

Cyclooxygenase-2 (COX-2) mRNA Expression Levels in Normal Lung Tissues and Non-small Cell Lung Cancers

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One of the cyclooxygenase (COX) isoforms, COX-2, is overexpressed in various human cancers. In this study, we examined the gene expression levels of COX-2 in primary non-small cell lung cancers (NSCLC), metastatic lymph nodes, and normal lung tissues. The expression levels of the COX-2 gene were assessed by means of the reverse transcription polymerase chain reaction in 76 autopsy samples (29 primary NSCLC, 29 corresponding normal lung tissues, and 9 metastatic lymph nodes). The expression levels in NSCLC (both adenocarcinomas and squamous cell carcinomas) were significantly higher than in normal lung tissues and were significantly higher in adenocarcinomas than in squamous cell carcinomas. Differences between the levels of expression of COX-2 in primary tumors and their corresponding metastatic lymph nodes in 9 adenocarcinoma patients were not significant. Our results indicate that COX-2 may be associated with carcinogenesis of NSCLC, and that it may be a target for the treatment of NSCLC.

Key words: Cyclooxygenase-2 (COX-2) — Non-small cell lung cancer — RT-PCR

Cyclooxygenase (COX), which catalyzes arachidonate metabolism, has two isoforms, COX-1 and COX-2. COX-1 is expressed constitutively in many mammalian tissues, while COX-2 is induced by several stimuli associated with inflammation. Although the two isoforms have very similar structure, they play independent roles in arachidonate metabolism.¹⁾

Previous reports showed that nonsteroidal antiinflammatory drugs (NSAIDs) reduced the risk of colon cancer and decreased the number and size of colorectal adenomas in patients with familial adenomatous polyposis (FAP).^{2,3)} Recently, COX-2 was shown to be induced in the polyps of a mouse FAP model. The number and size of the intestinal polyps were reduced dramatically when COX-2 was inactivated.⁴⁾ Furthermore, several reports showed that COX-2-specific inhibitors inhibited cell growth of colon cancers and had chemopreventive activity.⁵⁻⁷⁾ These results suggest that COX-2 overexpression is associated with colon carcinogenesis.

Recently, overexpression of COX-2 was shown in colorectal cancer,⁸⁾ gastric cancer,⁹⁾ and breast cancer.¹⁰⁾ However, the role and expression of COX-2 in lung cancers are still uncertain. Therefore, in this study we compared the gene expression levels of COX-2 in non-small cell lung cancers (NSCLC) with those in normal lung tissues and their metastatic lymph nodes.

MATERIALS AND METHODS

Patients and samples We studied 67 autopsy samples (29 primary NSCLC, 29 corresponding normal lung tissues, 9 lymph nodes) from 29 patients with lung cancer admitted to Hiroshima University Hospital and Chugoku Rousai General Hospital between September 1993 and October 1997. Fresh specimens of primary lung tumors, normal lung tissues, and metastatic tissues were obtained during autopsy after written informed consent had been obtained. The tissues were frozen in liquid nitrogen and stored at -80°C until analysis.

Reverse transcription polymerase chain reaction (RT-PCR) Total cellular RNA was extracted using the guanidinium isothiocyanate-phenol method described previously.¹¹⁾ We confirmed by ethidium bromide staining that almost the same quantity of total cellular RNA was extracted from each sample. cDNA was synthesized using random hexamer (Amersham, Buckinghamshire, UK) with Superscript RNase H-reverse transcriptase (GIBCO-BRL, Bethesda, MD), as described previously.¹¹⁾

First, to confirm the quality of the harvested RNA, the reverse-transcribed cDNA synthesized from equal amounts of total RNA was subjected to PCR amplification using β -actin primers. The sequences of the primers were: forward 5'-AAGAGAGGCATCCTCACCT-3' and reverse 5'-TACATGGCTGGGGTGTGAA-3'. The PCR conditions were as described previously.¹¹⁾ Twenty amplification cycles using these primers were carried out, and the PCR products were 218 bp long, corresponding to β -actin

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cDNA. We found that the expression levels of β -actin from the samples were essentially the same as determined by ethidium bromide staining, even when samples were obtained from patients whose intervals between death and autopsy differed. Therefore, we considered that the quality of harvested RNA from our samples was acceptable for molecular analysis.

