p37^-$ $EpCAM^-$ $E\text{-}cadherin^-$	33/38 (86.8%)	315/321 (98.1%)
$p37^+$ $EpCAM^+$ $Vimentin^+$	13/38 (34.2%)	74/471 (15.7%)
$p37^+$ $EpCAM^+$ $Vimentin^-$	22/38 (57.9%)	134/471 (28.5%)
$P37^+$ $EpCAM^-$ $Vimentin^+$	14/38 (36.8%)	151/471 (32.1%)
$p37^+$ $EpCAM^-$ $Vimentin^-$	25/38 (65.8%)	112/471 (23.8%)
$p37^-$ $EpCAM^+$ $Vimentin^+$	2/38 (5.3%)	3/317 (0.9%)
$p37^-$ $EpCAM^+$ $Vimentin^-$	2/38 (5.3%)	6/317 (1.9%)
$p37^-$ $EpCAM^-$ $Vimentin^+$	4/38 (10.5%)	7/317 (2.2%)
$p37^-$ $EpCAM^-$ $Vimentin^-$	33/38 (86.8%)	301/317 (95.0%)

Markers in healthy donors	Healthy donors with marker-positive cells	Marker-positive cells/ cells examined
$DAPI^+p37^+$	4/10 (40%)	15/204 (7.4%)
$DAPI^+p37^-$	6/10 (60%)	189/204 (92.6%)
$p37^+$ $EpCAM^+$ $Vimentin^+$	1/10(10%)	1/15 (6.7%)
$p37^+$ $EpCAM^+$ $Vimentin^-$	3/10(30%)	14/15 (93.3%)
$P37^+$ $EpCAM^-$ $Vimentin^+$	0/10(0%)	0/15 (0%)
$p37^+$ $EpCAM^-$ $Vimentin^-$	0/10(0%)	0/15 (0%)
$p37^-$ $EpCAM^+$ $Vimentin^+$	0/10(0%)	0/189 (0%)
$p37^-$ $EpCAM^+$ $Vimentin^-$	1/10(10%)	1/189 (0.5%)
$p37^-$ $EpCAM^-$ $Vimentin^+$	0/10(0%)	0/189 (0%)
$p37^-$ $EpCAM^-$ $Vimentin^-$	10/10(100%)	188/189 (99.5%)

Columns on the right represent the number of HCC patients and healthy donors with circulating cells that stain positive for a given marker and number of circulating cells scoring positive for each marker. Data are presented as no. (%).

2.5. Culture of mycoplasma-infected cells and detection of mycoplasmas

M. hyorhinis-infected or uninfected Huh7, HepG2 and A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution (Life Technologies, Korea) and were cultured for more than 3 years with occasional freezing for a short period of time [19,20]. Titer of mycoplasmas was performed using Mycoplasma IST2 kit (bioMeÂrieux, Marcyl'Etoile, France) and blood agar plates as described previously [20]. Mycoplasmas (1×10^5 CFU/ml) were added to mycoplasma-free

