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Pivotal role of epithelial cell adhesion molecule in the survival of lung cancer cells

Tetsunari Hase¹, Mitsuo Sato^{1,6}, Kenya Yoshida¹, Luc Girard⁵, Yoshihiro Takeyama¹, Mihoko Horio¹, Momen Elshazley¹, Tomoyo Oguri¹, Yoshitaka Sekido^{2,3}, David S. Shames⁴, Adi F. Gazdar⁵, John D. Minna⁵, Masashi Kondo¹, and Yoshinori Hasegawa¹

¹Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya

²Department of Cancer Genetics, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya

³Division of Molecular Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya, Japan

⁴Oncology Diagnostics, Genentech Inc., South San Francisco, California

⁵Hamon Center for Therapeutic Oncology Research and the Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA

Abstract

Epithelial cell adhesion molecule (EpCAM) is overexpressed in a wide variety of human cancers including lung cancer, and its contribution to increased proliferation through upregulation of cell cycle accelerators such as cyclins A and E has been well established in breast and gastric cancers. Nevertheless, very little is known about its role in supporting the survival of cancer cells. In addition, the functional role of *EpCAM* in the pathogenesis of lung cancer remains to be explored. In this study, we show that RNAi-mediated knockdown of *EpCAM* suppresses proliferation and clonogenic growth of three *EpCAM*-expressing lung cancer cell lines (H3255, H358, and HCC827), but does not induce cell cycle arrest in any of these. In addition, *EpCAM* knockdown inhibits invasion in the highly invasive H358 but not in less invasive H3255 cells in a Transwell assay. Of note, the *EpCAM* knockdown induces massive apoptosis in the three cell lines as well as in another *EpCAM*-expressing lung cancer cell line, HCC2279, but to a much lesser extent in a *cdk4/hTERT* immortalized normal human bronchial epithelial cell line, HBEC4, suggesting that EpCAM could be a therapeutic target for lung cancer. Finally, *EpCAM* knockdown partially restores contact inhibition in HCC827, in association with p27^{Kip1} upregulation. These results indicate that *EpCAM* could contribute substantially to the pathogenesis of lung cancer, especially cancer cell survival, and suggest that *EpCAM* targeted therapy for lung cancer may have potential.

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⁶To whom correspondence should be addressed. msato@med.nagoya-u.ac.jp.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of cells used for microarray analysis.

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Lung cancer is the leading cause of cancer deaths worldwide with a 5-year survival rate of approximately 15%.⁽¹⁾ The high mortality rate is due to late detection and the disease's inherent resistance to available therapeutics.^(2,3) There are a number of novel therapeutics designed to target common molecular changes in lung cancer, some of which have shown clinical benefits.⁽⁴⁾ These recent data, along with the advent of large-scale cell line screening efforts now underway in academia and the pharmaceutical industry, herald a new era for lung cancer therapy and will hopefully improve the situation for patients with lung cancer.⁽⁵⁾

The transmembrane glycoprotein, epithelial cell adhesion molecule (EpCAM), was the first tumor-associated antigen identified by means of mAbs.^(6,7) Subsequently, it was shown to be overexpressed in a great variety of human cancers, including lung, esophagus, gastric, breast, colorectal, and hepatocellular carcinomas,⁽⁸⁾ and therefore several anti-EpCAM antibodies have been developed and tested on patients with breast or colorectal cancer in clinical trials.⁽⁹⁾ Nevertheless, clinical benefits of anti-EpCAM antibodies were not determined in these studies, possibly because the EpCAM expression was not used as a biomarker for selection or stratification purposes.⁽¹⁰⁾

