Carbonyl Reductase Expression and Its Clinical Significance in Non–Small-Cell Lung Cancer

Kazumasa Takenaka, Eiji Ogawa, Hiroki Oyanagi, Hiromi Wada, and Fumihiro Tanaka

Department of Thoracic Surgery, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan

Abstract

Carbonyl reductase (CBR) is a cytosolic NADPH-dependent oxidoreductase metabolizing prostaglandins, steroids, quinines, and anthracycline antibiotics. Many experimental studies have shown that CBR plays important roles in the regulation of tumor progression, but clinical significance of CBR status remains unclear. Thus, we conducted a retrospective study on CBR mRNA expression in lung cancer. Tumor tissues obtained from 59 non–small-cell lung cancer patients were analyzed by quantitative real-time reverse transcription-PCR assay to reveal clinical significance of CBR expression. Angiogenesis was measured immunohistochemically as intratumoral microvessel density (IMVD) using anti-CD34 monoclonal antibody (CD34-IMVD) and anti-CD105 monoclonal antibody (CD105-IMVD). CBR mRNA expression was significantly reduced along with progression of primary tumors (the mean CBR mRNA/GAPDH mRNA, 3.288 × 10⁻² for pT₁, 1.628 × 10⁻² for pT₂, and 1.175 × 10⁻² for pT₃-₄ disease, respectively; P = 0.02). Moreover, CBR mRNA expression in tumor with nodal involvement seemed to be reduced as compared with that in tumor without nodal involvement (the mean CBR mRNA/GAPDH mRNA, 1.446 × 10⁻² and 2.531 × 10⁻², respectively), whereas the difference did not reach a statistical significance (P = 0.09). The mean CD105-IMVD for CBR-high tumor was 59.2, which was significantly lower than that for CBR-low tumor (130.6, P = 0.02), whereas no significant difference between the mean CD34-IMVDs for CBR-high tumor and CBR-low tumor was found. The 5-year survival rate of CBR-high patients was 68.3%, significantly higher than that of CBR-low patients (36.5%; P = 0.03). A multivariate analysis confirmed that CBR-high expression was a significant factor to predict a favorable prognosis (P = 0.04; relative risk, 0.39; 95% confidence interval, 0.16-0.98). Expression of CBR mRNA was a significant prognostic factor in non–small-cell lung cancer and was inversely associated with tumor progression and angiogenesis. (Cancer Epidemiol Biomarkers Prev 2005;14(8):1972–5)

Introduction

Lung cancer is a major cause of cancer-related deaths in industrialized countries, and non–small-cell lung cancer accounts for ~80% of lung cancer. The 5-year survival rate after surgery remains poor despite recent advances in the diagnosis and treatment. The tumor-node-metastasis staging classification is the principal prognostic factor for management of non–small-cell lung cancer patients (1-3). However, the development of reliable biological markers is important to improve the assessment of prognosis because the tumor-node-metastasis classification does not provide information on the biological features of tumor. Although many possible biological markers have been reported, none of them has been recommended as a practical clinical marker in the diagnosis or therapy of non–small-cell lung cancer (2, 4).

Carbonyl reductase (CBR) is a cytosolic monomeric, NADPH-dependent oxidoreductase reducing a wide variety of endogenous and xenobiotic carbonyl compounds, which exert toxic effects on biological systems (5). CBR shows ubiquitous distribution in human tissues (6, 7). The substrates and the roles of CBR are not fully understood, although CBR has been found to be involved not only in many areas of metabolism of prostaglandins, steroids, quinines, and anthracycline antibiotics, such as daunorubicine (8), but also in mutagenesis and carcinogenesis (7). In particular, the roles of CBR expression in drug metabolisms have been investigated, whereas CBR expression in drug metabolisms have been investigated, whereas little has been reported on the levels of CBR expression in tumor tissues.