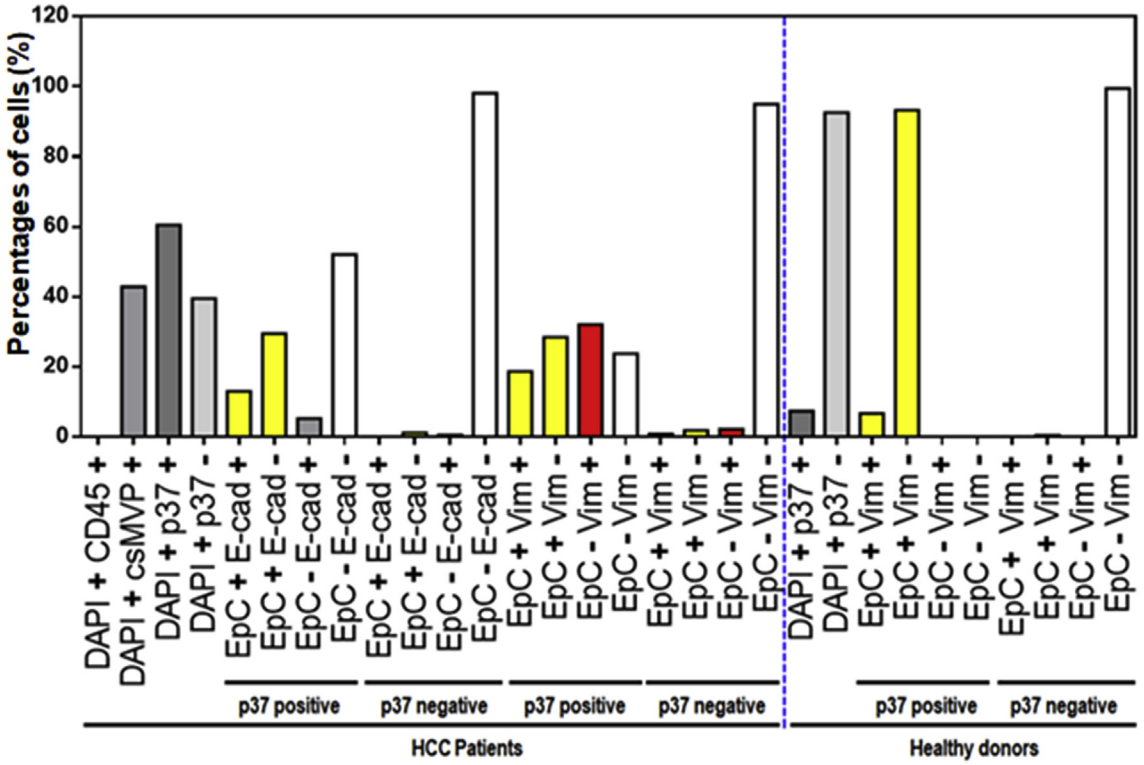

Fig. 2. Graphical presentation of the values listed in Table 2. Yellow-colored bars represent EpCAM-positive circulating cells while red-colored bars represent vimentin-positive circulating cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

HCC cells (2×10^5 cells/well) in 12 well plates as described previously [20]. To detect mycoplasmas by polymerase chain reaction (PCR) technique, infected cells were subjected to PCR using e-Myco™ VALid-Q qPCR kit (Intron, Korea) as described previously [20]. Amplified PCR products were visualized by agarose gel electrophoresis.

2.6. Flow cytometry and cell proliferation assay

Flow cytometry was performed using CA27, anti-EpCAM, anti-E-cadherin antibodies and other antibodies as described previously [19,23]. Cell proliferation of HCC cells infected with mycoplasmas was measured using CCK-8 (Dojindo, Japan) according to the manufacturer's instruction. Briefly, the Huh7 and mycoplasma-infected Huh7 cells were seeded in on to 96 well plates at 2×10^3 cells per well. After culture for 48 h, 10 µl of CCK-8 (5 mg/ml) were added to the culture medium, and the OD values were read using a microplate reader.

2.7. Migration assay

For transwell assay, Huh7 and Mycoplasma infected-Huh7 cells (1×10^4 cells) were seeded in the upper chamber of a transwell with serum-free medium. RPMI-1640 medium containing 10% FBS was placed in the lower chamber. After 24 h, the cells that invaded to the lower surface of the filters were washed with PBS (pH7.4) three times, fixed in 2% PFA, and stained with 0.1% crystal violet. Non-invasive cells in the upper chamber were removed by cotton swab. Photographs were taken using an inverted microscope, and the stained cells were analyzed by a cell counter program.

2.8. Western blot

Mycoplasma infected Huh7 cells were lysed in ice-cold immunoprecipitation buffer (150 mM NaCl, 1% NP 40, 0.5% deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 µg/ml aprotinin, 100 µg/ml PMSF, 5 µg/ml leupeptin, 1 mM NaF and 1 mM $NaVO_3$) at 4 °C for 30 min. Cell lysates were boiled with sample buffer of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cell lysates (each 60 µg) were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was performed as described previously [24,25]. The primary antibodies used were CA27 (Youngin Frontier), CA63 [20], CA279 [20], anti-annexin A2, anti-EGFR, anti-vimentin and anti-EpCAM antibodies (all Santa Cruz Biotechnology).

2.9. Immunoprecipitation

Mycoplasma infected Huh7 cells were lysed in ice-cold immunoprecipitation buffer (1% NP-40, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, aprotinin (2 µg/ml), leupeptin (5 µg/ml), 1 mM phenylmethanesulfonyl fluoride, 5 mM NaF and 1 mM Na_3VO_4) and incubated at 4 °C for 30 min. Pellets were precipitated by centrifugation at 12000 rpm for 40 min, and supernatants were collected. 30 µl of protein G agarose beads (Amicogen, Korea) were added to the supernatants (each 2.5 mg) and incubated at 4 °C for 3 h on a rocker. Detailed protocols were described previously [22].

