

## Expression of 5-Lipoxygenase in human colorectal cancer

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### Abstract

**AIM:** To evaluate the 5-lipoxygenases (Loxs) expression level in human colorectal cancer specimens in order to determine its clinicopathologic significance in human tumorigenesis.

**METHODS:** The relative quantity of 5-Lox mRNA in paired 91 colorectal tumor and adjacent normal mucosa samples was determined by real time quantitative PCR. Additionally, the expression of 5-Lox and cyclooxygenase (Cox)-2 proteins was also examined using immunohistochemical staining methods.

**RESULTS:** There was a marked increase in 5-Lox mRNA levels in the tumor compared with paired normal mucosa samples ( $P < 0.0001$ ). Sixty six (72.5%) tumors showed high 5-Lox mRNA levels. The positivity rate of 5-Lox and Cox-2 protein expression was 68.7% and 79.1% respectively. There was a significant association between tumoral 5-Lox mRNA level and tumor size (Rho = 0.392,  $P = 0.0002$ ), depth or vessel invasion.

**CONCLUSION:** These results suggest that 5-Lox is up-regulated in colorectal cancer and that inhibition of its expression might be valuable in the prevention and treatment of colorectal cancer.

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**Key words:** Arachidonic acid; 5-Lipoxygenase; Cyclooxygenase-2; Real time PCR; Immunohistochemistry; Colorectal cancer

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### INTRODUCTION

Evidence from epidemiological and animal studies suggests that a high-fat consumption is associated with an increased incidence and growth of tumors at several specific organ sites, including breast, pancreas, colon, and prostate<sup>[1]</sup>. In this respect, it has been demonstrated that the metabolism of arachidonic acid, a polyunsaturated fatty acid by either the Cyclooxygenase (Cox) pathway or the Lipoxygenase (Lox) pathway, generates a host of pro-inflammatory substances called eicosanoids including prostaglandins, thromboxanes, leukotrienes, *etc.*, that act as potent autocrine and paracrine regulators of cell biology<sup>[2]</sup>. These substances are known to modulate diverse physiological and pathological responses, including growth and invasiveness of tumor cells as well as suppression of immune surveillance<sup>[2]</sup>.

To date, two Cox isozymes have been identified, the constitutive Cox-1, and the inducible Cox-2<sup>[3-5]</sup>. For Lox, three major isoforms have been characterized in human tissue, according to the oxygenation sites in the substrate arachidonic acid. These enzymes are named, 5-, 12-, and 15-Lox<sup>[4,7]</sup>. Over the last two decades, a large body of evidence from experimental and clinical studies has shown that Cox-1/-2 play an important role in the promotion and progression of many types of human neoplasms<sup>[3,8-11]</sup>. And most importantly, specific inhibition of these enzymes is reported to exhibit a dramatic anti-neoplastic activity<sup>[12-15]</sup>. Although most attention has focused on prostaglandins (PGs) and other Cox-derived metabolites, emerging evidence now suggests that Lox-catalyzed products, Leukotrienes (LTs) and Hydroxyeicosatetraenoic acids (HETEs) also exert profound biological effects on the development and progression of human cancers<sup>[7]</sup>. An increase in expression of Lox and their metabolites has been detected in a variety of human cancer cell lines and tissues including those of the prostate<sup>[16]</sup>, bladder<sup>[17]</sup>, breast<sup>[18]</sup>, lung<sup>[18]</sup>, colon<sup>[5,19]</sup>, pancreas<sup>[1,20]</sup>, oral<sup>[21]</sup> and esophagus<sup>[22]</sup>; and this over-expression was reported to be significantly associated with tumor cell proliferation, resistance to apoptosis, and angiogenesis<sup>[23-25]</sup>. Moreover, the direct inhibition of 5-Lox or 12-Lox, was found to markedly suppress the tumor cell growth<sup>[2,17,18,26]</sup>. Nonetheless, some investigators also have demonstrated a

down-regulation of 15-Lox-1/-2 in human colorectal<sup>[27,28]</sup> and prostate tumors<sup>[29]</sup> compared with adjacent normal tissues. The clinicopathologic significance of Lox expression is less clearly defined in human colorectal cancer. This observation prompted us to evaluate the expression level of 5-Lox and, determine its correlation with clinical and pathological features of colorectal cancer.

## MATERIALS AND METHODS

### Patients and tissue samples

Ninety one patients who underwent surgical resection for colorectal cancer in our institution between January and December 2004 were included in this study. These consisted of 53 men and 38 women with a mean age of  $64 \pm 11$  years; 49 colon cancers and 42 rectal cancers. The clinicopathologic parameters were determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification of malignant tumors<sup>[30]</sup> and, the tumor size represents the greatest dimension measured after formalin fixation. Immediately after surgery, a small piece of colorectal tumor samples and matched adjacent normal mucosa (taken from the borders of the surgical specimen), were separately placed directly in RNA stabilization reagent (RNAlater, Qiagen) and stored frozen at  $-80^{\circ}\text{C}$  until further analysis. Additionally, 67 formalin-fixed, paraffin-embedded tumor tissue blocks obtained from the same patients were also available for immunohistochemical studies.