Ismail et al. (9) first showed that CBR played important roles in modulating metastatic behavior of tumor cells in a mouse model, whereas the mechanisms remain unclear. CBR has been found to be identical to prostaglandin 9-ketoreductase, which inactivates prostaglandin E₂ (PGE₂) by converting PGE₂ to prostaglandin F₂α (10-12). PGE₂, which is produced by cyclooxygenase enzymes, can promote development and progression of malignant tumors (13, 14) by promoting angiogenesis through enhancement of vascular endothelial growth factor (VEGF) expression (15, 16), inhibition of apoptosis (17), regulation of cytokines (18), and activation of the epidermal growth factor receptor (19). Although these data suggest that CBR plays important roles in tumor progression through various mechanisms including stimulation of tumor angiogenesis, few clinical studies have been undertaken to evaluate the clinical significance of CBR expression in malignant tumors. Only one clinical study has documented a significant correlation between CBR expression and postoperative survival as well as nodal metastasis in resected ovarian cancer (20), and no clinical study on CBR status in non–small-cell lung cancer has been reported.

In the present study, we first assessed the clinical significance of CBR status in non–small-cell lung cancer, and revealed the following results: (a) CBR mRNA expression was down-regulated along with tumor progression; (b) enhanced CBR mRNA expression was correlated with a favorable prognosis in correlation with inhibiting tumor angiogenesis. These results strongly suggested that CBR could be a new target in the diagnosis and treatment of non–small-cell lung cancer.
Patients and Methods

Patients and Tissue Samples. To reveal the clinical significance of CBR status, primary lung cancer tissues were obtained from 59 patients with pathologic (p-) stage I to VI non–small-cell lung cancer operated at Kyoto University Hospital between 1996 and 1998. P-stage was determined by the current tumor-node-metastasis classification (1). Histologic type and grade of cell differentiation were determined according to WHO classification. All samples were obtained after informed consent was taken and were immediately snap-frozen in liquid nitrogen and stored at −80°C until use. For histologic examination, samples were fixed in 10% (v/v) formalin and then embedded in paraffin. Serial 4-μm sections were prepared from each sample and were used for H&E and immunohistochemical stainings. This study was reviewed and approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University.

RNA Isolation and cDNA Synthesis. Total RNA was isolated using RNeasy mini kit (Qiagen Sciences, Tokyo, Japan) following the protocol of the manufacturer. To digest contaminated DNA, extracted RNA residual DNA was incubated with RNase-free DNase Set (Qiagen GmbH, Hilden, Germany). Reverse transcription of total RNA was done using the Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences, Uppsala, Sweden) and random hexomer (Amer- sham Pharmacia Biotech, Piscataway, NJ) following the protocol of the manufacturer.

Quantification of CBR Expression by Real-time PCR. To quantify gene expression of CBR, real-time PCR was done using the LightCycler thermal cycler system (Roche Diagnostics Japan, Tokyo, Japan) following the protocol of the manufacturer. The sense and antisense primers used for the quantitative amplification of CBR gene were 5′-AATCGAGTGAGGCAGAGGAA-3′ and 5′-ACCACTCAGAAAGCTGTA-3′, which amplified a 258 bp fragment. PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, used as an internal control, were forward 5′-TGAGGAGGAGCTACTCAGG-3′ and reverse 5′-TCCACCACTCTGTGTGTA-3′, which amplified a 307 bp fragment. A 20 μL reaction mixture containing 3 mmol/L MgCl₂, 0.5 μmol/L sense and antisense primers, and 0.1 μg of cDNA in FastStart DNA SYBR Green I mix (Roche Diagnostics Japan) was prepared. PCR amplification was carried out with an initial 10-minute preincubation at 95°C to activate the FastStart Taq DNA Polymerase, followed by 40 cycles of the following profile: denaturation at 95°C for 10 seconds and annealing at 55°C for 10 seconds. After the final extension at 72°C for 10 seconds, detection of fluorescence products was done by a 95°C step for 0 second, a 65°C step for 15 seconds, and a 95°C step for 0 second. The identities of PCR products were confirmed by DNA sequencing. The quantification data were analyzed with the LightCycler software (ver. 3.5). Expression level of CBR gene was normalized and represented as the ratio of CBR mRNA value to GAPDH mRNA.

Quantification of Angiogenesis (Intratumoral Microvessel Density). Immunohistochemical staining for CD34 or CD105 monoclonal antibody were counted under light microscopy with a 200-fold magnification. The average counts were recorded as the CD34 intratumoral microvessel density (CD34-IMVD) or CD105-IMVD for each case.