2.10. Microarray analysis

Microarray gene analysis was done using tools contained in the ebiogen (Seoul, Korea). Total RNAs were extracted using the RNA iso plus reagent (TaKaRa, Japan) and the synthesis of target cRNA probes and hybridization were performed using Agilent's Low Input Quick Amp Labeling Kit (Agilent, USA) according to the manufacturer's instructions. The labeled cRNAs were hybridized to Agilent human GE $4 \times 44K$ v2 Microarray. Arrays were scanned with DNA microarray scanner running Feature Extraction Software (Agilent). Raw expression values were normalized using GeneSpring GX software (Agilent).

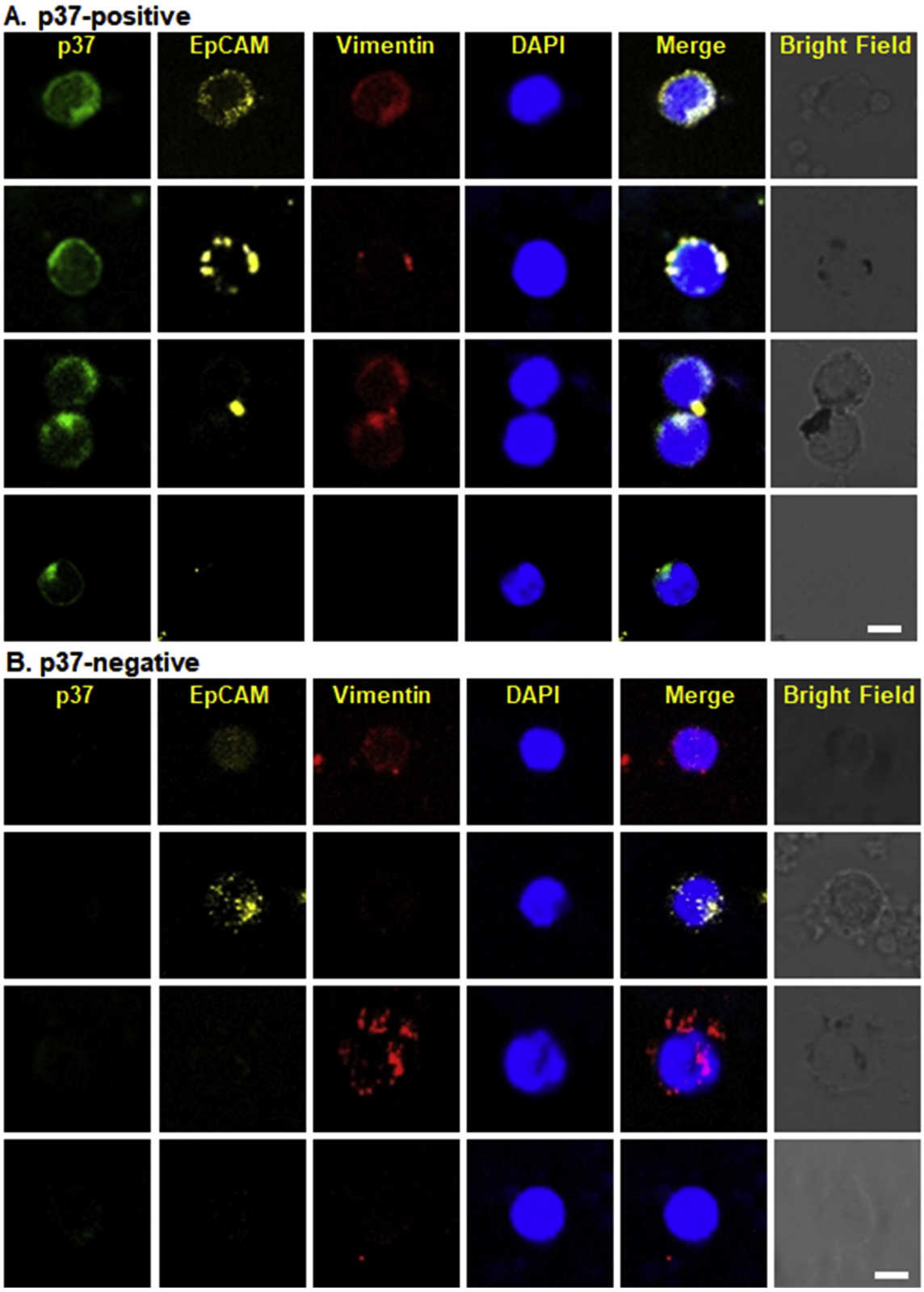

Fig. 3. Mycoplasma-infected CTCs shows EpCAM- or vimentin-positive phenotype. **(A, B)**: Immunocytochemical analysis of p37 (green), EpCAM (yellow), and vimentin (red) in mycoplasma-infected CTCs from HCC patients. Colocalization of the three proteins is shown in the merge. p37-positive (**A**) and p37-negative CTCs (**B**) are shown. Nuclei were stained with DAPI (blue). The scale bars are 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