This study was approved by the Institutional Review Board of the Tokyo Medical and Dental University, and written informed consent was obtained from all patients.

### RNA extraction and cDNA synthesis

Total RNA for each sample was extracted using the RNeasy mini kit (Qiagen) according to the manufacturers' instructions. RNA was quantified by measurement of  $A_{260}$  and  $A_{280}$  using a UV spectrophotometer (Beckman, Life Science); and, its quality was determined by electrophoresis through agarose gels and visualization of the 18S and 28S RNA bands under UV light. For cDNA synthesis, 10  $\mu\text{g}$  of total RNA was reverse-transcribed into cDNA samples using Superscript II RNase H-Reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

### Real time PCR

Real time quantitative Polymerase Chain Reaction (RTQ-PCR) analysis was done by using the 7300 Real Time PCR System (Applied Biosystems). Primers and probe sequences were designed using the Primer Express software (Applied Biosystems) as follows: 5-Lox primers: GGGCATGGAGAGCAAAGAAG and ACCTCGGCCGTGAACGT, probe: TACTTCTACCGGGACGACGGGCTCCT; ACTB-1 primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTACGCACGATTT; probe: ACCACCACGGCCGAGCGG.

The PCR reaction was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1  $\mu\text{L}$  of cDNA in a 24  $\mu\text{L}$  final reaction volume. The thermal cycling conditions were as follows:  $50^{\circ}\text{C}$  for 2 min,

$95^{\circ}\text{C}$  for 10 min and 45 cycles of 15 seconds denaturation at  $95^{\circ}\text{C}$  and 1 min annealing at  $60^{\circ}\text{C}$ . Each sample was run in duplicate for both target gene and endogenous gene.

The amount of 5-Lox (target) normalized to ACTB-1 (endogenous control) and relative to HCT15 (calibrator), was determined by the comparative  $C_T$  method<sup>[31]</sup> using the Relative Quantification (ddCt) Study Software (7300 Sequence Detection System version 1.2.1, Applied Biosystems).

The human colon adenocarcinoma cell line HCT15 used as a calibrator in this study, was kindly provided by Cell Resource Center for Biomedical Research Institute (Tohoku University, Miyagi, Japan).

### Immunohistochemical evaluation of 5-Lox expression

A Universal Immuno-enzyme Polymer method (Nichirei simple staining) was performed for immunohistochemical staining as we previously described<sup>[32]</sup>. Anti-5-Lox polyclonal antibody (Cayman Chemical, Ann Arbor, MI; dilution: 1:250) and anti-Cox-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI; dilution: 1:250) were used as the primary antibody for 2 h incubation at room temperature.

The immunoreactivity of 5-Lox/Cox-2 protein in the tumor cells was determined according to the procedure previously described<sup>[32]</sup>. Briefly, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), 3 (strong). Extent of staining was scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%) according to the percentage of positive staining area in relation to the whole carcinoma area. Then, the sum of intensity and extent score was regarded as the final staining score for 5-Lox or Cox-2; and, tumors having a final score of  $\geq 3$  were considered to be positive<sup>[32]</sup>.

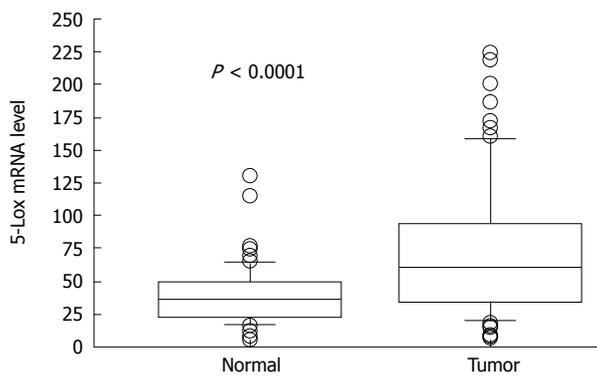
### Statistical analysis

Median value of normal samples was used as the cut-off point to distinguish between low and high levels of mRNA expression. Nonparametric Wilcoxon and Mann-Whitney  $U$  tests were used to evaluate differences in 5-Lox mRNA expression levels between groups. The correlations with clinicopathologic parameters were assessed with a Chi-square test for categorical variables and a Spearman rank test for continuous variables. The strength of association between 5-Lox and Cox-2 staining scores was determined by the Spearman rank test. The difference was considered significant at  $P < 0.05$ . All analyses were performed with the statistical software package Stat View (version 5.0).

