Overexpression of Gelsolin-Like Actin-Capping Protein Is Associated with Progression of Lung Adenocarcinoma

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Gelsolin-like actin-capping protein (CapG), a ubiquitous actin-binding protein, has been shown to play a critical role in regulating the migration ability of cells. In this study, we investigated CapG expression in lung cancer cell lines under hypoxia and evaluated the effect of CapG on the migration ability of these cells. We also analyzed the expression of CapG in a total of 75 patients with lung adenocarcinoma by immunohistochemistry. Our results showed that hypoxia increased the expression of CapG in the human lung cancer cell lines, A549 and H358. Knockdown of CapG expression with small interfering RNA led to a decrease in the migration ability of these cell lines. These results indicate that CapG expression is upregulated in lung cancer cell lines under hypoxia and that CapG may contribute to the migration ability of lung cancer cells. Moreover, the excised lung adenocarcinoma tissues showed significantly increased immunoreactivity for CapG, compared to the adjacent tumor-free tissues. Importantly, overexpression of CapG is significantly associated with male sex ($\chi^2 = 5.195$, $p = 0.033$) and lymph node metastasis ($\chi^2 = 5.58$, $p = 0.021$). Likewise, CapG overexpression was observed with advanced tumor stages (III and IV, 16/31), compared with early tumor stages (I and II, 14/44), but the difference was not statistically significant. These results suggest that overexpression of CapG may be associated with progression of lung adenocarcinoma. In conclusion, CapG may be a promising target for therapy and a potential biomarker for predicting the prognosis of lung adenocarcinoma.

Keywords: CapG; hypoxia; lung adenocarcinoma; metastasis; migration

Lung cancer is the most common cancer as well as the leading cause of cancer-related mortality in males (with 0.95 million deaths per year) and the second in females (with 0.43 million deaths per year). Metastatic lung cancer is responsible for more than 90% of lung cancer-related deaths (Jemal et al. 2011). As the most common type of lung cancer, lung adenocarcinoma has demonstrated an increasing frequency in recent years. Approximately 60% to 70% of patients with lung adenocarcinoma already have malignant pleural diffusion or distant metastasis at the time of diagnosis (Toh 2009). Therefore, the major risk faced by such patients is the development of metastasis. Intensive efforts are currently underway to develop therapeutics for the treatment of lung cancer metastasis.

Owing to uncontrolled growth and aberrant vascular function, a deficiency of oxygen and nutrients occurs in the local environment of most solid tumors (Kunz and Ibrahim 2003). This intrahypoxia can initiate multifaceted cellular responses via hypoxia-inducible factors (HIFs) and result in activation of specific genes that are implicated in several aspects of tumor biology, involving the metastatic process, survival, cellular growth, and apoptosis (Vaupel 2004; Arvelo and Cotte 2009). Accumulating clinical and experimental studies have shown a central role for intrahypoxia in promoting metastatic progression (Subarsky and Hill 2003; DeClerck and Elble 2010); however, the mechanism associated with this phenomenon is not fully understood. Hypoxia-associate proteins that may be involved in the metastasis process have thus become a central issue in research of tumor physiology and treatment.

Gelsolin-like actin-capping protein (CapG) was first isolated as a ubiquitous actin-binding protein of the gelsolin/villin superfamily that was able to control actin microfilament turnover by capping barbed ends and generate propulsive force (Silacci et al. 2004; Barzik et al. 2005). There is evidence that CapG contributes to the regulation of actin-based cell migration of nonmuscle benign cells in vivo and in vitro (Cunningham et al. 1991; Sun et al. 1995; Witke et al. 2001; Parikh et al. 2003). Overexpression of the CapG protein in endothelial cells and

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fibroblasts leads to increased motility. Moreover, bone marrow-derived macrophages from CapG-deficient mice exhibit distinct motility defects and inhibition of receptor-mediated ruffling. Van den Abbeele et al. (2007) showed that downregulation of gelsolin family proteins counteracts cancer cell invasion in vitro. Our previous report showed that CapG is upregulated under hypoxia in a HIF-1α-dependent manner in human pulmonary artery smooth muscle cells and that CapG contributes to the migration ability of cells (Zhang et al. 2009). In the current study, we investigated CapG expression under hypoxia in the lung cancer cell lines A549 and H358. The effect of CapG on the migration ability of these cells under normoxia and hypoxia was also observed. Furthermore, the clinical associations between CapG expression patterns and lung adenocarcinoma were analyzed.

Materials and Methods

Cell lines and cell culture

A549 (American Type Culture Collection (ATCC) number: CCL-185) and H358 (ATCC number: CRL-5807) were obtained commercially from the cell bank of the China Science Academy (Shanghai, China). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and penicillin-streptomycin (100 IU/ml) and then incubated in a Heraeus CO2 incubator set at 37°C in an atmosphere of 5% CO2. Exponentially growing cells were removed from the culture flasks using trypsin/EDTA, centrifuged at 800 rpm for 5 min, resuspended, and counted for use in subsequent experiments. The cells were routinely passaged every 2-3 days when they had reached approximately 80-90% confluence.