EpCAM was initially recognized as a disease marker, and subsequent studies revealed that in many types of cancers its expression correlated with poor patient prognosis, suggesting that it might contribute to carcinogenesis.⁽¹¹⁾ This finding has prompted investigators to explore the molecular basis of EpCAM-induced carcinogenesis. Munz *et al.*⁽¹²⁾ first showed that EpCAM directly contributes to carcinogenesis by inducing proliferation in breast cancer cells by upregulating c-Myc and cyclins A and E. These findings were confirmed by Osta *et al.*,⁽¹³⁾ who also showed that EpCAM contributes to proliferation, migration, and invasion of breast cancer cells. Furthermore, a study by Gires' group indicated that EpCAM is activated by intramembrane proteolysis, further elucidating the molecular mechanism of EpCAM-induced proliferation.⁽¹⁴⁾ These results show that EpCAM increases cellular proliferation as well as the invasiveness of human cancer. However, it is unclear whether EpCAM has any role in the resistance of cancer cells to apoptotic signaling, which is another important ability of cancer cells, in addition to increased proliferation.⁽¹⁵⁾

Epithelial cell adhesion molecule is frequently overexpressed in lung cancer⁽⁸⁾ and thus could be a promising therapeutic target for this disease. Nevertheless, its functional role in the pathogenesis of lung cancer remains to be thoroughly explored. One published report studying the contribution of EpCAM expression to the invasive phenotype of lung cancer showed that EpCAM expression inhibited tumor invasion and progression, suggesting a tumor-suppressive function.⁽¹⁶⁾ This result, however, contradicts the oncogenic role of EpCAM in other types of cancers reported by several studies.^(12,13) Thus, the functional role of EpCAM in lung cancer has been a matter of debate.

We designed this study to examine the contribution of EpCAM to the pathogenesis of lung cancer with particular focus on the anti-apoptotic phenotype. We found that RNAi-mediated *EpCAM* knockdown suppressed proliferation and the clonogenic growth of lung cancer cells in both anchorage-dependent and -independent conditions. Moreover, *EpCAM* knockdown induced massive apoptosis in lung cancer cell lines but to a much lesser extent in an immortalized normal bronchial epithelial cell line. These findings show that *EpCAM* has an essential role in the malignant phenotype of lung cancer and thus could serve as an attractive therapeutic target for this highly lethal disease.

Materials and Methods

Cell culture

Cell lines used in this study were purchased from ATCC (Manassas, VA, USA) or obtained from the Hamon Center collection (University of Texas Southwestern Medical Center, Dallas, TX, USA). Cell lines used for quantitative real-time PCR and/or functional assays included 18 non-small-cell lung cancer (NSCLC) cell lines, PC9, A549, NCI-H157, NCI-H358, NCI-H460, NCI-H820, NCI-H838, NCI-H1155, NCI-H1299, NCI-H1666, NCI-H1650, NCI-H1975, NCI-H3255, HCC827, HCC2279, HCC2935, HCC4006, and HCC4011 (cells with mutations in the epidermal growth factor receptor [*EGFR*] gene are underlined), and the *cdk4/hTERT*-immortalized normal human bronchial epithelial cell line HBEC4.^(17,18) In addition, 103 NSCLC cell lines and 51 normal controls (11 normal human lung cultures and 40 immortalized normal human bronchial epithelial cell lines), some of which overlap with the above 19 cell lines, were used for microarray expression analysis. These cell lines are listed in Table S1. Lung cancer cell lines were cultured in RPMI-1640 supplemented with 10% FBS, and normal control cells were cultured in K-SFM medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 50 ng/mL bovine pituitary extract and 5 ng/mL epidermal growth factor.

Quantitative real-time PCR analysis

Total RNA (5 µg) isolated using Trizol (Invitrogen, Carlsbad, CA, USA) was reverse transcribed with a SuperScript III First-Strand Synthesis System using a Random primer system (Invitrogen). Quantitative real-time PCR analysis of *EpCAM* was carried out as described previously.⁽¹⁹⁾ *GAPDH* (Assayon-Demand; Applied Biosystems, Foster City, CA, USA) was used as an internal control.