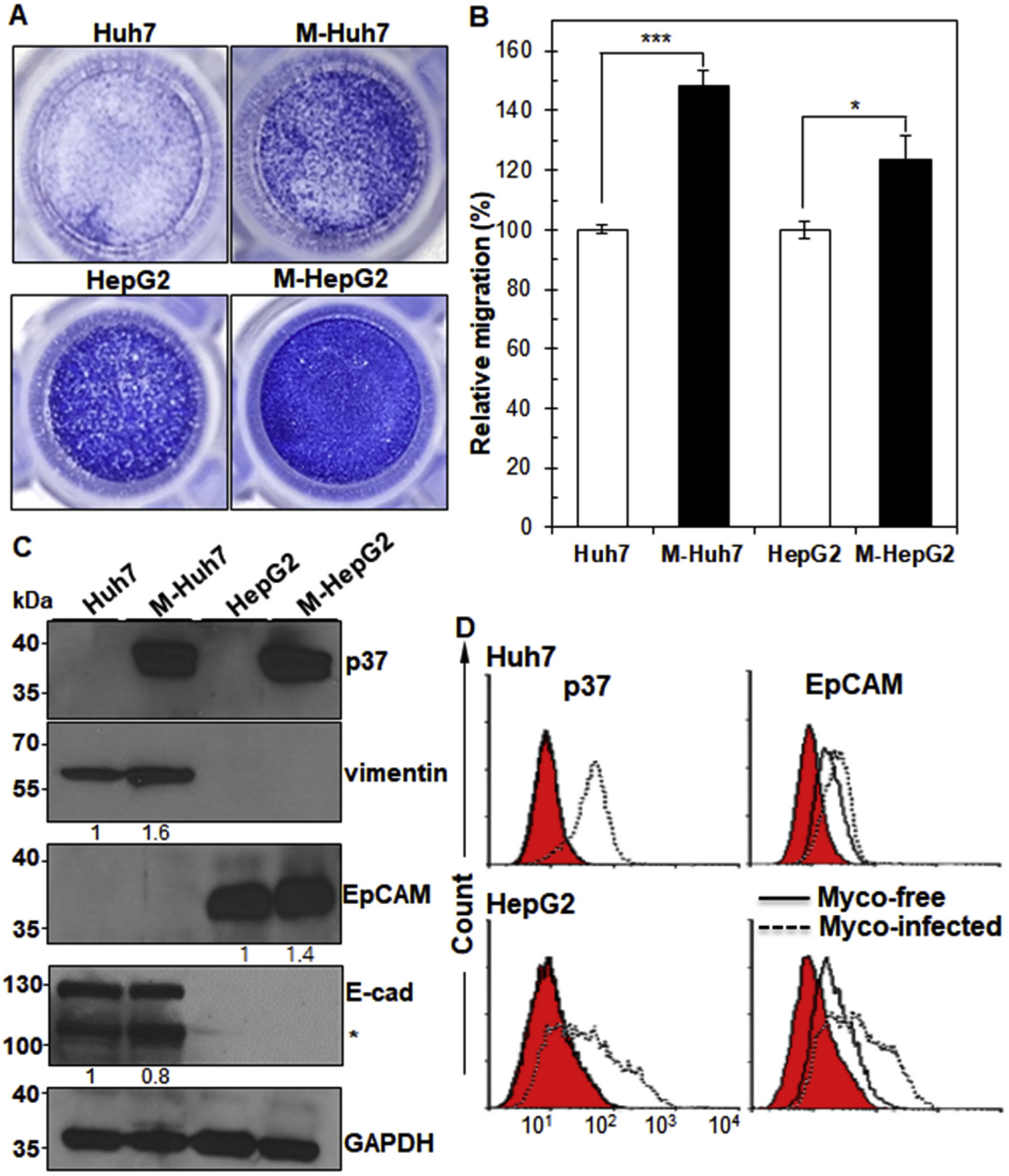

Fig. 4. Mycoplasma infection promotes migratory capacity of HCC cells with increased EpCAM and vimentin expression. **(A)**: Transwell migration assay of mycoplasma-free and infected Huh7 and HepG2 cells. **(B)**: Statistical analysis of **A**. Migrated and stained cells were counted by the cell counter program. Data are presented as mean and standard deviation from three experiments with triplicate for each sample. *, $p < 0.05$; ***, $p < 0.001$. **(C)**: Expression analysis of p37, vimentin, EpCAM, and E-cadherin in mycoplasma-free and infected Huh7 and HepG2 cells by Western blot analysis. The signal intensities of the Western blots were measured quantitatively using the Image J software, and GAPDH was used as a loading control. The asterisk indicates a cleaved product of E-cadherin. **(D)**: Mycoplasma infection increases expression of EpCAM in HCC cells. Mycoplasma-free or -infected Huh7 and HepG2 cells were subjected to flow cytometric analysis for the expression of p37 and EpCAM. Red-colored populations indicate FITC-conjugated secondary antibody staining as a control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.11. Statistical analysis

Statistical analysis was performed with the SPSS software, Version 21 (IBM Corp., USA). Chi-square tests were used to examine the associations among p37 expression and clinicopathologic parameters of tumor size, histologic grade, tumor focality, presence of small vessel invasion, presence of large vessel invasion, presence of vascular invasion, which includes both small and large vessels, presence of