Heterotopic production of ceruloplasmin by lung adenocarcinoma is significantly correlated with prognosis

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Abstract

Objectives: Ceruloplasmin (CP) is a well-known copper binding protein synthesized mainly in the liver, but its expression is known to be elevated in the serum of cancer patients and in malignant tumor cells. Lung cancer is the leading cause of cancer-related death worldwide, and adenocarcinoma is the main histological type of lung cancer. However, the role of CP in lung adenocarcinoma is still unclear. Here we examined and compared the expression of CP in various histological subtypes of lung adenocarcinoma and its correlation with clinical-pathological parameters.

Materials and methods: We examined CP expression in lung adenocarcinoma samples and cell lines using quantitative real-time RT-PCR and Western blot analysis. Immunohistochemistry for CP was carried out using 196 specimens of lung adenocarcinoma.

Results: CP expression was significantly higher in invasive adenocarcinoma than in adenocarcinoma in situ (AIS), and was significantly correlated with poorer outcome, pathological stage, pT, and pN. Multivariate analysis showed that CP expression was an independent prognostic factor for lung adenocarcinoma patients. Furthermore, Western blot analysis using protein extracted from lung adenocarcinoma cell lines revealed the secreted form of CP.

Conclusion: CP is produced heterotopically in lung adenocarcinoma cells and its expression is associated with tumor progression. In view of the presence of the secreted form of CP in tumor cells, CP may be a useful biomarker for lung adenocarcinoma.

1. Introduction

Lung cancer has a poor prognosis and is the leading cause of cancer-related death worldwide [1]. The most common histological type of lung cancer can be differentiated into mainly five histological subtypes: lepidic, acinar, papillary, solid and micropapillary. Recently, the WHO histological classification has proposed the new concepts of “adenocarcinoma in situ” (AIS) and “minimally invasive adenocarcinoma” (MIA) [2]. AIS and MIA show an extremely favorable prognosis, whereas invasive adenocarcinoma has a poor outcome [3–7]. AIS and MIA are thought to be adenocarcinomas at a very early stage, progressing thereafter to lepidic adenocarcinoma or other histological subtypes that are invasive [8].

In 2011, Shiba-Ishii et al. showed that the level of mRNA for ceruloplasmin (CP) was increased in invasive lung adenocarcinoma relative to AIS [9]. CP is a 132-kDa α2-glycoprotein produced mainly by liver hepatocytes. CP binds up to 95% of copper present in serum, and transports it to tissues; it is also the major ferroxidase in serum, catalyzing the conversion of Fe2+ to Fe3+ [10], and playing a role in angiogenesis [11]. CP is also known to be an important inflammatory reactive protein, its synthesis and secretion by hepatocytes being stimulated by interleukin-1 and interleukin-6, allowing it to scavenge oxygen radicals that are released by immune cells at inflammatory sites [12–15]. On the other hand, over the last decade, many reports have indicated that patients with malignant tumors have a high level of CP in their serum [16–19] and tumor tissue [19–22] including lung.
adenocarcinoma [23]. However, CP expression in lung adenocarcinoma and its association with clinicopathological factors have remained unclear. In the present study we investigated the expression of CP in lung adenocarcinoma cell lines and clinical samples using quantitative real-time RT-PCR and Western blotting. We also performed immunohistochemistry for CP in a large number of lung adenocarcinoma cases to clarify the correlation between CP expression and clinicopathologic parameters and prognosis.

2. Materials and methods

2.1. Patients and tissue specimens

Samples of 196 lung adenocarcinomas were obtained from patients who had undergone surgical resection at the University of Tsukuba Hospital (Ibaraki, Japan) between 1999 and 2007. All tissue specimens were fixed with 10% formalin and embedded in paraffin. Additionally, 28 frozen samples were obtained from patients who had undergone surgical resection at the University of Tsukuba Hospital and Ibaraki Higashi National Hospital (Ibaraki, Japan), and used for quantitative real-time RT-PCR. Six out of the 28 frozen samples were also used for Western blot analysis. All cases were classified histologically according to the WHO classification (4th edition) and TNM staging was performed in accordance with the UICC (8th edition). Serum samples were collected from 16 healthy adult individuals (volunteers). This research was approved by the ethics committee of Tsukuba University hospital, Ibaraki Higashi National Hospital, and the National Institute of Advanced Industrial Science and Technology. We explained the use of their clinical specimens to all participants, and obtained their informed consent.

