Biochemical and Biophysical Research Communications 446 (2014) 620–625

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

# Detection of mycoplasma infection in circulating tumor cells in patients with hepatocellular carcinoma

Hong Seo Choi [a,1], Hyun Min Lee [a,1], Won-Tae Kim [a], Min Kyu Kim [a], Hee Jin Chang [d], Hye Ran Lee [e], Jae-Won Joh [c], Dae Shick Kim [b,*], Chun Jeih Ryu [a,*]

[a] Department of Bioscience and Biotechnology, Institute of Bioscience, Sejong University, Seoul, Republic of Korea
[b] Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea
[c] Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea
[d] Center for Colorectal Cancer, Research Institute and Hospital of National Cancer Center, Goyang-si, Republic of Korea
[e] Department of Internal Medicine, Inje University Ilsan Paik Hospital, Goyang-si, Republic of Korea

ARTICLE INFO

*Article history:*
Received 24 February 2014
Available online 15 March 2014

*Keywords:*
Circulating tumor cell
Mycoplasmal p37 protein
Monoclonal antibody CA27
Hepatocellular carcinoma
Metastasis

ABSTRACT

Many studies have shown that persistent infections of bacteria promote carcinogenesis and metastasis. Infectious agents and their products can modulate cancer progression through the induction of host inflammatory and immune responses. The presence of circulating tumor cells (CTCs) is considered as an important indicator in the metastatic cascade. We unintentionally produced a monoclonal antibody (MAb) CA27 against the mycoplasmal p37 protein in mycoplasma-infected cancer cells during the searching process of novel surface markers of CTCs. Mycoplasma-infected cells were enriched by CA27-conjugated magnetic beads in the peripheral blood mononuclear cells in patients with hepatocellular carcinoma (HCC) and analyzed by confocal microscopy with anti-CD45 and CA27 antibodies. CD45-negative and CA27-positive cells were readily detected in three out of seven patients (range 12–30/8.5 ml blood), indicating that they are mycoplasma-infected circulating epithelial cells. CA27-positive cells had larger size than CD45-positive hematological lineage cells, high nuclear to cytoplasmic ratios and irregular nuclear morphology, which identified them as CTCs. The results show for the first time the existence of mycoplasma-infected CTCs in patients with HCC and suggest a possible correlation between mycoplasma infection and the development of cancer metastasis.

© 2014 Elsevier Inc. All rights reserved.

## 1. Introduction

It has been suggested that persistent infections of bacteria can initiate many cancers [1–3]. One class of bacteria that has attracted increasing attention from oncologists is mycoplasmas. Mycoplasmas are the smallest bacteria capable of independent replication. They are tiny, pleomorphic, wall-free, prokaryotic organisms that can survive well, either attached to eukaryotic membrane or inside the cells. Because these bacteria reside well with eukaryotes without producing noticeable symptoms, mycoplasma infection is a major concern for animal cell cultures. *Mycoplasma hyorhinis* is generally considered to be a common bacterial commensal in the respiratory tract of pigs [4]. *M. hyorhinis*-encoded protein p37 is part of a high-affinity transport system and is found in many mycoplasmas [5]. The mycoplasmal p37 protein was originally found to promote invasiveness in mouse sarcoma cells [6,7]. Many studies have indicated that persistent exposure to mycoplasmas such as *M. hyorhinis* is closely associated with oncogenic transformation in human cancers [3,8–10], and have found that the p37 protein alone is sufficient to increase the invasiveness and metastases of cancer cells [11–13].

The number of circulating tumor cells (CTCs) in peripheral blood is correlated with worse prognosis for metastatic cancer patients [14–17]. However, the detection of CTCs is a big technical hurdle due to their rarity in the peripheral blood, and to the absence of specific markers. Epithelial to mesenchymal transition (EMT) is a well-recognized mechanism by which adherent epithelial cancer cells transiently convert into motile and invasive

*Abbreviations:* CTC, circulating tumor cell; MAb, monoclonal antibody; HCC, hepatocellular carcinoma; EMT, epithelial to mesenchymal transition; PBMC, peripheral blood mononuclear cell; DAPI, 4,6-diamidino-2-phenylindole; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PI, propidium iodide; RT, room temperature; HRP, horse radish peroxidase.

* Corresponding authors.
*E-mail addresses:* oncorkim@skku.edu (D.S. Kim), cjryu@sejong.ac.kr (C.J. Ryu).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.bbrc.2014.03.024
0006-291X/© 2014 Elsevier Inc. All rights reserved.

mesenchymal cancer cells during cancer metastasis [18,19]. Studies have suggested that a major portion of CTCs may have a semi-mesenchymal phenotype because the EMT process is reversible and transitory [20]. Lung cancer cell line A549 seems to have a semi-mesenchymal phenotype among many cancer cell lines because it expresses EMT-associated markers at the intermediate level [21,22]. In this study, we generated a panel of monoclonal antibodies (MAbs) against surface molecules on A549 cells to apply them on searching for CTC-specific surface markers. CA27, one of the MAbs, bound to A549 and HepG2 cells but not to peripheral blood mononuclear cells (PBMCs). We found that the target antigen of CA27 is the *M. hyorhinis* p37 protein, and the p37 protein is detected in CTCs in patients with hepatocellular carcinoma (HCC). These results represent the first evidence for the presence of mycoplasma-infected CTCs in patients with HCC.