Microarray analysis

RNA quality and concentration were checked by the Experion Bioanalyzer (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Total RNA (500 ng) from each sample was used to label the cRNA probes by the Illumina TotalPrep RNA Amplification kit (Cat# IL1791; Ambion, Austin, TX, USA). Amplified and labeled cRNA probes (1.5 µg) were hybridized to Illumina Human WG-6 v3.0 Expression BeadChip (Cat# BD-101-0203; Ambion) overnight at 58°C, then washed, blocked, and detected by streptavidin-Cy3 following the manufacturer's protocol. After drying, the chips were scanned by an Illumina iScan system (Ambion). Bead-level data were obtained, and pre-processed using the R package Model-Based Background Correction (MBCB) for background correction and probe summarization. Pre-processed data were then quantile-normalized and log-transformed.

Analysis of EpCAM expression by flow cytometry

Cultured cells were prepared in single cell suspensions and incubated with an appropriate dilution of the control or specific antibody. The cells were incubated for 30 min with mouse monoclonal anti-EpCAM (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then washed and incubated with Alexa Fluor 488-conjugated (Molecular Probes, Eugene, OR, USA) secondary antibody for 30 min and washed again before analysis using a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Transfection with siRNA

H3255, H358, HCC827, or HCC2279 cells (4.0×10^5), or HBEC4 cells (1.0×10^5) were plated in 10 cm dishes. The next day, cells were transiently transfected with either 10 nM solution of two predesigned non-overlapping siRNA (Stealth Select RNAi, Invitrogen)

targeting *EpCAM* or control siRNA oligos (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. After 48 h, the transfected cells were harvested for further analyses or plated for cell growth assays.

Viral vectors and viral transduction

The lentiviral vectors directing expression of shRNA specific to *EpCAM* (TRCN0000073733) (Sigma-Aldrich, St. Louis, MO, USA) or *GFP* were used. Viral production and transduction were done as described previously.⁽²⁰⁾

Western blot analysis

Western blot analysis was done as described previously using whole cell lysates.⁽²⁰⁾ Primary antibodies used were mouse monoclonal anti-*EpCAM*, (Thermo Fisher Scientific), mouse monoclonal anti-E-cadherin (BD Biosciences), rabbit polyclonal anti-p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-actin (Sigma-Aldrich) antibodies. Actin protein levels were measured as a control for the adequacy of equal protein loading. Anti-rabbit or anti-mouse (GE Healthcare, Buckinghamshire, England, UK) was used at a 1:2000 dilution as the second antibody. Protein expression levels were quantified using densitometry.

In vitro cell growth assay

Colorimetric proliferation assay was carried out using a WST-1 assay kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Liquid and soft agar colony formation assays were done as described previously.⁽²⁰⁾

Cell cycle analysis

Cells were harvested 5 days after the transfection of siRNA oligos, fixed with 70% ethanol, resuspended in 0.5 mL PBS containing 200 µg RNase per mL and 20 µg propidium iodide per mL, incubated at 37°C for 30 min, and maintained at 4°C until flow cytometry analysis on a FAC-SCalibur. Results were analyzed using a Mod-Fit cell cycle analysis program (Verity Software House, Topsham, ME, USA).

Apoptosis analysis

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in apoptotic cells using a phycoerythrin (PE)-Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were harvested 5 days after the transfection of siRNA oligos, treated according to the manufacturer's instructions, and measured with PE/7-amino-actinomycin D (7-AAD) staining using flow cytometry on a FACSCalibur.

In vitro invasion assay

Invasion of cells was examined using 24-well BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions. Cells (2.5×10^4) stably transfected with shGFP- or sh*EpCAM*-expressing vectors were suspended in medium supplemented with 10% FBS and added to inserts containing 8-µm pore PET membranes layered with Matrigel. After being incubated for 48 h at 37°C, the cells that passed through the membrane were stained using a Diff-Quik kit (Sysmex, Kobe, Japan), and all invading cells were counted. Experiments were repeated twice in triplicate.