Exposure of cells to hypoxia

Cells were cultured in a hypoxia incubator (Thermo Electron; Forma Scientific, Marietta, OH, USA) under hypoxia (1% O2, 94% N2, and 5% CO2) and maintained at 37°C. The incubator chamber was sealed and purged with 1% O2, 5% CO2, and balance N2 for 60 min. Cells were exposed to hypoxia for 12, 24, or 48 h.

Western blot analysis

Cells were scraped from culture dishes, and the final protein concentration of the cell lysates was determined using the bicinchoninic acid (BCA) method with bovine serum albumin as the standard. Equivalent cell extracts (20-40 µg of protein) were boiled in 5x sodium dodecyl sulfate (SDS) at 95°C for 5 min, cooled on ice, and then resolved on a 10% SDS-polyacrylamide gel using a mini-gel apparatus (Bio-Rad, Hercules, CA, USA). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was blocked in Tris Buffered Saline (TBS) containing 5% non-fat dry milk and 0.1% Tween 20 for 1 h at room temperature (RT) and subsequently incubated with a rabbit anti-CapG polyclonal (1: 1,000; Proteintech Group, Chicago, IL, USA) or anti-HIF-1α monoclonal antibody (1:1,000; Abcam, Cambridge, UK) at 4°C overnight. The blots were then washed with Tris Buffered Saline Tween-20 (TBST) for 30 min (3 x 10 min) and then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary (1: 5,000) for 1 h at RT. Following washing, standard immuno-staining was carried out using an enhanced chemiluminescence kit to detect the signal. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. The band intensity was analyzed using Quality One software (Bio-Rad, Hercules, CA, USA).

Inhibition of CapG expression by small interfering RNA (siRNA)

A CapG-specific siRNA and scrambled CapG control siRNA were designed based on references and synthesized by Shanghai GenePharma (Shanghai, China). The sequence of the CapG-specific siRNA was: forward, 5’-GGG GGA GCC UGC UGA GAU G dTdT-3’ and reverse, 5’-CAU CUC AGC AGG CUC CUC C dTdT-3’. The corresponding control siRNA sequence was: forward, 5’-UCG GAA CGU GUC ACU dTdT-3 and reverse, 5’-ACG UGA CAC GUU CGG AGA A dTdT-3’. Cells were treated with the siRNA targeting CapG (100 nM) and incubated under normoxia or hypoxia. siRNA transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Migration assay

A549 and H358 cells were grown in six-well plates, transfected with the CapG siRNA or control siRNA for 6 h, and then cultured for another 24 h. Cells were trypsinized and finally resuspended in RPMI 1640 medium supplemented with 1% FBS. The cell suspension was adjusted to a concentration of 1 x 10⁶/ml for A549 and 2.5 x 10⁶/ml for H358, and 100 µl of cells was pipetted into the upper chamber of a Transwell plate (Costar, Cambridge, MA, USA). The lower chamber was filled with 600 µl of RPMI 1640 medium containing 10% FBS. After 24 h of incubation under normoxia or hypoxia at 37°C, the filter side of the upper chamber was cleaned with a cotton swab. The cells that had migrated across the filters were fixed in dehydrated alcohol for 5 min and stained with crystal violet solution for 20 min. The filter was gently cut from the chamber, and the number of cells that had migrated through the filter pores from the underside of the filter was counted in four high-power fields per insert (10 x 40). For each migration condition, three identical replicates were performed.

Wound-healing assay

The cell monolayers transfected with the CapG siRNA or control siRNA were manually scraped from one end of the well to the other end with a sterile p200 pipet tip. The medium and cell debris were aspirated away and replaced with 2 ml of fresh serum-free medium. The wound was marked with a pen and photographed via phase contrast microscopy, and this representing 0 h. The width of the wound distance was calculated as the basic width. The same field was photographed after the plates were incubated under normoxia or hypoxia conditions for 24 h, and three randomly selected points along each wound were marked. The horizontal distance between the migrating cells and the initial wound was measured. Wound closure (%) was determined as the distance migrated after 24 h relative to the basic width. For each migration condition, three identical replicates were performed.

Patients and Specimens

A total of 75 patients with lung adenocarcinoma were included in the current study from August 2006 to the December 2008. Matched tumor tissues and adjacent tumor-free tissues were obtained. Tissue samples for immunohistochemistry (IHC) were fixed in formalin and paraffin embedded. Patient clinicopathological data, includ-
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ing sex, age, pathological TNM stage of the tumor, histology, and grade, were retrieved from medical records. The study was approved by the Hospital Ethical Committee. Written informed consent was obtained from all participants.