## 2. Materials and methods

### 2.1. Cell culture

Human lung carcinoma cell lines (H358, A549 and H1703) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotic–antimycotic solution (Life Technologies, Seoul, Korea). Mycoplasma-free control A549 was also purchased from the Korean Cell Line Bank. Human liver cancer cell line HepG2 and FO myeloma cells were maintained in DMEM medium supplemented with 10% FBS and antibiotic–antimycotic solution (Life Technologies).

### 2.2. Research ethics and patient consent

Human PBMCs were isolated by the Ficoll-Paque Plus method (GE Healthcare, Seoul, Korea). Peripheral blood samples were obtained in heparin-containing blood collection tubes with informed consent from patient who underwent curative hepatectomy for primary HCC in the Department of Surgery, Samsung Medical Center (Seoul, Korea). The study protocol was approved by the Institutional Review Board of Samsung Medical Center. Cancer staging by the American Joint Committee on Cancer (AJCC, 2010) was used.

### 2.3. Generation, purification and biotinylation of MAbs

Murine MAbs were generated as previously described [23]. Briefly, $2 \times 10^6$ cells of H358 and A549 cells were injected into the hind footpads of 6 female Balb/c mice (DBL, Chungbuk, Korea). To generate a panel of hybridomas producing MAbs that bind to A549 cells but not to H358 cells, H358 cells were immunized into the right hind footpads of mice as decoy immunogen, while A549 cells were immunized into the left hind footpads of the same mice 3 days later as target immunogen. The lymphocyte suspension from the left popliteal lymph nodes was fused to FO myeloma cells. Isotype analysis of selected antibodies was carried out by Mouse Immunoglobulin Isotyping Kit (BD Biosciences, Seoul, Korea), according to the supplier's protocol. MAbs were purified from the culture supernatants of hybridomas by Protein G-Sepharose column chromatography as described before [23,24]. Biotinylation of MAbs was carried out with DSB-X-Biotin Protein Labeling Kit (Life Technologies) according to the supplier's protocol.

### 2.4. Flow cytometry

Flow cytometric analysis was performed as described previously [25]. Briefly, cells were treated with 0.05% trypsin–EDTA (WellGene, Daegu, Korea) and suspended in PBA (1% bovine serum albumin (BSA), 0.02% $NaN_3$ in phosphate buffered saline (PBS), pH 7.4). Cells were then incubated with MAbs or biotinylated MAbs for 30 min at 4 °C and further incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing twice with PBA, propidium iodide (PI)-negative cells were analyzed for the antibody binding using FACSCalibur and Cell Quest software (BD Biosciences).

### 2.5. Immunoprecipitation and Western blot

A549 or HepG2 cells were lysed in ice-cold immunoprecipitation buffer (25 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% NP-40, 2 μg/ml aprotinin, 100 μg/ml phenylmethanesulfonyl fluoride, 5 μg/ml leupeptin, 1 mM NaF and 1 mM $NaVO_3$) at 4 °C for 30 min. After preclearing with Protein G agarose (Millipore, Seoul, Korea), cell lysates were incubated with approximately 1 μg of MAbs at 4 °C overnight and were further incubated with Protein G agarose. The beads were washed with ice-cold immunoprecipitation buffer, and bound proteins were eluted from the beads by boiling in SDS–PAGE sample buffer. Precleared and eluted proteins were resolved by 10% polyacrylamide gel under denaturing conditions and transferred to nitrocellulose membrane. Western blotting was performed as described previously [24,26]. Membrane was incubated with rabbit anti-E-cadherin, rabbit anti-snail, mouse anti-vimentin, mouse anti-slug, anti-hnRNP A2/B1 (all from Santa Cruz Biotechnology) or purified MAbs for 1 h at room temperature (RT) and further incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). Antibody binding was visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Seoul, Korea).

### 2.6. Mass spectrometry

The protein of interest was enzymatically digested in-gel in a manner similar to that described previously [27]. The search program, ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting [28]. Spectra were calibrated with trypsin auto-digestion ion peak *m/z* (842.510, 2211.1046) as internal standards.