Statistics

SPSS version 18 software (SPSS, International Business Machines [IBM], Armonk, NY, USA) was used for all statistical analyses in this study. The Mann-Whitney *U*-test was used

for analyzing the difference between two groups. Pearson's correlation coefficients with associated *P*-values were calculated between mRNA and protein expression levels of EpCAM in lung cancer cell lines.

Results

Lung cancer cell lines express higher levels of *EpCAM* than normal lung epithelial cell lines

We carried out quantitative real-time PCR analyses of *EpCAM* in 18 NSCLC cell lines including 10 adenocarcinoma-derived cell lines that have mutations in the *EGFR* gene as well as one immortalized normal human bronchial epithelial cell line (HBEC4). *EpCAM* mRNA expression varied broadly across the lung cancer cell lines and 13 (72%) NSCLC cell lines expressed higher levels of *EpCAM* than HBEC4 (Fig. 1A). The *EGFR* mutant cell lines expressed higher levels of *EpCAM* than *EGFR* wild-type lines (60.3 vs 7.9, Mann–Whitney *U*-test, *P* = 0.003). We also used EpCAM Western blot analysis for the same set of cell lines and found good correlation between mRNA and protein expression of EpCAM (Pearson's correlation coefficient = 0.61, *P* < 0.003). We determined the cell surface expression of EpCAM by flow cytometry for eight cell lines selected from the above 19 cell lines. All the eight cell lines expressed EpCAM surface protein at levels consistent with their mRNA and total protein expression of *EpCAM* (Fig. 1B). Furthermore, we examined a large panel of NSCLC (*n* = 103) and normal lung controls (normal human lung cultures and immortalized normal human bronchial epithelial cell lines; *n* = 51) for *EpCAM* mRNA expression using microarray expression analysis. *EpCAM* expression was elevated significantly by 18.3-fold (Mann–Whitney *U*-test, *P* < 0.001) in NSCLC cell lines compared to normal lung controls (Fig. 1C). These results indicate that the majority of NSCLC cell lines overexpress *EpCAM*.

EpCAM knockdown suppresses growth of lung cancer cells

To test the role of EpCAM expression in the pathogenesis of lung cancer cells, we carried out RNAi-mediated gene silencing of *EpCAM*. A number of studies have shown that *EGFR* mutant and *EGFR* wild-type lung cancers are very different in biological as well as clinicopathological characteristics.⁽²⁾ Thus, we chose one *EGFR* mutant and one *EGFR* wild-type cell line for initial analysis: H3255 representing *EGFR* mutant cell lines; and H358 representing *EGFR* wild-type cell lines. To minimize the possibility of “off-target” effects, we used two non-overlapping synthesized oligos targeting *EpCAM*, purchased from Invitrogen. We obtained a nearly complete knockdown of EpCAM at both the mRNA and protein levels (Fig. 2A,B). To evaluate the effect of *EpCAM* knockdown on cellular proliferation, we used WST-1 colorimetric assays and found that the *EpCAM* knockdown suppressed proliferation to 40–60% in both cell lines (Fig. 2C). The effects of the *EpCAM* knockdown on clonal growth were measured by a liquid colony formation assay. The *EpCAM* knockdown suppressed colony formation to 20–40% in H3255 cells and to 40–60% in H358 cells (Fig. 2D). Next, to evaluate the effects of the *EpCAM* knockdown on anchorage-independent growth, we carried out a soft agar colony formation assay for H358 (parental H3255 cells do not form colonies in soft agar). The *EpCAM* knockdown significantly inhibited growth of H358 in soft agar (Fig. 2E). To find additional cell lines that could be used for the soft agar assay we tested three more EpCAM-expressing cell lines (HCC2279, HCC4006, and HCC4011) for their colony forming ability in soft agar, but none of them formed colonies. These results suggest that EpCAM expression does not necessarily confer anchorage-independent growth ability on lung cancer cells, but a subset of lung cancer cells may require EpCAM expression for their anchorage-independent growth.