**IHC and scoring methods**

For IHC detection of CapG, 5-μm thick microarray sections containing matched tumor tissues and adjacent tumor-free tissues cores from 75 patients were prepared from the paraffin-embedded tissues, and standard methods were applied. Briefly, slides were deparaffinized in xylene and then rehydrated in a series of alcohol. Antigen retrieval was carried out by boiling the slides in a pressure cooker containing 6.5 mmol/L sodium citrate (pH 6.0) for 3 min and cooling for 20 min at RT. Endogenous peroxidase activity was blocked in 3% H2O2 in phosphate buffered saline (PBS) for 30 min at RT. Slides were blocked with 10% bovine serum albumin/1x PBS at RT for 30 min to reduce nonspecific background staining. Serum was removed from the slides, and the slides were then incubated with the CapG polyclonal antibody at 4°C overnight in a humidity chamber. The slides were then washed in PBS and incubated with biotinylated secondary antibodies for 30 min in PBS buffer, followed by three washes in PBS for 5 min each. Finally, the slides were incubated with biotinylated alkaline phosphatase-streptavidin (StreptAB Complex/AP; DAKO, Glostrup, Denmark) for 20 min according to the manufacturer’s instructions (Dako, Glostrup, Denmark), developed with 3,3’ diaminobenzidine (DAB) substrate, and counterstained with hematoxylin before being mounted and examined by light microscopy. Routine negative controls using nonimmune serum instead of the primary antibody were included to verify specificity.

The immunoreactions were blindly evaluated independently by two experienced pathologists to quantify the state of CapG protein expression. The intensity of CapG staining in both the cytoplasm and nuclei of the tumor cells was scored on a 4-point scale: negative, 0; weak, 1; intermediate, 2; and strong, 3. Cells with a staining intensity score of 1, 2, or 3 were regarded as positive immunoreactivity, while those with scores of 0 were regarded as negative immunoreactivity. The percentage of positive tumor cells (0% = negative, 1-50% = 1, 51-75% = 2, and ≥ 76% = 3) was assessed by counting more than 1000 cancer cells in 10 randomly selected high-power fields (10 × 40). A combined staining score for each compartment was obtained as the product of intensity and extent of staining, and a score of < 4 was considered to be low expression or a score of ≥ 6 was considered to be high expression.

**Statistical analysis**

Statistical analysis was performed using SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA). Comparisons between two different groups were analyzed using the Student’s t test. Correlations between CapG IHC scores and clinicopathologic features were evaluated by Fisher’s two sided exact test. p < 0.05 was considered statistically significant.

**Results**

**Effects of hypoxia on CapG expression in lung cancer cell lines**

A549 and H358 cells were exposed to normoxia or hypoxia (1% O2) for 12 h, 24 h, or 48 h (Fig. 1). The expression levels of CapG protein were remarkably increased in both cell lines after incubation under hypoxia for 24 h, compared with normoxia controls, and the expression levels remained elevated at 48 h.

**Inhibition of CapG expression results in impaired cell migration**

As shown in Fig. 2, the expression level of CapG protein was effectively decreased in A549 and H358 cells by treatment with the CapG-specific siRNA, compared to the control with a scramble CapG siRNA. Compared to controls, cells transfected with the CapG-specific siRNA showed slower rates of wound healing,
especially under hypoxia conditions (Fig. 3). At 24 h after wounding, cells transfected with the scrambled control siRNA had completely healed, with at least 80% wound closure under both conditions. In contrast, cells transfected with the CapG-specific siRNA showed at most 50% wound closure.

The results of the cell migration assays were consistent with the results of the wound healing assays. As shown in Fig. 4, CapG-specific siRNA reduced the migration ability of A549 cells by 28% and 39% under normoxia and hypoxia, respectively. For H358 cells, the proportion was 50% and 67.3%, respectively. In addition, we found that the migration ability of cells transfected with the scrambled control siRNA was improved under hypoxia.