### 2.7. Isolation of CTCs from peripheral blood of cancer patients

PBMCs were separated from peripheral blood of healthy donor or patients with HCC by Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. CTCs were directly stained with fluorescent CA27 and anti-CD45 antibodies in PBMCs without further isolation, or further isolated from PBMCs by using Dynabeads FlowComp™ Flexi Kit (Life Technologies) according to the protocol provided by supplier. Control experiments were performed in parallel using 100 cells of A549 cell line spiked in blood from healthy donors. Briefly, PBMCs were suspended in 500 μl of cold isolation buffer (PBS, pH 7.4, 0.1% BSA and 2 mM EDTA) in microcentrifuge tube and incubated with 10 μg of biotinlyated CA27 antibody for 30 min at 4 °C with rolling and tilting. Cells were harvested after addition of 1 ml of cold isolation buffer. Cells were resuspended in 1 ml of cold isolation buffer and incubated for 15 min at 4 °C with rolling and tilting after addition of 10 μl ($1.5 \times 10^7$ beads) of Dynabeads (Life Technologies). Target cells were collected by placing the tube in a magnet for 2–3 min. The supernatant containing non-target cells was carefully removed in the magnet, and the bead/cell complexes were cleaned up by a total of three washes. Cells were then removed from the magnet by incubation of 1 ml of FlowComp Release Buffer (Life Technologies) for 10 min at RT with rolling and tilting. The supernatant with target cells was carefully transferred to a new tube and put on the magnet again to remove residual beads. The supernatant with

target cells was subjected to centrifugation at 600*g* for 10 min, and the bead free-cells were resuspended in 200 μl of cold isolation buffer. Isolated cells were finally collected on poly-L-lysine-coated glass slide by cytospin centrifugation at 300*g* for 6 min.

### 2.8. Immunocytochemistry

CTCs on poly-L-lysine-coated glass slide were fixed in 4% paraformaldehyde for 15 min at RT. Cells were washed, blocked with blocking solution (10% horse serum, 0.1% BSA in PBS, pH 7.4) and incubated with CA27 overnight at 4 °C. Antibody-bound cells were further incubated with Dylight649-conjugated anti-mouse IgG (Vector Laboratories, Seoul, Korea) and Alexa488-conjugated CD45 antibody at RT in the dark for 1 h. Between each step cells were washed with PBS containing $Ca^{2+}$ and $Mg^{2+}$. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole). Fluorescence signals were detected with a Leica TCS SP5 confocal microscope (Leica Microsystems, Seoul, Korea).

### 2.9. Mycoplasma detection

Mycoplasma detection of cultured cells was carried out by e-Myco™ Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Seongnam, Korea) according to the protocol provided by supplier. A mycoplasma-specific PCR primer set that is complementary to conserved regions of 16S ribosomal RNA genes from 51 mycoplasma species was used for the detection of mycoplasma infection. To get rid of mycoplasmas from infected cells, cells were treated with BM-cyclin (Roche, Seoul, Korea) for 2 weeks according to the supplier's protocol.

## 3. Results and discussion

### 3.1. Generation of MAb CA27

Studies have suggested that a major population of CTCs undergoes the EMT process and has a semi-mesenchymal phenotype [19,20]. Among lung cancer cell lines H358, A549 and H1703, A549 seems to have a semi-mesenchymal phenotype because it expresses EMT-associated markers at the intermediate level [21,22]. To find whether A549 cells have intermediate expression levels of EMT markers such as E-cadherin, vimentin, slug and snail, we compared the expression levels of the EMT markers in 3 cancer cell lines by Western blot analysis (Supplementary data 1). Mesenchymal markers were strongly expressed in H1703, suggesting that H1703 cells have a fixed mesenchymal phenotype. The pan-epithelial marker E-cadherin was lower in A549 than in H358, while the mesenchymal marker vimentin was higher in A549 than in H358. hnRNP A2/B1 plays a role in EMT in nonepithelial lung cancer cell lines through the regulation of E-cadherin expression [22]. Expectedly, hnRNP A2/B1 expression was only detected in A549 and H1703. Hence, Western blot analysis suggests that A549 cells have a semi-mesenchymal phenotype, while H358 cells have an epithelial phenotype. Based on literature and our Western analysis, we postulate that A549 cells may have a CTC-mimic phenotype. Therefore, we generated a panel of MAbs recognizing cell surface molecules on A549 cells to apply them on searching for CTC-specific surface markers by using the decoy immunization strategy [23]. A549 cells were injected into left footpads of mice as target immunogen, while epithelial H358 cells were injected into right footpads of the same mice 3 day earlier as decoy immunogen. Fusion of left popliteal lymph node cells to myeloma cells generated 75 hybridomas secreting MAbs specific to A549 but not to H358 (data not shown). CA27 ($IgG_3$,κ), one of the MAbs, bound to A549 and HepG2 but not to H358 and PBMCs (Fig. 1A). Western blot analysis showed that CA27 recognized an approximately 40 kDa protein on A549 and HepG2 cells (Fig. 1B).