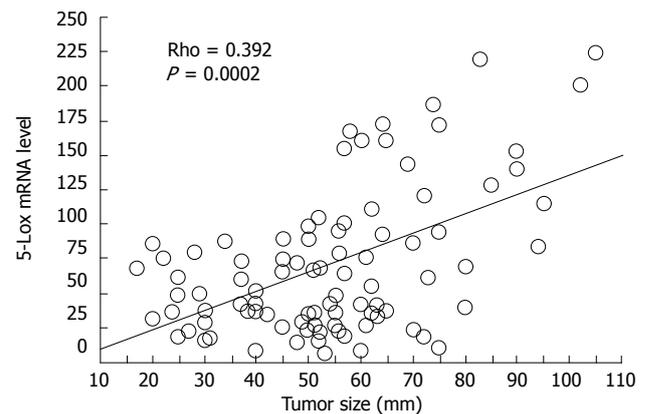
## RESULTS

### 5-Lox mRNA expression and its association with clinicopathologic parameters

The median value of 5-Lox mRNA expression (relative quantity, RQ) in normal and tumor samples was 36.094 (minimum: 5.966, maximum: 131.859) and 61.113 (minimum: 6.228, maximum: 224.773), respectively. Both Wilcoxon and Mann-Whitney  $U$  tests, showed a significant difference in mRNA levels between the two groups of samples ( $P < 0.0001$ ; Figure 1). Using the median RQ of normal samples as the cut off point, we found that 66 of



**Figure 1** Differential 5-Lox mRNA relative quantity between matched normal mucosa and tumor tissue samples. Boxes: lower and upper quartiles (median inside); bars: at end of whiskers; individual plots: values outside the range of the whiskers.



**Figure 2** Linear regression plot showing a significant positive correlation between tumoral 5-Lox mRNA level and tumor size.

**Table 1** Correlation of clinicopathologic parameters with 5-Lox mRNA expression

Clinicopathologic parameters	n	5-Lox expression		P
		Low	High	
All cases:	91	25 (27.47%)	66 (72.53%)	
Age:	91	Rho = 0.018		NS <sup>1</sup>
Gender:				
Male	53	15	38	
Female	38	10	28	NS
Tumor site:				
Colon	49	13	36	
Rectum	42	12	30	NS
Tumor size:	91	Rho = 0.392		0.0002 <sup>1</sup>
Histology:				
Well	49	11	38	
Moderate	34	13	21	
Poor	8	1	7	NS
Depth of invasion:				
pT1	11	7	4	
pT2	18	6	12	
pT3	43	9	34	
pT4	19	3	16	0.021
Lymph node metastasis:				
Yes	45	16	29	
No	46	9	37	NS
Lymphatic invasion:				
Yes	66	14	52	
No	25	11	14	0.030
Venous invasion:				
Yes	72	15	57	
No	19	10	9	0.006
TNM stage:				
I	12	2	10	
II	31	7	24	
III	38	15	23	
IV	10	1	9	NS

<sup>1</sup>Spearman rank test P value; NS: Not Significant ( $P > 0.05$ ).

91 tumor specimens (72.5%) showed high 5-Lox mRNA level. In Table 1, the associations between 5-Lox mRNA expression status and various clinicopathologic parameters are shown. 5-Lox expression was significantly correlated

with tumor size (Rho = 0.392,  $P = 0.0002$ ; Figure 2), depth of invasion ( $P = 0.0208$ ), lymphatic invasion ( $P = 0.0297$ ), and venous invasion ( $P = 0.0057$ ). There was no significant correlation with patient age and gender, tumor site, histological type, lymph node metastasis and TNM stage.