perineural invasion and staging. Cancer staging was used according to the AJCC staging system [26]. Disease-free survival and overall survival were determined using the Kaplan-Meier method, and the log-rank test was used to compare groups according to p37 expression. A Cox proportional hazard regression model was used to evaluate the prognostic significance in univariable and multivariable analyses. Two-sided p values < 0.05 were considered statistically significant.

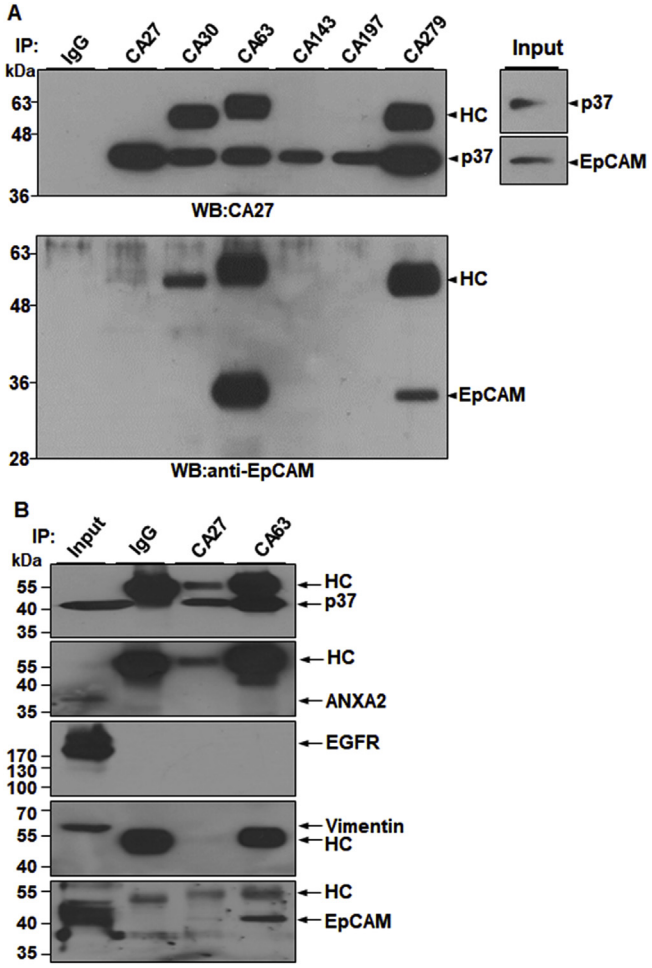

Fig. 5. p37 interacts with EpCAM in mycoplasma-infected cancer cells. (**A**): Immunoprecipitation analysis of mouse IgG (IgG) and various anti-p37 antibodies (CA27-CA279) in mycoplasma-infected non-small cell lung carcinoma cells (A549). Cell lysates from mycoplasma-infected A549 cells were subjected to immunoprecipitation with the indicated antibodies. Immunoprecipitated proteins were examined by Western blotting with CA27 and anti-EpCAM antibodies. Right panels show the input of protein as used in the immunoprecipitation. (**B**): Immunoprecipitation analysis of IgG, CA27, and CA63 antibodies in mycoplasma-infected HCC cells (Huh7). Mycoplasma-infected Huh7 cells were extracted and immunoprecipitated with mouse IgG (IgG) and monoclonal anti-p37 antibodies (CA27 and CA63). The immunoprecipitates were analyzed by Western blots with anti-p37, anti-annexin A2 (ANXA2), anti-EGFR, anti-vimentin, and anti-EpCAM antibodies. CA63, anti-p37 monoclonal antibody, was able to immnuoprecipitate EpCAM but was not able to immunoprecipitate ANXA2, EGFR, and vimentin. HC, immunoglobulin heavy chain.