### 3.2. CA27 recognizes the mycoplasmal p37 protein

To identify the cell surface antigen recognized by CA27, A549 cell lysate was immunoprecipitated with CA27, and the 40 kDa protein were excised and subjected to mass spectrometry. Mass spectrometric analysis of the 40 kDa protein repeatedly identified

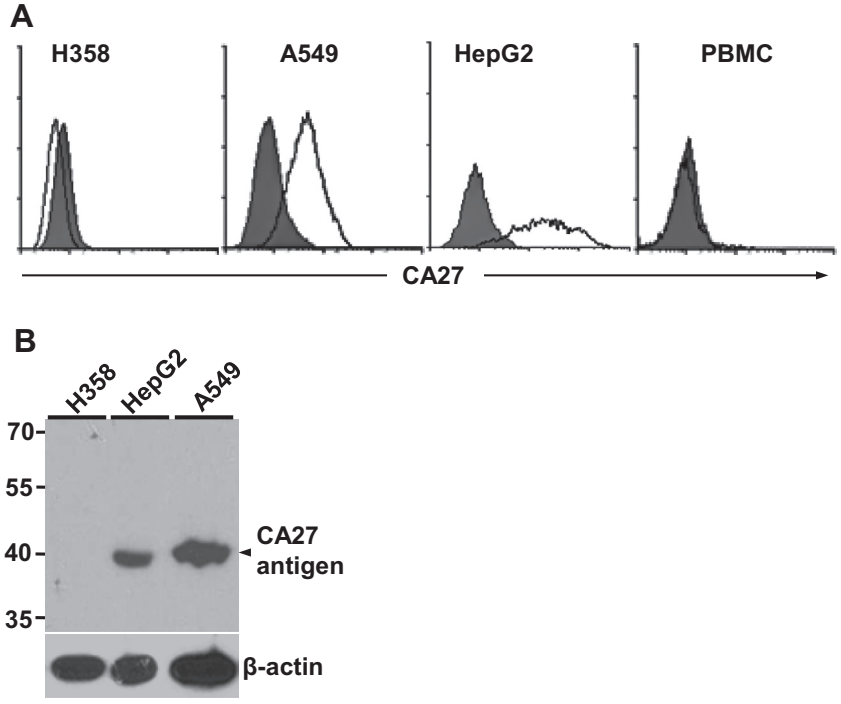


**Fig. 1.** CA27 recognizes an approximately 40 kDa surface protein on A549 and HepG2 cells. (A) Flow cytometric analysis of H358, A549, HepG2, and PBMCs with CA27. (B) Western blot analysis of CA27 antigen in H358, A549 and HepG2 cells.

it as the *M. hyorhinis* p37 protein from a protein database search (Fig. 2). The result suggested that A549 cells we used in this study were infected with *M. hyorhinis*. The p37 protein is found in at least 11 other species of mycoplasmas, and structural alignments reveal the similarity of the p37 protein to various p37-like proteins [5]. To confirm whether CA27 was generated against the mycoplasmal p37 protein originated from mycoplasma infection, therefore, we treated A549 cell with the anti-mycoplasma compound BM-cyclin. When BM-cyclin was added to the A549 cell culture, CA27 antigen expression was drastically suppressed (Supplementary data 2a and 2b). Mycoplasma DNA was also detected from the A549 culture (Supplementary data 2c), indicating that the antigen target of CA27 is the mycoplasmal p37 protein. Expectedly, CA27 expression was not detected in mycoplasma-free control A549 cells (Supplementary data 2a and 2c). The mycoplasma DNA was also detected in HepG2, and removed in BM-cyclin-treated HepG2 cells (Supplementary data 2d). The results indicate that that CA27 recognizes the mycoplasmal p37 protein in A549 and HepG2 cells we used in this study.

### 3.3. Detection of CA27-positive CTCs in the peripheral blood in patients with HCC

Persistent exposure to mycoplasmas such as *M. hyorhinis* is closely associated with oncogenic transformation in human cancers [3,8–10], and the mycoplasmal p37 protein alone increases the invasiveness and metastases of cancer cells [11–13]. The previous studies drove us to study whether the mycoplasmal p37 protein is involved in CTCs in patient with HCC. To analyze CA27-positive cells in the peripheral blood in patients with HCC, we investigated the peripheral blood from 7 patients with HCC (Table 1). PBMCs from 3 patients and 1 healthy donor were directly analyzed after cytospin centrifugation. Double fluorescent labeling with CA27 and anti-CD45 antibodies (hematological lineage cell marker) was performed to detect CTCs in the PBMCs. Confocal microscopic analysis showed that CA27-positive cells were readily detected in patient 2, while they were not detected in healthy donor and patient 1 and 3 (red arrowheads in Supplementary data 3). The result indicates that mycoplasma-infected cells are circulating in the peripheral blood in patient 2 with HCC. However, it was difficult to dissect CTCs by the direct staining method because of excessive numbers of PBMCs.