Knockdown of *EpCAM* induces massive apoptosis in lung cancer cells

Whether *EpCAM* supports the survival of lung cancer cells is unknown. In addition to the H3255 and H358 cell lines used above, we added two cell lines for this analysis: the HCC2279 *EGFR* mutant line and the *cdk4/hTERT*-immortalized human bronchial epithelial cell line HBEC4. Both HCC2279 and HBEC4 express comparable levels of *EpCAM*, which in turn are less than those expressed by H3255 or H358 (Fig. 1A). We carried out transient *EpCAM* knockdown in HCC2279 and HBEC4 and obtained clear *EpCAM* knockdown in both cell lines (Fig. 3A,B). We determined the cell cycle profiles of cells transfected with *EpCAM* or control siRNA and found that the *EpCAM* knockdown resulted in a dramatic increase in cells in the sub-G₁ population in the three lung cancer cell lines (Fig. 3C). By contrast, the *EpCAM* knockdown in HBEC4 resulted in only a subtle increase in the sub-G₁ population. Flow cytometric analysis of cells stained with 7-AAD and annexin V showed that *EpCAM* knockdown resulted in a significant increase in the population of the high annexin V and low 7-AAD cells, suggesting that *EpCAM* knockdown leads to the induction of apoptosis in all the three lung cancer cell lines analyzed (Fig. 3D). By contrast, only a small population of HBEC4 underwent apoptosis after the *EpCAM* knockdown. These results indicate that lung cancer cell lines, but not a normal bronchial epithelial cell line, depend largely on *EpCAM* expression for their survival.

EpCAM knockdown does not induce G₁ cell cycle arrest in lung cancer cell lines

Previous studies have shown that silencing *EpCAM* causes G₁ cell cycle arrest primarily by upregulating cyclin D1.⁽²¹⁾ H3255 and H358 cells expressing *GFP* or *EpCAM* shRNA were grown in 0.1% serum medium for 24 h for synchronization, then their cell cycle profiles were analyzed. Cell cycle analysis revealed that the *EpCAM* knockdown does not induce G₁ cell cycle arrest (data not shown).

Knockdown of *EpCAM* restores contact inhibition in lung cancer cells in part through upregulating p27^{Kip1} cyclin-dependent kinase inhibitor

Next, we examined whether *EpCAM* knockdown restores contact inhibition in lung cancer cell lines lacking contact inhibition. To this end, we first tested three lung cancer cell lines for their contact inhibition. After reaching confluence, H358 and H3255 cells stopped growing and remained as a monolayer culture, whereas HCC827, which expressed *EpCAM* protein at high levels (Fig. 1A), continued to grow, indicating contact inhibition was lost in HCC827 cells. Therefore, we used HCC827 for evaluating the effects of *EpCAM* knockdown on contact inhibition. By transfecting *EpCAM* shRNA-expressing vector we obtained clear *EpCAM* knockdown in HCC827 cells (Fig. 4A). HCC827 cells expressing *GFP* shRNA almost completely lost their contact inhibition, as shown by their continuous growth after reaching confluence (Fig. 4B, lower). By contrast, cells expressing *EpCAM* shRNA dramatically reduced their growth rate, remaining a monolayer (Fig. 4B, upper). Cell cycle analysis with propidium iodide staining, followed by FACS, revealed that in confluent culture the percentage of cells in S phase decreased in HCC827 cells expressing *EpCAM* shRNA compared to HCC827 cells expressing *GFP* shRNA (23% vs 37%; Fig. 4C), suggesting increased contact inhibition in HCC827 cells expressing *EpCAM* shRNA. A previous study of thyroid carcinomas showed that the loss of contact inhibition due to functional abrogation of E-cadherin occurs in part through downregulation of p27^{Kip1} cyclin-dependent kinase inhibitor,⁽²²⁾ thus we examined whether p27^{Kip1} was upregulated in cells with restored contact inhibition after *EpCAM* knockdown. We found p27^{Kip1} to be increased in *EpCAM* shRNA-transfected cells (Fig. 4D), suggesting that *EpCAM* knockdown-induced contact inhibition might occur through p27^{Kip1} upregulation. We evaluated the effects of *EpCAM* knockdown on growth and apoptosis in HCC827 cells. As seen in H3255 and H358, *EpCAM* knockdown significantly suppressed proliferation and clonogenic growth of HCC827, as well as induced apoptosis (Fig. 4E–G).