3. Results

3.1. Correlation between p37 expression and clinicopathologic parameters in HCC

To investigate the presence of mycoplasma infection in HCC tissues, p37 immunostaining was examined with the TMA method on tumor samples from 204 patients, and 133 cases (65.2%) showed p37 positivity (Fig. 1A–D). The correlation between p37 expression and the clinicopathologic parameters in HCC patients is shown in Table 1 p37 expression showed significant correlations with higher histologic grade ($p < 0.001$), small vessel invasion ($p = 0.010$), large vessel invasion ($p = 0.047$), vascular invasion ($p = 0.006$) and higher AJCC stage ($p = 0.027$). p37 expression also tended to show correlation with perineural invasion (100%), although the sample size was small (n = 5, $p = 0.098$). However, p37 expression showed no correlation with tumor size and tumor focality. The results suggest that mycoplasma infection drives HCC progression by promoting the vascular invasion of HCC cells.

3.2. p37 expression predicts poor disease-free survival of HCC patients

In the Kaplan-Meier survival analysis, p37 expression showed a significant association with poor disease-free survival ($p = 0.018$, log-rank test, Fig. 1E). Univariate survival analysis for disease-free survival showed that p37 expression ($p = 0.020$), histologic grade ($p = 0.017$), large vessel invasion ($p < 0.001$) and AJCC stage ($p < 0.001$) were associated with poor prognosis (Supplementary Table S1). Multivariate Cox regression analysis revealed that only the AJCC stage ($p < 0.001$) was an independent predictor for poor disease-free survival (Supplementary Table S1). There was no statistical significance between p37 expression and overall survival ($p = 0.467$, Fig. 1F). Thus, HCC patients with mycoplasma infection showed the worse disease-free survival, suggesting that mycoplasma infection associates with HCC progression.

3.3. Detection of p37-positive circulating cells in the peripheral blood in HCC patients

To study the characteristics of mycoplasma-infected CTCs in the peripheral blood of 47 HCC patients, CD45-depleted live cells were collected and analyzed (Supplementary Fig. 1A). [22]. DAPI-positive cells were not detected in 2 out of 47 patients, and subsequent analyses were done with DAPI-positive cells (Table 2). CD45-positive cells were not detected in CD45-depleted cells (Table 2 and Supplementary Fig. 1B). Cell surface major vault protein (csMVP) positivity was also examined as a marker of CTCs in HCC patients, because csMVP-positive CTCs were detected in HCC patients (approximately 90%) but not in healthy donors [22]. csMVP-positive CTCs were readily detected in CD45-depleted cells (Table 2 and Supplementary Fig. 1B).

p37-positive cells were detected in 42 (89.4%) out of 47 patients, although p37 positivity was not significantly associated with some clinicopathologic characteristics in HCC patients (Supplementary Table S2). The number of p37-positive cells ranged between 0.1 and 14.8 per ml, and the average number of p37-positive cells was 3.18 per ml (Table 2 and Supplementary Table S3). p37-positive circulating cells were also detected in 4 out of 10 healthy donors (40%), and the cell number ranged between 0 and 1.4 per ml (Table 2, Supplementary Table S3 and Fig. 1C). The cell size of p37-positive cells averaged 23 μm with a range between 14.3 and 42.9 μm (Supplementary Table S4). The results suggest that approximately 89% of HCC patients have CTCs with mycoplasma infection. Interestingly, p37-positive circulating cells were also detected even in healthy donors, although the average number of p37-positive circulating cells (0.75/ml) was significantly low ($p = 0.001$) (Supplementary Table S3 and Fig. 2).