2.2. Cell lines and culture conditions

PL16T is an immortalized cell line that was established from human lung atypical adenomatous hyperplasia (AAH) in our laboratory [24]. PL16T cells were cultured in MCDB 133 HAA medium (Wako, Osaka, Japan) containing 2% fetal bovine serum (FBS), 0.5 ng/ml EGF (Toyobo, Tokyo, Japan), 5 μg/ml insulin (Wako), 72 mg/ml hydrocortisone (Wako), and 10 μg/ml transferrin (Sigma-Aldrich, St. Louis, MO). The lung adenocarcinoma cell lines H1650, HCC827 and H1975 were cultured in RPMI 1640 medium (GIBCO; Thermo Fisher Scientific, MA) supplemented with 10% FBS, 100 units/ml penicillin (GIBCO; Thermo Fisher Scientific) and 100 μg/ml streptomycin (GIBCO; Thermo Fisher Scientific). To prepare cell lysates, cells were cultured to 60–80% confluence and then harvested. Cell lysates were prepared using RIPA buffer (150 mM NaCl, 10 mM Tris-HCl pH7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA) containing protease inhibitor (Complete™ Protease Inhibitor Cocktail, Roche, Switzerland), with collected cell pellets (0.5-1.0 × 10^7 cells/pellet). To obtain the culture supernatant, the cells at 60–80% confluence were washed 3 times with D-PBS and further cultured in PBS-free RPMI 1640 for 48 h. The final media were harvested and the supernatants were filtered using a 0.45-μm disc filter (Millipore, MA). Protein concentrations were determined using the BCA method with BSA as the standard.

2.3. Quantitative real-time RT-PCR

Expression of CP mRNA was evaluated by quantitative real-time RTPCR. Total RNA was prepared from 28 frozen specimens of lung adenocarcinoma and 4 lung adenocarcinoma cell lines using an RNasey Mini Plus Kit (QIAGEN, Hilden, Germany). The quality of the isolated total RNA was assessed by detection of 28S and 18S ribosomal RNA with an Agilent 2100 Bioanalyzer. Real-time PCR was performed with SYBR® Premix Ex Taq™ (Perfect Real Time; Takara Bio, Tokyo, Japan) on a GeneAmp® 7300 Sequence Detection System (Thermo Fisher Scientific) according to the manuals supplied. Primers used in this study are listed in Supplementary Table 1. All assays were performed three times.

2.4. Western blot analysis

Six frozen specimens of lung adenocarcinoma and 4 cell lines were solubilized in RIPA buffer. The culture supernatants were concentrated 100-fold using an Amicon™ Ultra 10 K column (Merck Millipore, MA). After protein assay, 2-μg samples of lung tissue protein, 5-μg samples of cell line culture supernatant protein, and 40-μg samples of cell line lysate protein were electrophoresed under reducing conditions on 10% SDS polyacrylamide gel (PANTERA XV gel, DRC, Inc., Tokyo, Japan), and transferred to Immuno-Blot™ PVDF membranes (Bio-Rad Laboratories, CA). For Western blot analysis of CP, the membranes were blocked with BLOCK ACE reagent (DS Pharma Biomedical, Osaka, Japan) in TBS containing 0.1% Tween-20 (TBS-T), and were then incubated with 0.1 mg/ml HRP-labeled anti-CP antibody (1:2000 dilution, A80-124A, Bethyl Laboratories, TX) in TBS-T. HRP labeling was performed with a Dojindo Peroxidase Labeling Kit-NH2 (DOJINDO Laboratories, Kumamoto, Japan). The membranes were washed 3 times with PBS-T and then developed with Western Lightning™ chemiluminescence reagent (Perkin Elmer, MA). For Western blot analysis of β-actin, the membranes were blocked with 3% BSA in PBS containing 0.1% Tween-20 (PBS-T), and incubated with anti-β-actin antibody (1:5000 dilution, clone AC-15, SIGMA, MO). After washing, the membranes were incubated with the secondary antibody (HRP-conjugated anti-mouse IgG, 1:3000 dilution, NA931, GE Healthcare, PA). The membranes were then washed 3 times with PBS-T and developed with the Western Lightning™ chemiluminescence reagent (PerkinElmer). As control samples, pooled normal human serum (NHS) for Western blot analysis was prepared by mixing equal amounts of serum obtained from the 16 healthy individuals. CP purified from human plasma (Merck, Germany) was used as a standard protein.