Statistical Analysis. The StatView 5.0 statistical software package was used for all statistical analyses. The χ² was used to compare counts. Continuous data between two groups were compared using Student’s t test if the distribution of samples was normal, or Mann-Whitney U test if the sample distribution was asymmetrical. Continuous data among three or more groups were compared using ANOVA if the distribution of samples was normal, or Kruskal-Wallis H test if the sample distribution was asymmetrical. The postoperative survival rate was analyzed by the Kaplan-Meier method, and the differences in survival rates were assessed by the log-rank test. Multivariate analysis of prognostic factors was done using Cox’s proportional hazard model. Differences were considered significant when P < 0.05.

Results

CBR mRNA Expression in Tumor Tissues from Lung Cancer Patients. Tumor tissues obtained from 59 non–small-cell lung cancer patients were reviewed to assess the correlation between CBR mRNA expression and the clinicopathologic features. No significant correlation was revealed between CBR mRNA expression and age, sex, grade of tumor differentiation, or p-stage (Table 1). CBR mRNA expression in adenocarcinoma was significantly higher than that in other histologic types (P = 0.02; Table 1).

CBR mRNA expression was associated with tumor status including pathologic (p) T factor and pN factor. CBR mRNA expression was significantly reduced along with progression of primary tumors (the mean CBR mRNA/GAPDH mRNA, 3.288 × 10⁻² for pT1, 1.628 × 10⁻² for pT2, and 1.175 × 10⁻² for pT3-4 disease, respectively; P = 0.02). CBR mRNA expression in tumor with nodal involvement seemed to be reduced compared with tumor without nodal involvement (the mean CBR mRNA/GAPDH mRNA, 1.446 × 10⁻² for pN-positive, and 2.531 × 10⁻² for pN-negative, respectively), whereas the difference did not reach a statistical significance (P = 0.09).

Subsequently, to assess the relationship between tumor angiogenesis and CBR mRNA expression, IMVD, a measurement of angiogenesis, was examined by immunohistochemical

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients and CBR mRNA expression</th>
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<tr>
<td>No. patients (%)</td>
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<tr>
<td>All patients 59 (100) 2.091 ± 2.421 0.09</td>
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<tr>
<td>Age &lt;68 y (median) 30 (50.8) 2.618 ± 3.157 0.28</td>
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<tr>
<td>≥68 y 29 (49.2) 1.547 ± 1.102 0.28</td>
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<tr>
<td>Gender Male 42 (71.2) 1.874 ± 2.605 0.28</td>
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<tr>
<td>Female 17 (28.8) 2.628 ± 1.851 0.28</td>
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<tr>
<td>Histologic type Adenocarcinoma 41 (69.5) 2.672 ± 2.695 0.28</td>
</tr>
<tr>
<td>Squamous cell carcinoma 13 (22.0) 0.842 ± 0.531 0.28</td>
</tr>
<tr>
<td>Large cell carcinoma 5 (8.5) 0.576 ± 0.267 0.28</td>
</tr>
<tr>
<td>Tumor differentiation Well 27 (45.8) 2.494 ± 1.998 0.28</td>
</tr>
<tr>
<td>Moderate 19 (32.2) 1.934 ± 3.397 0.28</td>
</tr>
<tr>
<td>Poor 13 (22.0) 1.411 ± 1.219 0.28</td>
</tr>
<tr>
<td>Postage I 26 (44.1) 2.695 ± 3.094 0.13</td>
</tr>
<tr>
<td>II 8 (13.6) 0.982 ± 0.668 0.13</td>
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<tr>
<td>III 12 (20.3) 1.102 ± 0.919 0.13</td>
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<tr>
<td>IV 13 (22.0) 2.480 ± 2.114 0.13</td>
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NOTE: Data are means ± SD.
staining; as a marker of endothelial cells, CD34, a marker of pan-endothelial cells (CD34-IMVD), or CD105, a marker of proliferation-related endothelial cells (CD105-IMVD), was used. The mean CD105-IMVD for CBR-high tumor was significantly lower than that for CBR-low tumor (59.2 and 130.6, respectively; \( P = 0.02 \)), whereas no significant difference in the mean CD34-IMVD according to the status of CBR mRNA expression was documented (298.0 and 313.0, respectively; \( P = 0.74 \)).

CBR mRNA Expression and Postoperative Survival. Univariate analyses of postoperative survivals of 59 non–small-cell lung cancer patients were shown in Table 2. Five-year survival rates of CBR-high patients and CBR-low patients were 68.3% and 36.5%, respectively, demonstrating a significant favorable prognosis of CBR-high patients (log-rank, \( P = 0.03 \); Fig. 1; Table 2).