Knockdown of *EpCAM* suppresses invasiveness in H358 with highly invasive phenotype but not in H3255 with poorly invasive phenotype

We performed *in vitro* tumor invasion assays for H3255 and H358 cells stably transfected with *EpCAM* shRNA-expressing vectors. We obtained clear *EpCAM* knockdown in H3255 and H358 by transfecting *EpCAM* shRNA-expressing vector (Fig. 5A). H3255 and H358 showed different invasive capabilities: H358 was highly invasive, whereas H3255 was less so. H358 expressing *EpCAM* shRNA showed significantly decreased invasiveness compared to H358 expressing *GFP* shRNA (Fig. 5B), but this effect was not seen in H3255. These results suggest that *EpCAM* expression plays a role in the invasive phenotype of lung cancer, at least in highly invasive cells.

Discussion

In the present study, we showed for the first time that *EpCAM* substantially contributes to survival of lung cancer cells. Importantly, *EpCAM* knockdown induced massive apoptosis in lung cancer cell lines but not in an immortalized normal human bronchial epithelial cell line, suggesting that *EpCAM*-targeted therapy for lung cancer could have a high therapeutic value. In addition, we found that *EpCAM* knockdown suppressed proliferation, colony formation in both liquid (anchorage-dependent) and soft agar (anchorage-independent), as well as invasiveness in a Transwell assay, and partially restored their contact inhibition through upregulating p27^{Kip1} in lung cancer cell lines. These findings indicated that *EpCAM* expression is important to lung cancer pathogenesis and may be an attractive therapeutic target for lung cancer.

Knockdown of *EpCAM* resulted in massive apoptosis in HCC2279 but not in normal control HBEC4, although both cell lines express similar levels of *EpCAM* (in both cases, lower than those in H3255 and H358). These findings suggest that HCC2279 but not HBEC4 depends on *EpCAM* expression for survival. This observation might be explained by the concept of “oncogene addiction,” which was first introduced by Weinstein⁽²³⁾ to describe the phenomenon that cancer cells depend on one or few oncogenes for the maintenance of malignant phenotype. Thus, our finding suggests that *EpCAM* functions as an oncogene in lung cancer.

We found that *EGFR* mutant lines expressed higher levels of *EpCAM* than *EGFR* wild-type lines. Recently, we and another research group showed that *EGFR* mutant tumors frequently show an epithelial phenotype, as shown by high E-cadherin (epithelial marker) and low vimentin (mesenchymal marker) levels.^(19,24) Epithelial cell adhesion molecule is also an epithelial marker, and therefore the observed high *EpCAM* expression in *EGFR* mutant lines might reflect their epithelial phenotype. However, considering the critical role of *EpCAM* in the pathogenesis of lung cancer shown in this study, it is possible that *EGFR* mutant lines may be more dependent on *EpCAM* expression for their malignant phenotype than *EGFR* wild-type lines.

In the present study, *EpCAM* knockdown resulted in a significant apoptotic phenotype but did not induce cell cycle arrest in lung cancer cells. Although a number of published reports have established the role of *EpCAM* as a cell cycle accelerator in human cancers, the contribution of *EpCAM* to cell survival has not been determined. To our knowledge, only one published report showed that *EpCAM* knockdown caused apoptosis in a colon cancer cell line, HCT-8.⁽¹⁴⁾ A recent paper has shown that *EpCAM* knockdown induced G₁ cell cycle arrest but not apoptosis in gastric cancer.⁽²¹⁾ These findings suggest that *EpCAM* knockdown-induced apoptosis might be specific for lung cancer cells. Alternatively, it is possible that the degree of *EpCAM* depletion could determine which type of cell fates cancer cells undergo, cell cycle arrest or apoptosis. Although we obtained nearly complete