The reverse-transcribed cDNA from each sample was subjected to PCR amplification with primers based on the *COX-2* and β -actin (internal control) gene sequences. After pre-denaturation at 94°C for 5 min, the cDNA was added to 5 μ l of PCR mixture, comprising 1 μ l of 10 \times PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl), 1 μ l of 15 mM MgCl₂, 2 μ l of distilled water, 0.2 μ l of 20 mM dNTPs (Takara, Tokyo), 0.2 μ l of 50 μ M forward primer, 0.2 μ l of 50 μ M backward primer, and 0.4 μ l (0.2 U) of *Taq* polymerase (Promega, Madison, WI). We used the *COX-2* forward primer 5'-TCCAAATGAGAT-TGTGGGAAAATTGCT-3' and reverse primer 5'-AGAT-CATCTCTGCCTGAGTATCTT-3'.¹²⁾ Amplification was carried out using a thermal cycler (Geneamp PCR System 2400; Perkin Elmer Applied Biosystems Division, Norwalk, CO). An amplification cycle for the reactions using the *COX-2* primers comprised denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. The PCR products of *COX-2* were 305 base pairs long. In order to determine the optimal number of cycles, the accuracy of the quantitative PCR procedure was tested in titration experiments, as described previously.¹¹⁾ The optimal number of cycles for *COX-2* was 24. We used the β -actin gene as an internal control; the sequences of its primers and amplification cycles were as described above.

Quantification of mRNA expression The PCR products were electrophoresed using 2% w/v agarose gels, transferred to nylon membranes (Hybond N+; Amersham), and subjected to hybridization analysis with ³²P-labeled cDNA probes using the procedures described previously. After

washing each filter, the radioactivity level was measured with a laser imaging analyzer (BAS-2000; Fuji Photo Film, Tokyo). The products of *COX-2* described above were used as cDNA probes and the β -actin probe was as described previously.¹¹⁾ The radioactivity associated with gene expression in each sample is expressed as the yield of the target gene relative to that of the β -actin gene (Fig. 1).

Statistical analysis Contingency table analyses based on χ^2 statistics were used to determine the significance of associations between categorical variables. Differences between the expression levels of each gene in tissue samples were analyzed with the Mann-Whitney *U*-test. All statistical tests were two-sided, the data were expressed as medians and ranges, and differences of *P* values of less than 0.05 were considered to be significant.

RESULTS

Patients' characteristics We analyzed the expression levels of *COX* genes in 76 autopsy samples (29 primary NSCLC, 29 corresponding normal lung tissues, and 9 lymph nodes) from 29 patients, whose characteristics are presented in Table I. There were 24 men and 5 women, ranging in age from 44 to 86 years old (median 65 years). Nineteen had adenocarcinoma and 10 had squamous cell carcinoma. Almost all of them (26 of 29) had been smokers. The intervals between death and autopsy were 1 to 16 h (median 3 h).

Expression levels of *COX-2* gene There was considerable variability between tumors or between normal lung tissues in the level of expression of *COX-2*. The median levels of *COX-2* expression were 0.108 (range 0.001–0.462) in tumors and 0.002 (0.001–0.115) in normal lung tissues. The expression levels in tumors were significantly higher than those in normal lung tissues (*P*<0.0001, Fig. 2). We also compared the levels of expression

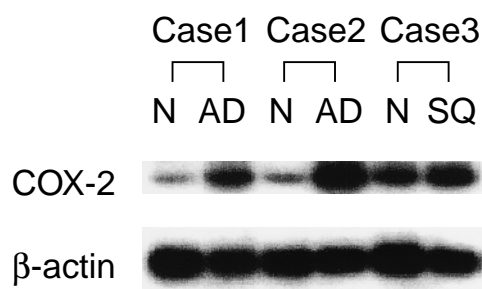


Fig. 1. Expression of *COX-2* relative to that of β -actin evaluated by RT-PCR.

Table I. Patients' Characteristics

All patients	29
Age (years)	
median	65
range	44–86
Sex (male/female)	24/5
Smoker (yes/no)	26/3
Histology	
adenocarcinoma	19
squamous cell carcinoma	10
Intervals between death to autopsy (h)	
median	3
range	1–16

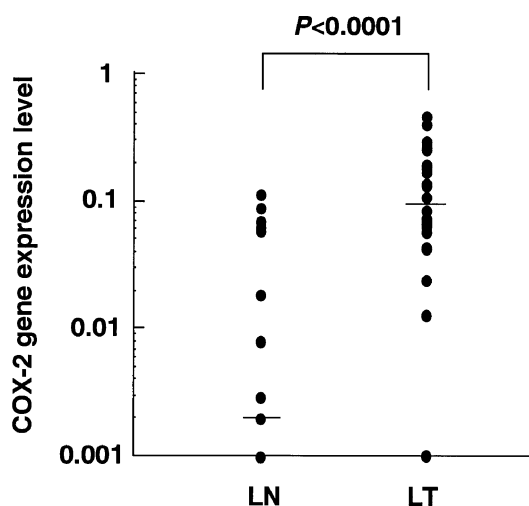


Fig. 2. Expression of COX-2 in primary NSCLC tissues (LT) compared with that in normal lung tissues (LN). Bars denote median gene expression levels.