**Table 1**
Counts of CA27-positive CTCs in patients with HCC.

| Patient | Primary tumor | Pathologic tumor stage | Total CTCs (per 8.5 ml) | Isolation |
|---|---|---|---|---|
| Control | Normal | – | 0 | Direct |
| #01 | HCC | pT1 | 0 | Direct |
| #02 | HCC | pT2 | 24 | Direct |
| #03 | HCC | pT2 | 0 | Direct |
| #04 | HCC | pT2 | 1 | Dynabead |
| #05 | HCC | pT3a | 12 | Dynabead |
| #06 | HCC | pT2 | 30 | Dynabead |
| #07 | HCC | pT2 | 0 | Dynabead |
| A549 (100 cells) | Cell line | Spiked | 22 | Dynabead |
| A549 (100 cells) | Cell line | Spiked | 26 | Dynabead |

To further enrich CA27-positive cells from PBMCs, CA27-positive cells were isolated from PBMCs in four patients by CA27-conjugated magnetic beads. Control experiments were performed in parallel using 100 cells of A549 cell line spiked in blood from healthy donors. The number of isolated A549 cells was ranged from 22 to 26, indicating that isolation efficiency by CA27-conjugated magnetic beads is approximately 24% (Table 1). Confocal microscopic analysis was able to detect CA27-positive cells in 2 patients with HCC (range 12–30/8.5 ml blood) after the isolation of cells by CA27-conjugated-magnetic beads (Table 1, Fig. 3). Some CD45-positive cells were also detected (green arrowheads in Fig. 3), suggesting that the isolation procedure does not remove all hematological lineage cells. Some residual magnetic beads were also detected (black arrowheads and small DAPI spots in Fig. 3). All CA27-positive cells were CD45-negative (red arrowheads in Fig. 3), indicating that they are mycoplasma-infected circulating epithelial cells. When CA27-positive nuclei were morphocytologically

Match to : **gi | 423263247** Score : **153** Expect : 1.2e-08
**High affinity transport system protein p37 precursor**

Matching peptide

| Start-end | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Sequence |
|---|---|---|---|---|---|---|
| 32–44 | 1505.6655 | 1504.6582 | 1504.6794 | -0.0212 | 0 | K.QEDVSVSQGQWDK.S |
| 45–57 | 1451.7216 | 1450.7144 | 1450.7456 | -0.0313 | 0 | K.SITFGVSEAWLNK.K |
| 67–78 | 1481.7646 | 1480.7573 | 1480.7926 | -0.0353 | 1 | K.EVINTFLENFKK.E |
| 100–129 | 3172.6745 | 3171.6672 | 3171.5936 | 0.0736 | 0 | K.VTPIQDSTVLLNNLSTDNPELDFGINASGK.L |
| 130–135 | 748.2812 | 747.2739 | 747.4531 | -0.1792 | 0 | K.LVEFLK.N |
| 136–158 | 2536.2679 | 2535.2607 | 2535.2857 | -0.0250 | 1 | K.NNPGIITPALETTTNSFVFDKEK.D |
| 181–196 | 1936.9060 | 1935.8988 | 1935.9003 | -0.0015 | 0 | K.IFVETPYASWTDENHK.W |
| 234–244 | 1452.6473 | 1451.6400 | 1451.6582 | -0.0182 | 1 | K.AWNDKDWNTFR.N |
| 245–252 | 885.3870 | 884.3797 | 884.4868 | -0.1072 | 0 | R.NFGILHGK.D |
| 259–267 | 1120.6031 | 1119.5958 | 1119.6539 | -0.0581 | 1 | K.FKLEETILK.N |
| 274–281 | 995.3959 | 994.3887 | 994.4720 | -0.0833 | 0 | K.FTTLNEDR.S |
| 292–318 | 2996.3563 | 2995.3490 | 2995.3624 | -0.0134 | 0 | K.SADTLGTLDDFHIAFSEEGSFAWTHNK.S |
| 351–369 | 2172.1669 | 2171.1596 | 2171.1950 | -0.0354 | 0 | K.SVNQLEQNLIVQTFINLAK.N |

**gi|423263247 High affinity transport system protein p37 precursor**

```
  1 MLKKFKNFIL FSSIFSPIAF AISCSNTGVV KQEDVSVSQG QWDKSITFGV SEAWLNKKKG
 61 DKEVNKEVIN TFLENFKKEF NKLKNANDKT KNFDDVDFKV TPIQDSTVLL NNLSTDNPEL
121 DFGINASGKL VEFLKNNPGI ITPALETTTN SFVFDKEKDK FYVDGTDSDP LVKIAKEINK
181 IFVETPYASW TDENHKWNGN VYQSVYDPTV QANFYRGMIW IKGNDETLAK IKKAWNDKDW
241 NTFRNFGILH GKDNSSSKFK LEETILKNHF QNKFTTLNED RSAHPNAYKQ KSADTLGTLD
301 DFHIAFSEEG SFAWTHNKSA TKPFETKANE KMEALIVTNP IPYDVGVFRK SVNQLEQNLI
361 VQTFINLAKN KQDTYGPLLG YNGYKKIDNF QKEIVEVYEK AIK
```

**Fig. 2.** Mass spectrometric identification of CA27 antigen after immunoprecipitation with CA27. The 40 kDa band from A549 lysate was excised from-SDS–PAGE gels and treated with trypsin, and resulting peptides were analyzed by MALDI peptide mass fingerprinting. Peaks were searched against the NCBInr database. 13 tryptic peptides (177 amino acids, underlined) originating from the 40 kDa protein matched the *M. hyorhinis* p37 protein.