A multivariate analysis using Cox's proportional hazard model showed that p-stage was the most significant prognostic factor (relative risk, 4.15; 95% confidence interval, 1.72-10.0; \( P < 0.01 \)), whereas the grade of tumor differentiation had no independent influence on postoperative survival (Table 2). Finally, CBR-high expression was an independent and significant factor to predict a favorable prognosis (relative risk, 0.39; 95% confidence interval, 0.16-0.98; \( P = 0.04 \); Table 2).

Discussion

In the present study, we showed that high expression of CBR mRNA was a significant factor to predict a favorable prognosis in non–small-cell lung cancer. In addition, we, for the first time, have revealed that the expression of CBR mRNA is significantly correlated with tumor angiogenesis as well as progression in malignant tumor. Although experimental studies have revealed a potential importance of CBR in the development and progression of malignant tumors, only a few clinical studies on CBR expression in malignant tumors have been reported (20, 22), and the clinical significance remains unclear. Only one study conducted by Umemoto et al. (20) assessed a prognostic significance of CBR status and revealed that immunohistochemically determined reduced CBR expression was significantly correlated with a poor prognosis in epithelial ovarian cancer tissues, which was consistent with our result demonstrating that low CBR expression is significantly correlated with a poor prognosis in non–small-cell lung cancer. Umemoto et al. (20) also showed that reduced CBR expression was significantly correlated with nodal involvement. The present study revealed similar results; reduced CBR mRNA expression was significantly correlated with progression of primary tumor and seemed to be correlated with nodal involvement although the difference was not statistically significant. However, in another clinical study on CBR expression in hepatocellular carcinoma, CBR status determined by immunohistochemical staining was not correlated with tumor progression (22). These conflicting results may be due to small number of patients. Larger

![Figure 1. Postoperative survival of non–small-cell lung cancer patients according to the status of CBR mRNA expression. CBR expression status was classified into CBR-low or CBR-high by using the median CBR mRNA/GAPDH mRNA value (1.30 × 10⁻²) as a cutoff.](http://example.com/fig1.png)
prospective studies should be conducted to clarify clinical significance of CBR expression in malignant tumors including non–small-cell lung cancer.

Although several experimental and clinical studies, including the present study, suggested that CBR played a role in progression of malignant tumors, the mechanisms of action remain unclear. It has been revealed that CBR is identical to prostaglandin 9-ketoreductase which inactivates PGE2 (10, 11). PGE2 can promote tumor angiogenesis (10, 11, 14-17, 23), and CBR may inhibit tumor angiogenesis through inactivation of PGE2. Tumor angiogenesis has been known to closely correlate with tumor progression and prognosis in a variety of malignant tumors (24) including non–small–cell lung cancer (21, 25, 26). Thus, we evaluated tumor angiogenesis by immunohistochemical staining using an anti-C3D4 antibody (CD34-IMVD) and an anti-CD105 antibody (CD105-IMVD) in correlation with CBR mRNA expression. Interestingly, CBR mRNA expression was inversely correlated with CD105-IMVD, but not with CD34-IMVD. As shown in previous experimental studies, CD105 is a proliferation-related endothelial cell marker, and anti-CD105 antibodies preferentially react with “activated” endothelial cells in tissues participating in angiogenesis such as tumor tissues (27, 28). In clinical studies as well, CD105-IMVD proved to be superior to CD34-IMVD in the evaluation of angiogenesis in breast carcinoma and non–small–cell lung cancer (21, 28). The significant correlation between reduced CBR mRNA expression and higher CD105-IMVD documented in the present study supports the experimental data showing that CBR may inhibit tumor angiogenesis. These results strongly suggest that enhanced CBR expression is correlated with a favorable prognosis as well as lower pt and/or pn factors partly through inhibition of tumor angiogenesis. To clarify the relationship between angiogenesis and CBR expression, PGE2 and vascular endothelial growth factor expression, along with CBR expression, should be assessed in future studies.

In conclusion, the present study revealed that reduced CBR mRNA expression in non–small–cell lung cancer was significantly correlated with active angiogenesis and tumor progression, and that CBR mRNA status was an independent and significant prognostic factor.

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References
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