depletion of EpCAM expression, the recent study mentioned above showed only partial *EpCAM* knockdown in gastric cancer cell lines.⁽²¹⁾ Also, it would be important to investigate the molecular mechanisms responsible for *EpCAM* knockdown-induced apoptosis. One of the key players in apoptosis, phosphatidylinositol 3-kinase (PI3K), may be implicated in this apoptosis because EpCAM was shown to modulate cadherins by modulating PI3K.⁽²⁵⁾

Knockdown of *EpCAM* caused HCC827 cells to partially restore their contact inhibition in association with p27^{Kip1} upregulation. Loss of contact inhibition is one of the hallmarks of cancer cells.⁽¹⁵⁾ However, the underlying mechanisms of contact inhibition have not been fully elucidated. Studies have shown that E-cadherin plays a central role in contact inhibition.⁽²⁶⁾ Previous studies have shown that EpCAM modulates E-cadherin function by reducing cytoskeleton-anchored E-cadherin without changing its total expression level.^(13,27) Another study has shown that functional loss of E-cadherin resulted in downregulation of p27^{Kip1}, leading thyroid carcinoma cells to lose contact inhibition.⁽²²⁾ Collectively, these findings suggest that the increased contact inhibition induced by *EpCAM* knockdown might be caused by p27^{Kip1} upregulation resulting from the functional abrogation of E-cadherin.

Invasiveness is key to malignant progression and strongly influences the ability of cancer cells to metastasize.⁽¹⁵⁾ Because EpCAM expression correlates with poor patient prognosis in many types of human cancers, it is plausible that EpCAM expression contributes to the invasive phenotype of cancer cells. However, results from published *in vitro* invasion assays have yielded contradictory results, although different types of cells were analyzed. Two studies showed that *EpCAM* knockdown suppressed invasiveness and migration in breast cancer cells.^(13,28) By contrast, Tai *et al.* showed that *EpCAM* knockdown increased invasiveness and *EpCAM* overexpression decreased invasiveness in a bladder carcinoma cell line.⁽¹⁶⁾ Furthermore, they found that EpCAM expression in lung adenocarcinoma tissues was inversely correlated with advanced clinical stages and lymph node involvement, suggesting that EpCAM expression may suppress invasiveness in lung cancer.⁽¹⁶⁾ In our study, *EpCAM* knockdown decreased invasiveness in the highly invasive cell line H358, suggesting that *EpCAM* expression plays an important role in the invasiveness of lung cancer. Because invasiveness has been studied in only a limited number of lung cancer cells, further analysis using more cell lines will be needed to confirm the role of EpCAM expression in the invasive phenotype of lung cancer cells.

In summary, we showed that *EpCAM* knockdown inhibited tumor growth and resulted in massive apoptosis of lung cancer cells, indicating that it is a promising therapeutic target for lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Lung cancer cell lines express higher levels of epithelial cell adhesion molecule (*EpCAM*) than normal lung epithelial cells. (A, Upper panel) Quantitative real-time PCR analysis of *EpCAM* in 18 non-small-cell lung cancer (NSCLC) cell lines and an immortalized normal human bronchial epithelial cell line (HBEC4). Expression levels are standardized by *GAPDH* expression. *Cell line with a mutation in the epidermal growth factor receptor gene (*EGFR*). The *EGFR* mutant cell lines expressed *EpCAM* at higher levels than *EGFR* wild-type lines (60.3 vs 7.9, Mann-Whitney *U*-test, $P = 0.003$). (Lower panel) Western blot analysis of *EpCAM* for 18 NSCLC cell lines and an immortalized normal human bronchial epithelial cell line. Actin was used as loading control. (B) Levels of *EpCAM* surface

expression determined by flow cytometry using anti-EpCAM antibody. Eight lung cancer cell lines were analyzed. (C) *EpCAM* mRNA expression determined by microarray analysis. Box shows median and interquartile range $\pm 95\%$ confidence interval. *** $P < 0.001$.