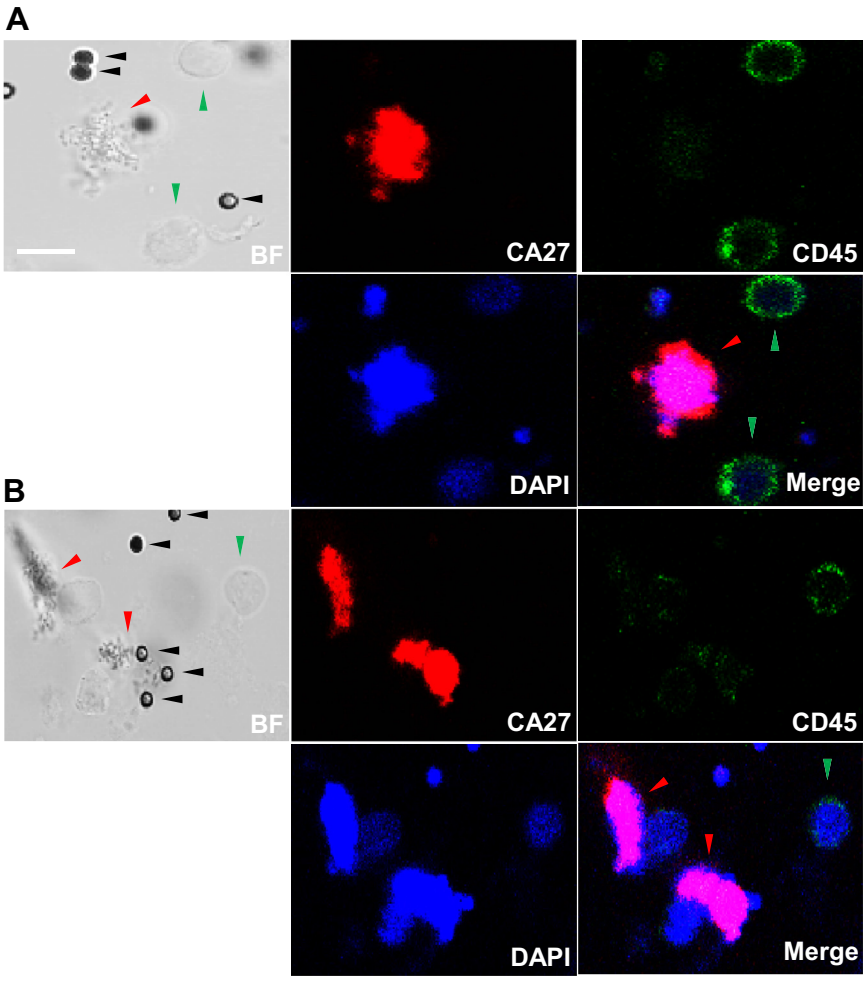


**Fig. 3.** Confocal microscopic analysis of CTCs detected in a patient with HCC. CA27-postive CTCs were enriched using CA27-conjugated magnetic beads and stained with CA27 (red arrowheads) and anti-CD45 antibodies (green arrowheads). Residual magnetic beads are also shown and nonspecifically stained with DAPI (black arrowheads). Nuclei were stained with DAPI. Two different images (A, B) from patient #06 are shown. Bright field images (BF) are also shown. The scale bar is 7.5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

examined by using bright field and DAPI-stained images, CA27-positive cells had larger size than CD45-positive hematological lineage cells. CA27-positive cells also had high nuclear to cytoplasmic ratios and irregular nuclear morphology, which are CTC characteristics [29]. The results indicate that CA27-positive and CD45-negative cells are CTCs.

This report describes the generation of MAb CA27 against the mycoplasmal p37 protein that led to the unintended finding about the presence of mycoplasma-infected CTCs in patients with HCC. Previous studies have shown that chronic mycoplasma infections are closely associated with oncogenic transformation in many human cancers [3,8–10]. Researchers have thought that mycoplasma invades cancer tissues after the disease has started. However, continuous infection of mycoplasma can lead to transformation of mammalian cells, up-regulating expression of oncogenes, and some biologic changes of tumor cells, suggesting close association of mycoplasma infection with tumorigenesis [10,30]. Furthermore, recent studies have shown that the mycoplasmal p37 protein alone increases the invasiveness and metastasis of cancer cells [11–13], suggesting that mycoplasma infection and its products can promote cancer metastasis. Many researchers still do not believe that mycoplasma infection itself is a direct cause of tumorigenesis and metastasis in the cancer patients. Instead, they prefer to believe that mycoplasma infection may play a potential exacerbating role in tumorigenesis and metastasis. Thus, the exact correlation between mycoplasma infection and cancer metastasis has still remained elusive.