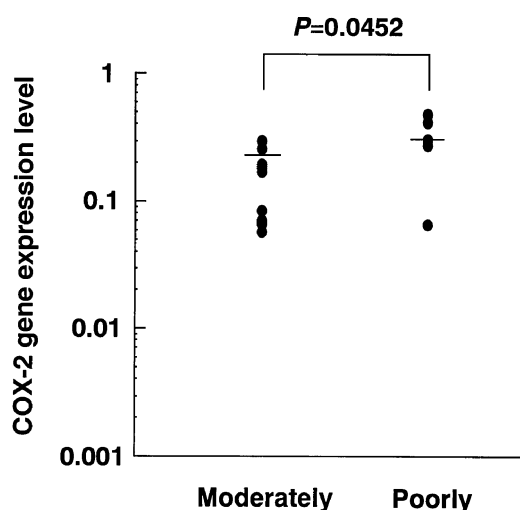


Fig. 4. Expression of COX-2 in poorly differentiated adenocarcinomas compared with that in moderately differentiated adenocarcinomas. Bars denote median gene expression levels.

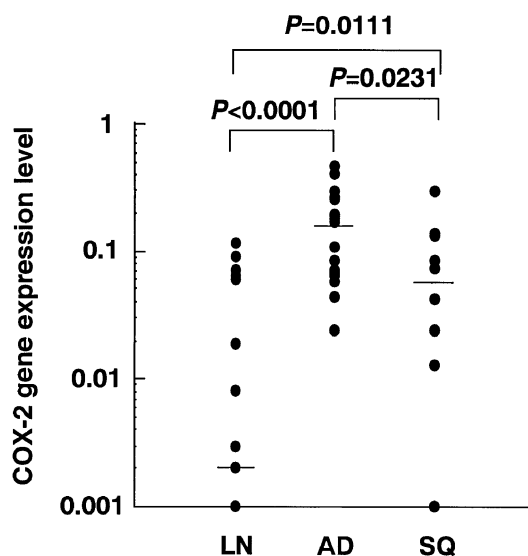


Fig. 3. Comparison of expression of COX-2 in normal lung tissues (LN), adenocarcinomas (AD), and squamous cell carcinomas (SQ). Bars denote median gene expression levels.

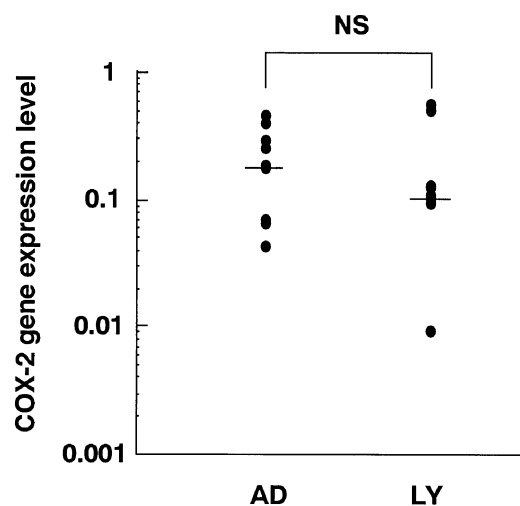


Fig. 5. Expression of COX-2 in primary adenocarcinomas (AD) compared with that in their lymph node metastatic tissues (LY) in 9 patients. Bars denote median gene expression levels. NS, not significant.

of COX-2 among adenocarcinomas, squamous cell carcinomas, and normal lung tissues. The expression levels of COX-2 were 0.182 (0.024–0.462) in adenocarcinomas and 0.058 (0.001–0.296) in squamous cell carcinomas. These were significantly higher than those in normal lung tissues (adenocarcinomas, $P < 0.0001$; squamous cell carcinomas, $P = 0.0111$; Fig. 3). Further, the levels of expression of COX-2 in adenocarcinomas were significantly

higher than those in squamous cell carcinomas ($P = 0.0231$, Fig. 3).

Six adenocarcinomas were poorly differentiated, 10 were moderately differentiated, and 3 were papillary adenocarcinomas. We compared the levels of expression of COX-2 between poorly and moderately differentiated adenocarcinomas. The median levels were 0.300 (range

0.063–0.462) in poorly differentiated types and 0.176 (0.024–0.291) in moderately differentiated types. There was a significant difference between the two groups ($P=0.0452$, Fig. 4).

We also compared the levels of expression of *COX-2* gene between primary tumors and their corresponding metastatic lymph nodes in 9 adenocarcinoma patients. The median levels of COX-2 expression were 0.190 (range 0.043–0.462) in primary tumors and 0.106 (0.009–0.553) in lymph nodes. There was no significant difference between the two groups (Fig. 5).

DISCUSSION

The present study shows that levels of *COX-2* gene expression not only in adenocarcinomas, but also in squamous cell carcinomas of the lung were significantly higher than those in normal lung tissues.