2.5. Immunohistochemistry and scoring

The tissue specimens were cut into sections 4 μm thick from formalin-fixed, paraffin-embedded (FFPE) blocks, and the sections were deparaffinized and subjected to antigen retrieval in Tris-EDTA buffer (pH 9.0) at 105°C for 10 min. Immunohistochemical staining was performed with anti-CP antibody (1:2000 dilution, 66156-1-lg, Protein Tech, IL) for 30 min. The sections were then incubated with the secondary antibody (EnVision+ Dual link system: Agilent Technologies, Santa Clara, CA). The sections were stained with DAB substrate kit (Agilent Technologies) solution, followed by counterstaining with hematoxylin. We evaluated the staining intensity as: 0, no staining; 1, weak; 2, intermediate; 3: strong (Supplementary Fig. 1), and the area of positive tumor cells in each section was calculated in units of 10%. The H-score was determined as the sum of each intensity score (0–3) x proportion percentage (0–100%). We then determined the cut-off point of the H-score by drawing the ROC curve.

2.6. Statistical analysis

Student’s t test, one-way ANOVA with Tukey’s honest significant difference (HSD) post hoc test, Pearson’s correlation coefficient analysis and chi-squared test were used to compare the results. Survival curves were calculated using the Kaplan–Meier method and then compared using log-rank test. The Cox proportional hazards model was used to analyze prognostic factors. Statistical analyses were carried out using SPSS Base, version 24, at a significance level of p < .05.
3. Results

3.1. Expression of CP in clinical samples of lung adenocarcinoma

Quantitative real-time RT-PCR assays were carried out to compare the levels of CP expression between AIS and invasive adenocarcinoma. First, we examined 22 clinical samples of lung adenocarcinoma (AIS 6 cases, invasive adenocarcinoma 16 cases). CP showed significantly higher expression in invasive adenocarcinoma than in AIS (Fig. 1A). We confirmed the expression of CP by immunohistochemistry using 22 cases and evaluated them using the H-score. H-score was significantly higher in invasive adenocarcinoma than in AIS (Fig. 1B). A significant correlation was found between the H-score and mRNA level using Pearson’s correlation coefficient analysis \( r = 0.701, p < .01 \) (Fig. 1C).

Next, we performed quantitative real-time RT-PCR assays and Western blot analysis, and immunohistochemistry for CP in 6 additional clinical samples of lung adenocarcinoma including AIS (3 cases) and invasive adenocarcinoma (3 cases) to compare the mRNA level and protein expression of CP. At the mRNA level, CP showed significantly higher expression in invasive adenocarcinoma than in AIS. CP expression was detected in all clinical samples, and several invasive adenocarcinomas showed a high level of CP expression by Western blotting. The H-score in Cases 1, 2, 3, 4, 5 and 6 was 10, 0, 80, 150, 120 and 210, respectively (Fig. 2A), and Pearson’s correlation coefficient analysis revealed a significant correlation between the H-score and the mRNA level \( r = 0.90, p < .01 \). Immunohistochemistry detected CP protein in not only tumor cells but also intra-alveolar spaces in Cases 1, 3, 4, 5, 6 (Fig. 2B).