Mycoplasma infection was reported in many cancer tissues such as gastric, colon, esophageal, lung, breast, glioma, renal and prostate carcinomas [8–10,12]. However, no study has been reported about the presence of mycoplasma infection in hepatocellular carcinoma. The present study is the first report about mycoplasma infection in the peripheral circulating epithelial cells in patients with HCC. Although a latest study found antibodies against the *M. hyorhinis* p37 protein in the serum of men with newly diagnosed prostate cancer [31], mycoplasma-infected circulating epithelial cells have not been reported yet. In this study, we could detect mycoplasma-infected CTCs in the peripheral blood in patients with HCC. Our results do not disclose the relationship between mycoplasma infection and cancer progression. However, the presence of mycoplasma-infected CTCs suggests that mycoplasma infection may accelerate cancer metastasis in patients with HCC, because the detection of CTCs is considered as an important indicator in the metastatic cascade. Thus, the present study provides a potential connection between mycoplasma infections and the progression of cancer. Further investigation in a larger cohort of HCC patients could verify its clinical relevance.

## Acknowledgments

This study was supported in part by the National Research Foundation of Korea (NRF-2006-2004097 and NRF-2013R1A1A 2012056) and the Converging Research Center Program funded by the Ministry of Education, Science and Technology (Project No. 2013K000272). This study was also supported by Samsung Biomedical Research Institute, grant C-B1-104-1/2/3.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.024.