Recently, COX-2 expression in human lung cancers was investigated by immunohistochemical analysis; the results showed frequent overexpression of COX-2 in adenocarcinomas.^{13,14} Our data confirmed these findings, and the present report is the first to document such differences based on molecular biological methods. Both colon cancers and gastric cancers that were histologically adenocarcinomas were also shown to overexpress COX-2.^{8,9} Our results indicate that adenocarcinomas of various cancer types show increased COX-2 expression.

The role of COX-2 in colorectal cancer has been intensively studied. Several reports showed that NSAIDs decrease the number and size of colorectal adenomas in patients with FAP and reduce the risk of colon cancer,^{2–4} and that COX-2-specific inhibitors play a role in the regression of the cell growth of colon cancers and have chemopreventive activity.^{5–7} These reports indicate that COX-2 overexpression is associated with colon carcinogenesis. However, the role of COX-2 in lung cancer is still uncertain. Recently, atypical epithelium, which is considered to be a precursor lesion for lung cancers, was also found to express increased COX-2.¹⁴ Furthermore, NSAIDs inhibited proliferation of NSCLC xenografts in nude mouse,¹² and a COX-2-specific inhibitor prevented lung carcinogenesis in A/J mice.¹⁵ These results suggest that COX-2 expression may be associated with lung carcinogenesis and its progression.

Interestingly, we also found greater expression of COX-2 in squamous cell carcinomas than in normal lung tissues. Hida *et al.*¹³ reported overexpression of COX-2 in adenocarcinomas, although they did not compare COX-2 expression between normal lung tissues and squamous cell carcinomas. On the other hand, Wolff *et al.*¹⁴ detected COX-2 expression in all squamous cell carcinomas of the lung but not in the normal counterparts. Zimmermann *et al.*¹⁶ showed immunohistochemically that most esophageal

squamous cell carcinomas express COX-2, and that expression of the COX-2 protein in some tumors was greater than in normal esophageal epithelia. In addition, greater COX-2 mRNA levels were found in squamous cell carcinomas of the head and neck than in normal oral mucosa from healthy volunteers and in normal-appearing epithelium adjacent to squamous cell carcinomas of the head and neck.¹⁷ These results indicate that COX-2 may play a role in carcinogenesis in squamous cell carcinomas. Furthermore, COX-2-specific inhibitors had chemopreventive activity,^{5–7} suggesting that COX-2-specific inhibitors may prevent cell growth not only in adenocarcinomas, but also in squamous cell carcinomas.

We found greater expression of COX-2 in poorly differentiated adenocarcinomas than in moderately differentiated adenocarcinomas. Hida *et al.*¹³ also found more intensive expression of COX-2 in poorly differentiated lesions than in well or moderately differentiated lesions. Several earlier reports showed that poorly differentiated adenocarcinomas had a higher metastatic potential and a poorer prognostic outcome than well or moderately differentiated adenocarcinomas.^{18–20} Furthermore, increased COX-2 expression was shown to be associated with a poor prognosis in stage I lung adenocarcinoma.²¹ These results indicate that COX-2 expression can be a marker for aggressive behavior of NSCLC. However, as adenocarcinomas frequently show an admixture of differentiated phenotypes in a single tumor, further studies are required to elucidate the role of COX-2 expression in the transition of differentiated phenotypes of adenocarcinomas.

Tsujii *et al.*²² showed that constitutive COX-2 expression increased the metastatic potential in colorectal cancers. Hida *et al.*¹³ compared the COX-2 expression in primary lung adenocarcinomas and their corresponding metastatic lymph nodes, finding much greater expression in metastatic lymph nodes than in primary tumors. Recently it was shown that COX-2 can modulate production of angiogenic factors.²³ Angiogenic factors are suggested to contribute not only to the progressive growth of primary cancers, but also to their metastatic potential.^{24,25} These results suggest that COX-2 may be associated with acquisition of metastatic potential. Although, we found no significant differences in COX-2 expression levels between metastatic lymph nodes and primary adenocarcinomas, the COX-2 expression levels in metastatic lymph nodes were significantly higher than those in normal lung tissues (data not shown). However, further studies are required to elucidate whether COX-2 expression is associated with metastatic potential.

One possible criticism of our study is that we used autopsy samples. However, all the autopsies were performed within 16 h of death, which has been proved to be acceptable for obtaining mRNAs and proteins of satisfactory quality.²⁶

Finally, several *in vitro* studies showed that COX-2 inhibitors can induce apoptotic cell death in human cancer cells.^{27–29} At present, neither chemotherapy nor radiotherapy is effective for NSCLC treatment. Although further studies are required to determine whether COX-2 is a suitable target for the treatment of NSCLC, COX-2 inhibitors may contribute to the development of new therapies for NSCLC.

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