3.4. p37-positive CTCs show EpCAM- or vimentin-positive phenotype

To investigate the characteristics of p37-positive CTCs in HCC patients, p37-positive CTCs were analyzed for EpCAM expression. E-cadherin, a representative epithelial marker, was also examined because EpCAM-positive CTCs are lowly detected in HCC patients [27,28]. In the p37/EpCAM/E-cadherin triple staining, p37-positive CTCs were 59% (468/789), while p37-negative CTCs were 41% (321/789) (Table 2, Fig. 2, and Supplementary Fig. 3). Approximately 29% (230/789) of CTCs were EpCAM- or E-cadherin-positive. Among p37-negative CTCs, approximately 98% (315/321) of CTCs were both EpCAM- and E-cadherin-negative. Among p37-positive CTCs, 42.5% (199/468) of p37-positive CTCs were positive for EpCAM, and 18.4% (86/486) of p37-positive CTCs were positive for E-cadherin. Approximately 52%

(244/468) of p37-positive CTCs were p37-single positive CTCs with neither EpCAM nor E-cadherin expression. The results suggest that most (98%) of p37-negative CTCs are EpCAM/E-cadherin-negative, while p37-positive CTCs consist of EpCAM/E-cadherin-positive (48%) and EpCAM/E-cadherin-negative cells (52%).

Successful metastasis depends on the generation of semi-mesenchymal or mesenchymal CTCs during the EMT process in breast, prostate, and colon cancers [29–33]. To analyze the mesenchymal characteristic of p37-positive circulating cells, p37-positive cells were further analyzed for the expression of vimentin and EpCAM simultaneously. Among p37-negative CTCs, intermediate phenotype with neither EpCAM nor vimentin expression was approximately 95% (301/317) in HCC patients (Table 2, Figs. 2 and 3). Among p37-positive CTCs, EpCAM-single positive CTCs were 28.4% (134/471), while vimentin-single positive CTCs were 32.1% (151/471). Both EpCAM/vimentin-positive and -negative CTCs were 15.7% (74/471) and 23.8% (112/471), respectively. Therefore, most p37-positive CTCs (76.3%) were EpCAM-positive (44.2%) or vimentin-single positive (32.1%) in HCC patients. Thus, EpCAM- and vimentin-positive CTCs were significantly higher in p37-positive cells than in p37-negative cells ($p < 0.001$ and $p < 0.001$, respectively) (Supplementary Figs. 4A and B), suggesting that EpCAM- or vimentin-positive CTCs are enriched with mycoplasma infection. Interestingly, all p37-positive circulating cells were EpCAM-positive in the healthy donors (Table 2 and Supplementary Table S3). The results further suggest that p37 may associate with host EpCAM on the cell surface during mycoplasma infection.

3.5. Mycoplasma infection promotes migratory capacity of HCC cells with increased EpCAM and vimentin expression

To study the effect of mycoplasma infection on HCC cells, we examined cell proliferation of mycoplasma-infected Huh7 cells. Mycoplasma-infected Huh7 cells showed a small and compact morphology and showed the same viability and proliferation capacity as compared with mycoplasma-free Huh7 cells (Supplementary Fig. 5), although Huh7 cells newly infected with mycoplasmas showed decreased cell survival and proliferation (Supplementary Fig. 6). Transwell assays showed that mycoplasma infection increased migratory capacity of Huh7 cells by 48% (Fig. 4A and B). Western blot analysis showed that vimentin expression was increased in mycoplasma-infected Huh7 cells while E-cadherin expression was slightly decreased (Fig. 4C). Similar results were also obtained with another HCC cell line HepG2, showing increased expression of EpCAM in mycoplasma-infected cells (Fig. 4A–C). Flow cytometry further revealed that mycoplasma infection increased cell surface expression of EpCAM in mycoplasma-infected HCC cells (Fig. 4D). Thus, mycoplasma infection promotes migratory capacity of HCC cells with increased EpCAM expression on the cell surface.