3.2. Expression of CP in lung adenocarcinoma cell lines

We performed quantitative real-time RT-PCR assays and Western blotting using one immortalized AAH cell line (PL16T) and 3 lung adenocarcinoma cell lines (H1650, HCC827 and H1975). Whole-cell lysates and supernatants were used for Western blot analysis to confirm the expression and secretion of CP.

At the mRNA level, H1650, HCC827 and H1975 showed significantly higher expression of CP than PL16T (Fig. 3A). The specific band of CP was detected in the supernatants of all the cell lines examined, and the expression level of PL16T was weaker than in the other cell lines (Fig. 3B). Interestingly, the lysates of all the cell lines showed weaker bands than the supernatant samples (Fig. 3C).

3.3. Correlation between CP expression and clinicopathological parameters in lung adenocarcinoma patients

We examined CP expression by immunohistochemistry and evaluated the level of expression in terms of the H-score. We examined 196 lung adenocarcinoma cases in total. ROC curve analysis indicated a coordinate of 90 (sensitivity 0.704, specificity 0.656) as the cut-off point in this study (data not shown). In accordance with this result, we
divided the cases into a high expression group (H-score > 90; 92 cases) and a low expression group (H-score ≤ 90; 104 cases). Eighty-nine of 164 invasive adenocarcinomas (54.3%), 2 out of 12 MIAs (16.7%) and 1 out of 20 AISs (0.5%) belonged to the high expression group. Chi-squared test showed that CP expression was significantly correlated with pathological stage, and pT and pN status (Table 1).

To clarify the correlation between CP expression and histological subtype, we categorized the histological subtypes into four groups: an AIS and MIA group, a lepidic adenocarcinoma group, a papillary and acinar adenocarcinoma group and a solid and micropapillary adenocarcinoma group. CP expression was significantly higher in the invasive adenocarcinoma groups than in the AIS and MIA group. Moreover, the solid and micropapillary adenocarcinoma group showed higher expression of CP than the lepidic adenocarcinoma group. Papillary and acinar adenocarcinoma group showed relatively higher expression of CP in comparison to lepidic adenocarcinoma group, but the difference was not significant. Invasive mucinous adenocarcinoma showed relatively higher expression of CP in comparison to AIS and MIA group, but the difference was not significant (Fig. 4A).

CP expression was also examined in relation to patient outcome. The patients were stratified according to CP expression, as described in Methods (n = 196; High: 92 cases, Low: 104 cases). The log-rank test was used to compare disease-free survival between the groups. The Kaplan-Meier curves indicated that the high expression group had a significantly poorer outcome than the low expression group (Fig. 4B). Furthermore, in the solid and micropapillary adenocarcinoma group, patients whose tumors showed high expression had a significantly poorer outcome than those with low expression (Fig. 4E). On the other hand, in the papillary and acinar adenocarcinoma group and lepidic adenocarcinoma group the outcome of patients with high expression was considerably worse than that of patients with low expression, although the difference was not significant (Fig. 4C-D).

Multivariate survival analysis was carried out using the Cox proportional hazards model employing 3 factors selected on the basis of the results of univariate analysis: histological subtype (AIS and MIA vs. invasive adenocarcinoma), pathological stage (p stage 0, I vs. II, III and IV), and the CP H-score (low vs. high). The results showed that histological subtype, pathological stage and CP H-score were independent

![Quantitative real-time RT-PCR, Western blotting and H-score of CP in 3 cases of AIS and 3 cases of invasive adenocarcinoma (A). CP immunohistochemical staining pattern in each of the 6 cases (B).](image1)

![Quantitative real-time RT-PCR of CP in 1 AAH cell line (PL16T) and 3 human lung adenocarcinoma cell lines (H1650, HCC827 and H1975) (A) and Western blots of the supernatant (B) and whole cell lysate (C) of each cell line.](image2)
4. Discussion