## References

[1] K. Oikonomopoulou, D. Brinc, K. Kyriacou, E.P. Diamandis, Infection and cancer: revaluation of the hygiene hypothesis, Clin. Cancer Res. 19 (2013) 2834–2841.
[2] S. Tsai, D.J. Wear, J.W. Shih, S.C. Lo, Mycoplasmas and oncogenesis: persistent infection and multistage malignant transformation, Proc. Natl. Acad. Sci. USA 92 (1995) 10197–10201.
[3] L.K. Dennis, C.F. Lynch, J.C. Torner, Epidemiologic association between prostatitis and prostate cancer, Urology 60 (2002) 78–83.
[4] S. Razin, D. Yogev, Y. Naot, Molecular biology and pathogenicity of mycoplasmas, Microbiol. Mol. Biol. Rev. 62 (1998) 1094–1156.
[5] K.H. Sippel, A.H. Robbins, R. Reutzel, S.K. Boehlein, K. Namiki, S. Goodison, M. Agbandje-McKenna, C.J. Rosser, R. McKenna, Structural insights into the extracytoplasmic thiamine-binding lipoprotein p37 of *Mycoplasma hyorhinis*, J. Bacteriol. 191 (2009) 2585–2592.
[6] C. Steinemann, M. Fenner, R.W. Parish, H. Binz, Studies of the invasiveness of the chemically induced mouse sarcoma FS9. I. Monoclonal antibodies to a 37,000 Dalton membrane glycoprotein inhibit invasion of fibroblasts in vitro, Int. J. Cancer 34 (1984) 407–414.
[7] R. Dudler, C. Schmidhauser, R.W. Parish, R.E. Wettenhall, T. Schmidt, A mycoplasma high-affinity transport system and the in vitro invasiveness of mouse sarcoma cells, EMBO J. 7 (1988) 3963–3970.
[8] M. Pehlivan, S. Pehlivan, H. Onay, M. Koyuncuoglu, Z. Kirkali, Can mycoplasma-mediated oncogenesis be responsible for formation of conventional renal cell carcinoma?, Urology 65 (2005) 411–414
[9] S. Huang, J.Y. Li, J. Wu, L. Meng, C.C. Shou, Mycoplasma infections and different human carcinomas, World J. Gastroenterol. 7 (2001) 266–269.
[10] J.Y. Ning, C.C. Shou, Mycoplasma infection and cancer, Ai Zheng 23 (2004) 602–604.
[11] C.M. Ketcham, S. Anai, R. Reutzel, S. Sheng, S.M. Schuster, R.B. Brenes, M. Agbandje-McKenna, R. McKenna, C.J. Rosser, S.K. Boehlein, P37 induces tumor invasiveness, Mol. Cancer Ther. 4 (2005) 1031–1038.
[12] S. Goodison, K. Nakamura, K.A. Iczkowski, S. Anai, S.K. Boehlein, C.J. Rosser, Exogenous mycoplasmal p37 protein alters gene expression, growth and morphology of prostate cancer cells, Cytogenet. Genome. Res. 118 (2007) 204–213.
[13] M. Gong, L. Meng, B. Jiang, J. Zhang, H. Yang, J. Wu, C. Shou, P37 from *Mycoplasma hyorhinis* promotes cancer cell invasiveness and metastasis through activation of MMP-2 and followed by phosphorylation of EGFR, Mol. Cancer Ther. 7 (2008) 530–537.
[14] W.J. Allard, J. Matera, M.C. Miller, M. Repollet, M.C. Connelly, C. Rao, A.G. Tibbe, J.W. Uhr, L.W. Terstappen, Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases, Clin. Cancer Res. 10 (2004) 6897–6904.
[15] K. Pantel, R.H. Brakenhoff, B. Brandt, Detection, clinical relevance and specific biological properties of disseminating tumour cells, Nat. Rev. Cancer 8 (2008) 329–340.
[16] S. Nagrath, L.V. Sequist, S. Maheswaran, D.W. Bell, D. Irimia, L. Ulkus, M.R. Smith, E.L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U.J. Balis, R.G. Tompkins, D.A. Haber, M. Toner, Isolation of rare circulating tumour cells in cancer patients by microchip technology, Nature 450 (2007) 1235–1239.
[17] M. Yu, A. Bardia, B.S. Wittner, S.L. Stott, M.E. Smas, D.T. Ting, S.J. Isakoff, J.C. Ciciliano, M.N. Wells, A.M. Shah, K.F. Concannon, M.C. Donaldson, L.V. Sequist, E. Brachtel, D. Sgroi, J. Baselga, S. Ramaswamy, M. Toner, D.A. Haber, S. Maheswaran, Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition, Science 339 (2013) 580–584.
[18] N. Tiwari, A. Gheldof, M. Tatari, G. Christofori, EMT as the ultimate survival mechanism of cancer cells, Semin. Cancer Biol. 22 (2012) 194–207.
[19] C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis, Science 331 (2011) 1559–1564.
[20] N. Bednarz-Knoll, C. Alix-Panabieres, K. Pantel, Plasticity of disseminating cancer cells in patients with epithelial malignancies, Cancer Metastasis Rev. 31 (2012) 673–687.
[21] S. Thomson, E. Buck, F. Petti, G. Griffin, E. Brown, N. Ramnarine, K.K. Iwata, N. Gibson, J.D. Haley, Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition, Cancer Res. 65 (2005) 9455–9462.
[22] J. Tauler, E. Zudaire, H. Liu, J. Shih, J.L. Mulshine, HnRNP A2/B1 modulates epithelial-mesenchymal transition in lung cancer cell lines, Cancer Res. 70 (2010) 7137–7147.
[23] H.S. Choi, H. Kim, A. Won, J.J. Kim, C.Y. Son, K.S. Kim, J.H. Ko, M.Y. Lee, C.H. Kim, C.J. Ryu, Development of a decoy immunization strategy to identify cell-surface molecules expressed on undifferentiated human embryonic stem cells, Cell Tissue Res. 333 (2008) 197–206.
[24] H.S. Choi, W.T. Kim, H. Kim, J.J. Kim, J.Y. Ko, S.W. Lee, Y.J. Jang, S.J. Kim, M.J. Lee, H.S. Jung, J. Kzhyshkowska, S.J. Um, M.Y. Lee, S.H. Lee, C.H. Kim, C.J. Ryu, Identification and characterization of adenovirus early region 1B-associated protein 5 as a surface marker on undifferentiated human embryonic stem cells, Stem Cells Dev. 20 (2011) 609–620.
[25] H.S. Choi, H.M. Lee, Y.J. Jang, C.H. Kim, C.J. Ryu, Heterogeneous nuclear ribonucleoprotein A2/B1 regulates the selfrenewal and pluripotency of human embryonic stem cells via the control of the G1/S transition, Stem Cells 31 (2013) 2647–2658.
[26] H. Jee, S.H. Lee, J.W. Park, B.R. Lee, K.T. Nam, D.Y. Kim, Connexin32 inhibits gastric carcinogenesis through cell cycle arrest and altered expression of p21Cip1 and p27Kip1, BMB Rep. 46 (2013) 25–30.
[27] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[28] W. Zhang, B.T. Chait, ProFound: an expert system for protein identification using mass spectrometric peptide mapping information, Anal. Chem. 72 (2000) 2482–2489.
[29] T. Fehm, E.F. Solomayer, S. Meng, T. Tucker, N. Lane, J. Wang, G. Gebauer, Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells, Cytotherapy 7 (2005) 171–185.
[30] K. Namiki, S. Goodison, S. Porvasnik, R.W. Allan, K.A. Iczkowski, C. Urbanek, L. Reyes, N. Sakamoto, C.J. Rosser, Persistent exposure to mycoplasma induces malignant transformation of human prostate cells, PLoS ONE 4 (2009) e6872.
[31] C. Urbanek, S. Goodison, M. Chang, S. Porvasnik, N. Sakamoto, C.Z. Li, S.K. Boehlein, C.J. Rosser, Detection of antibodies directed at *M. hyorhinis* p37 in the serum of men with newly diagnosed prostate cancer, BMC Cancer 11 (2011) 233.