3.6. p37 interacts with EpCAM in mycoplasma-infected HCC cells

All of the mycoplasma-infected circulating cells were EpCAM-positive in healthy donors (Table 2), suggesting the possible interaction between p37 and EpCAM on the HCC cell surface. In previous studies, we generated three monoclonal anti-p37 antibodies (CA27, CA63 and CA279) [19,20]. Among the three MAbs, we found that immunoprecipitation with CA63 and CA279 was able to immunoprecipitate EpCAM from mycoplasma-infected A549 cells (Fig. 5A), suggesting that p37 interacts with EpCAM. To gain mechanistic insight into how mycoplasma infection affects HCC progression, we investigated host binding molecules of p37 in mycoplasma-infected Huh7 cells by immunoprecipitation with CA27 and CA63 (Fig. 5B) [19,20]. Although p37 is known to interact with annexin A2 and EGFR in gastric cancer cells [15,34,35], annexin A2 and EGFR were not detected in the p37-immunoprecipitates. Vimentin was not detected in the p37-immunoprecipitates, as well (Fig. 5B, the fourth panel). However, EpCAM was detected in the p37-immunorecipitate with CA63 (Fig. 5B, the fifth panel), which is consistent with the results obtained from mycoplasma-infected A549 cells. The results suggest that mycoplasmal membrane protein p37 physically interacts with host EpCAM. Recent studies have shown that EpCAM regulates tumor progression via the AKT/mTOR pathway [36,37]. To look at the effect of mycoplasma infection on the AKT/mTOR pathway, we examined the expression of mTOR signaling molecules in mycoplasma-infected HCC cells. Microarray analysis revealed that the expression of Akt, mTOR, S6K, S6, and eIF4B were increased in mycoplasma-infected HCC cells while the expression of PTEN was slightly decreased (Supplementary Fig. 7). The results further suggest that the interaction between the mycoplasmal p37 and EpCAM during mycoplasma infection is involved in the Akt/mTOR pathway in HCC cells.

4. Discussion

We accidently found the presence of mycoplasma-infected CTCs in the peripheral blood of HCC patients by using MAb CA27 recognizing the p37 protein [19,20]. In this study, we found that p37 expression is also detected in approximately 65% of HCC tissues and associated with vascular invasion and higher histologic tumor stage (Fig. 1 and Table 1). p37 expression was also associated with poor disease-free survival in HCC patients (Fig. 1E). Therefore, we expected that p37 expression accelerates HCC progression. Most of p37-positive CTCs (76%) were EpCAM- (44%) or vimentin-positive (32%) in HCC patients, although approximately 95% of p37-negative CTCs were intermediate phenotype with neither EpCAM nor vimentin expression (Table 2, Figs. 2 and 3). EpCAM expression of p37-positive CTCs strongly drew our attention because all p37-positive circulating cells were EpCAM-positive in the healthy donors (Table 2 and Fig. 2). Although EpCAM has shown to prevent metastasis, recent studies have shown that EpCAM expression is associated with enhanced EMT and metastasis in breast, prostate and nasopharyngeal carcinomas [36,38–40]. EpCAM-positive HCC cells are also identified as cancer-initiating cells [41,42]. Furthermore, EpCAM-positive CTCs display EMT phenotype, cancer stem cell biomarkers, high tumorigenic potential, and low apoptotic propensity, and lead to decreased overall survival in HCC patients [27,43]. In addition, EpCAM-positive CTCs are positively associated with patients with intermediate and advanced HCC and are also closely correlated to tumor recurrence [27,43]. Therefore, it is possible to speculate that mycoplasma-infected EpCAM-positive CTCs are associated with decreased disease-free survival in HCC patients (Fig. 1E). p37 interacts with annexin A2 and EGFR protein and mediates mycoplasma-driven cell migration in gastric cancer cells [15]. However, we were not able to immunoprecipitate annexin A2 and EGFR with anti-p37 antibodies in HCC cells. Instead, anti-p37 antibodies were able to immunoprecipitate EpCAM (Fig. 5), although EpCAM expression was barely detectable by Western blot analysis in Huh7 cells (Figs. 4 and 5). Therefore, it is tempting to speculate that EpCAM-associated survival and migration capacity may be increased in mycoplasma-infected cancer cells during the process of tumorigenesis. Based on the results, we propose that mycoplasma infection accelerates tumor progression through interaction of the mycoplasmal membrane protein p37 with EpCAM.

Although HCC cells newly infected with mycoplasmas showed decreased cell survival and proliferation (Supplementary Fig. 6), HCC cells chronically infected with mycoplasmas showed increased cell survival and migration (Fig. 4 and Supplementary Fig. 5). The results are consistent with the previous studies in terms of oncogenic transformation and migratory potential [4–6,15,18,44]. It seems that chronic mycoplasma infection causes HCC cells to acquire increased survival and migratory potential, although the mechanistic effect of chronic mycoplasma infection remains still elusive. CTC analysis in HCC patients also suggested that mycoplasma infection enriches EpCAM- or vimentin-positive CTCs, which have higher survival and

migratory potential. Immunohistochemistry also revealed that mycoplasma infection drives vascular invasion of HCC cells and predicts poor disease-free survival of HCC patients. Based on the present study, therefore, the elimination of mycoplasma infection should be an important prerequisite for successful treatment of HCC patients in the clinical setting.

Conflicts of interest

The authors declare that they have no competing financial interests to disclose.

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Appendix A. Supplementary data

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