**Correlation of CapG protein expression and clinicopathological parameters in lung adenocarcinoma**

Representative results for CapG protein expression in tumor tissues and adjacent tumor-free tissues are shown in Fig. 5. For the purpose of analysis, CapG IHC scores were classified as low (score < 4) or high (score ≥ 4). Using this classification, the high expression rates of CapG in tumor tissues and adjacent tumor-free tissues were 40% (30/75) and 4% (3/75), respectively. Compared to the adjacent tumor-free tissues, lung adenocarcinoma tissues showed significantly increased immunoreactivity for CapG ($\chi^2 = 5.195$, $p < 0.01$). Associations between CapG expression and clinicopathological features of patients with lung adenocarcinoma are shown in Table 1. CapG expression was strongly associated with male sex ($\chi^2 = 5.195$, $p = 0.033$) and lymph node metastasis ($\chi^2 = 5.58$, $p = 0.021$). In addition, overexpression of CapG was observed with advanced tumor stages (III and IV, 16/31) compared with early tumor stages (I and II, 14/44), but this difference was not statistically significant ($\chi^2 = 2.969$, $p = 0.09$).
Fig. 4. The effect of decreasing CapG expression on the migration of cells under normoxia or hypoxia. Cells transfected with si-CapG or si-control were plated into millicell insert at a density of $2 \times 10^5$ per well for A549 or $5 \times 10^5$ per well for H358. The migrated cells were photographed and counted in 4 fields per insert after 24 h of incubation under normoxia or hypoxia. A transwell cell migration assay on A549. B transwell cell migration assay on H358. All assays were performed in triplicate and data were shown as mean ± SEM. *$p < 0.05$ compared with normoxia si-control, **$p < 0.01$ compared with hypoxia si-control, and *$p < 0.05$ compared with normoxia si-control.

Fig. 5. Immunostaining for CapG in lung tissues.
Serial sections of adjacent tumor free tissues were stained with antibody to CapG (A) × 200 and (B) × 400. Serial sections of lung adenocarcinoma tissues were stained with antibody to CapG (C) × 200 and (D) × 400. Immunoreactivity was indicated by arrows.
Discussion

The malignant progression of lung cancer usually culminates in distant metastasis, and this is the predominant cause of death, especially for patients with lung adenocarcinoma. Furthermore, hypoxia creates a stress environment for the evolution of metastasis through a multiplicity of mechanisms. There is evidence that promotion of cell migration might be modulated through upregulation of HIF-1–dependent protein expression (Choi et al. 2006), which is a key transcriptional regulation factor influencing many genes during hypoxia. However, the molecules involved in driving migration of hypoxic tumor cells are not fully understood. De Corte et al. (2004) showed that CapG may modulate the invasive properties of MDCK-AZ, HeLa, and HEK293T cells. Renz et al. (2008) also found that the import of CapG into the nucleus is increased in invasive breast cancer cells. Our results showed that reduction of CapG expression in A549 and H358 cells was accompanied by significantly impaired motility, especially under hypoxia. In addition, we showed that exposure to hypoxia stimulated the migration of A549 and H358 cells. These results suggest that the CapG protein could promote migration of lung cancer cells under hypoxia.

CapG is a member of the Ca²⁺- and polyphosphoinositiode-modulated gelsolin family of actin binding proteins and is able to control actin microfilament turnover by capping barbed ends and modulate the dynamic behavior of the actin cytoskeleton. The formation of protrusive structures and intracellular forces that power cell migration are generated from the actin cytoskeleton (Lambrechts et al. 2004), and there is evidence that regulation of the actin cytoskeleton is involved in cancer metastasis (Yamaguchi and Condeelis 2007; Hall 2009). The underlying mechanisms resulting in deregulation of the actin system are complex. The CapG protein may be involved in a mechanism linking intracellular signaling pathways with actin polymerization dynamics after receptor activation, and a dramatic increase in actin polymerization is observed in the presence of external stimuli, such as growth factors and chemoattractants.

CapG, an important marker of motility, may be valuable for assessing tumor progression and/or prognosis. Recent studies have also suggested that overexpression of CapG may be involved in promoting tumor progression. Significant overexpression of the CapG protein was detected in pancreatic cancer (Thompson et al. 2007) and oral squamous-cell carcinoma (Nomura et al. 2008), with associations with large tumor size and advanced staging in the case of oral squamous-cell carcinoma. CapG is also expressed at higher levels in ovarian carcinomas with possible correlations with advanced disease stage and survival (Partheen et al. 2008). Our results showed that lung adenocarcinoma tissues had significantly increased expression of CapG immunoreactivity compared to adjacent tumor-free tissues. The level of CapG expression also correlated with lymph node metastasis. In addition, high expression of CapG was often observed with advanced tumor stages (III and IV) compared with early tumor stages (I and II), but this was not statistically significant; however,
a limited number of samples were employed in this study, which limited the statistical power. Overall, our results suggest that CapG may be a potential biomarker for predicting the prognosis of lung adenocarcinoma. However, additional large-scale studies are warranted to validate our findings.

In summary, the present study is the first to demonstrate that CapG expression is upregulated in human lung cancer cell lines under hypoxia and that CapG contributes to the migration ability of these cells. In addition, expression of CapG was significantly increased in lung adenocarcinoma tissues, compared to adjacent tumor-free tissues. The level of CapG expression also correlated with lymph node metastasis. Accordingly, CapG may be a promising target for therapy and a potential biomarker for predicting the prognosis of lung adenocarcinoma.

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Conflict of Interest

None of the authors have a financial relationship with any commercial entity that has an interest in the subject of this manuscript.

References