Fig. 2.

Knockdown of epithelial cell adhesion molecule (*EpCAM*) inhibits proliferation and clonogenic growth of lung cancer cell lines in both anchorage-dependent and -independent conditions. (A) Quantitative real-time PCR and (B) Western blot analyses of *EpCAM* in cells transfected with *EpCAM*RNAi or control oligos. (C) WST-1, a cellular proliferation assay, for cells transfected with *EpCAM*RNAi or control oligos. (D) Liquid colony formation of cells transfected with *EpCAM*RNAi or control oligos. (E) Soft agar colony formation of cells transfected with *EpCAM*RNAi or control oligos. ** $P < 0.01$, Mann-Whitney U -test. Data are averages of three (C,D) or four (E) independent experiments done in triplicate.

Fig. 3.

Knockdown of epithelial cell adhesion molecule (*EpCAM*) causes massive apoptosis in lung cancer cell lines but to a much lesser extent in immortalized normal human bronchial epithelial cell line HBEC4. (A) Quantitative real-time PCR and (B) Western blot analyses of *EpCAM* in HCC2279 and HBEC4 transfected with *EpCAM* RNAi or control oligos. (C) FACS cell cycle profiles of cells transfected with *EpCAM* RNAi or control oligos. Cells were harvested 5 days after transfection, stained with propidium iodide, and analyzed by flow cytometer for cell cycle profiling. (D) FACS analysis of cells co-stained with anti-annexin V and 7-amino-actinomycin D (7-AAD). High annexin V and low 7-AAD cells are undergoing apoptosis. Percentages of apoptotic cells are shown in the plot graphs.

Fig. 4. Knockdown of epithelial cell adhesion molecule (*EpCAM*) increases contact inhibition in *EpCAM*-expressing HCC827 lung cancer cells. (A) Western blots of *EpCAM* and E-cadherin in HCC827 stably transfected with *EpCAM* shRNA expressing vector. (B) Photomicrographs of HCC827 cells stably transfected with *EpCAM* shRNA or GFP shRNA expressing vector. *EpCAM* shRNA-transfected HCC827 cells had significantly decreased cell density compared to GFP shRNA-transfected cells. (C) FACS analysis of cell cycle profiling of HCC827 cells transfected with *EpCAM* or GFP shRNA-expressing vector. In confluent culture the percentage of HCC827 cells in S phase was significantly decreased in *EpCAM* shRNA-transfected cells compared to GFP shRNA-transfected cells. (D) Western

blots of p27^{kip1} in HCC827 cells transfected with *EpCAM* or *GFP* shRNA-expressing vector. (E) WST-1 and (F) liquid colony formation for HCC827 cells transfected with *EpCAM*RNAi or control oligos. $**P < 0.01$. (G) FACS cell cycle profiles of HCC827 cells transfected with *EpCAM*RNAi or control oligos. Cells were harvested 5 days after transfection, stained with propidium iodide, and analyzed using a flow cytometer for cell cycle profiling.

Fig. 5. Stable knockdown of epithelial cell adhesion molecule (*EpCAM*) suppresses invasiveness of highly invasive H358 but not poorly invasive H3255 lung cancer cells. (A) Western blots of *EpCAM* and E-cadherin for HCC827 and H358 stably transfected with *EpCAM*- or *GFP*-shRNA expressing vector. (B) Invasion assay for cells stably transfected with *EpCAM* shRNA- or *GFP* shRNA-expressing vector. Cells were placed in Transwell chambers layered with Matrigel, and invading cells were stained and counted. Averaged data from two independent experiments carried out in triplicate are shown as the mean $\pm$ SD. * $P < 0.05$.