In this study, we examined for the first time CP expression in lung adenocarcinoma clinical samples and cultured cell lines. At the mRNA level, CP expression was significantly higher in invasive adenocarcinoma than in AIS in clinical samples, and a similar result was observed for cell lines. In clinical samples, there was a significant correlation between CP expression and H-score determined by immunohistochemistry. CP showed a dot-like staining pattern in the tumor cell cytoplasm, and also showed a positive immunoreaction in extracellular materials. The results of immunohistochemistry showed that CP expression was higher in invasive adenocarcinoma than in AIS and a similar result was observed in cell lysates (Fig. 3A–C). This might have been due to CP produced by tumor cells being totally secreted into the culture medium. Therefore, immunohistochemical staining of CP was evident not only in tumor cells but also in alveolar spaces. This phenomenon could explain the elevation of CP in serum of lung adenocarcinoma patients [16]. However, the usefulness of CP as a serum tumor marker of lung adenocarcinoma is limited, because CP level of serum elevates also in patients with inflammatory conditions, pregnancy, iron deficiency and renal failure [16–19,25,26,29], and these kinds of CP are synthesized by the liver, as discussed in the previous report [23]. Therefore the difference between CP produced from lung adenocarcinoma cells and hepatocyte should be investigated. As a solution, hepatocellular carcinoma marker, alpha-fetoprotein (AFP), might be a good model. Serum level of AFP was known to be elevated in patients with hepatocellular carcinoma, although serum levels of AFP is elevated even in those with benign liver diseases. Previous studies focused on the sugar chains profile of AFP and revealed that presence of a fucosylated carbohydrate chain of AFP is specific modification in patients’ serum with hepatocellular carcinoma [30,31]. This modified AFP, so called AFP-L3, is used as a diagnostic and prognostic marker for hepatocellular carcinoma [32–35]. As AFP, CP secreted from lung adenocarcinoma may not be completely the same as CP secreted by hepatocytes, and may undergo some modification. The post-translational cancer-specific modification of CP is one of the following goals of this study.

In this study, we have shown that lung adenocarcinoma cells produce CP, suggesting that CP could have potential as a specific biomarker for lung adenocarcinoma. However, our study had several limitations. First, when scoring the immunohistochemical staining of CP, we did not include CP expression in non-cancerous areas (e.g. alveolar spaces, parenchyma, and intravascular regions). Although the level of CP mRNA in AIS was lower than in invasive adenocarcinoma, Western blot analysis showed variable bands in AIS cases (Fig. 2A). The high signal level of CP in AIS revealed by Western blotting may have been attributable to CP secreted and located outside tumor cells. In order to detect CP in tumor cells, they must be purified by laser microdissection. Secondly, the sample size of Western blot analysis of clinical samples was too small, because there were only 6 cases which had sufficient amount of frozen sample to perform quantitative realtime RT-PCR and Western blot analysis. Therefore, to understand our result more correctly, we are planning to collect more cases. Lastly, we did not analyze the serum of lung adenocarcinoma patients. Comparison of the CP levels in tumor tissue and serum would yield more
Fig. 4. CP expression in each histological subtype of lung adenocarcinoma (A). Kaplan-Meier curve showing the disease-free survival of patients with lung adenocarcinoma: all patients (B), lepidic adenocarcinoma group (C), papillary and acinar adenocarcinoma group (D), and solid and micropapillary adenocarcinoma group (E). AIS: adenocarcinoma in situ, MIA: minimally invasive adenocarcinoma, Lep: lepidic adenocarcinoma, Pap: papillary adenocarcinoma, Aci: acinar adenocarcinoma, Sol: solid adenocarcinoma, Mpap: micropapillary adenocarcinoma, IMA: invasive mucinous adenocarcinoma, *p < .01 (compared with AIS and MIA group), §p < .01 (compared with Lepidic adenocarcinoma group)
information, and to this end, further studies should be performed.

In conclusion, we have demonstrated for the first time heterotopic expression of CP in lung adenocarcinoma, suggesting that CP could be a potentially useful prognostic biomarker of lung adenocarcinoma.

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Role of the funding source
The sponsor had no roles in the study design, data collection, data analysis, data interpretation or writing of the report.

Conflict of interest
The authors have no conflicts to disclose.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jungcan.2018.01.012.

References

Table 2
Multivariate analysis of disease-free survival of lung adenocarcinoma patients using the Cox proportional hazards model.

<table>
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