### 5-Lox and Cox-2 protein expression

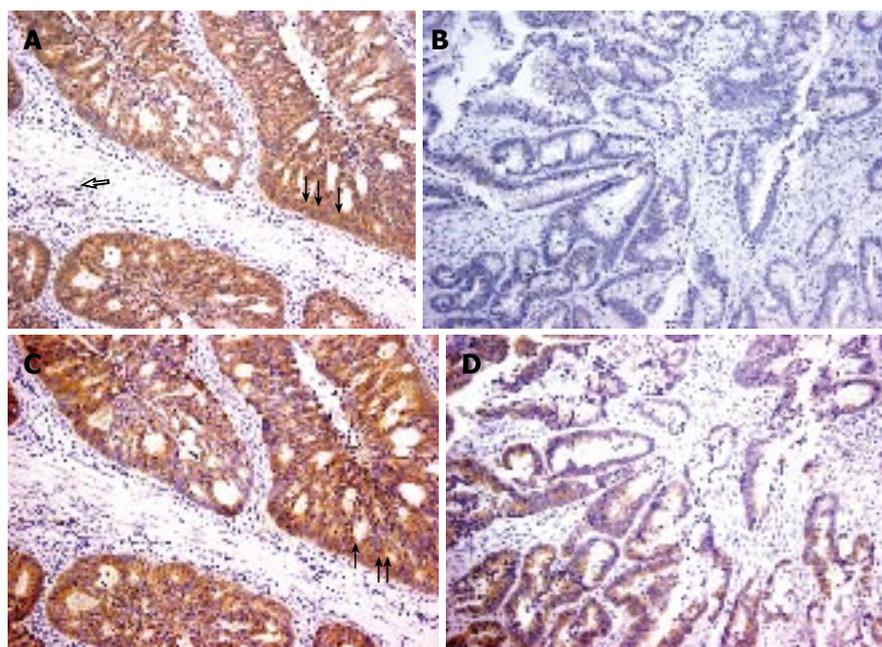
Immunoreactivity of 5-Lox protein was found in the tumor epithelial cells within the cytoplasm, nucleus and nuclear membrane. In contrast, Cox-2 expression was mainly cytoplasmic. Occasionally, little staining was found in the stromal cells or tissue and the adjacent normal mucosa particularly for 5-Lox. Representatives of the staining patterns are shown in Figure 3.

The positivity rate (staining score of 3-7) for 5-Lox and Cox-2 expression was 68.7% and 79.1%, respectively. The Spearman rank test, showed a significant correlation between 5-Lox and Cox-2 staining scores (Rho = 0.277,  $P = 0.0246$ ); and, 5-Lox mRNA levels versus protein staining scores (Rho = 0.380,  $P = 0.0025$ ). Forty four samples stained concurrently positive for both proteins, and had a greater mean tumor size when compared with the group of other samples ( $58.273 \pm 19.966$  vs  $46.391 \pm 20.221$ ,  $P = 0.0476$ ; Figure 4).

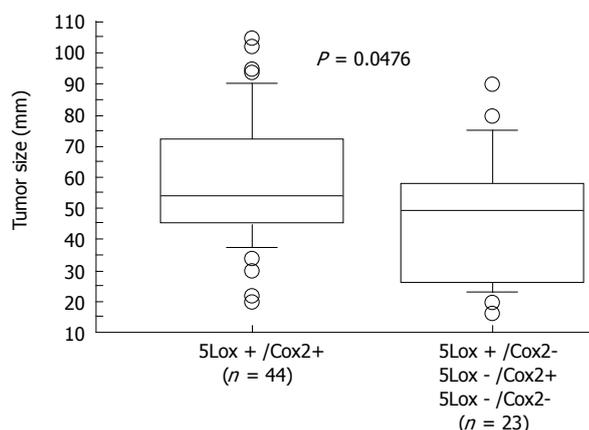
## DISCUSSION

In the current study, we demonstrated that 5-Lox expression is significantly up-regulated in human colorectal tumor compared with the adjacent normal mucosa. Similar results have been reported for various tumors including those of the colon and rectum<sup>[19]</sup>, esophagus<sup>[22]</sup>, pancreas<sup>[11]</sup>, oral<sup>[21]</sup>, bladder<sup>[17]</sup>, and prostate<sup>[16]</sup>. However, according to a PubMed search using the keywords 5-Lipoxygenase, and cancer, this is the first study to examine the expression levels and clinicopathologic significance of this gene in a large series of surgical specimens.

Our most important finding was the positive correlation found between 5-Lox expression level with tumor size, depth and vessels invasion. This observation is similar to our previous data indicating that gastric<sup>[33]</sup> or colorectal<sup>[32]</sup> tumors with higher Cox-2 level, grow



**Figure 3** Representative cases of 5-Lox/Cox-2 immunostaining in colorectal cancer (x 200). **A:** Positive, diffuse, strong cytoplasmic and focal nuclear membrane 5-Lox staining in epithelial tumor cells (black arrow) and stromal cells (white arrow); **B:** Negative, weak 5-Lox staining. The same samples are shown with strong (**C**) and moderate (**D**) Cox-2 staining.



**Figure 4** Differential tumor size between a group of concurrent 5-Lox/Cox-2 positive samples and a group of others (5Lox+/Cox2-, 5Lox-/Cox2+, 5Lox-/Cox2-).

more larger and deeper. It also provides further support to the concept that 5-Lox and Cox-2 display similarities in expression and function in human cancer such as proangiogenic and anti-apoptotic properties<sup>[3]</sup> and a substrate preference. In our series, the fact that concurrent 5-Lox and Cox-2 positive tumors had the largest mean size, may be considered as the consequence of a combined effect of the two metabolic pathways that remove a proapoptotic substrate, arachidonic acid<sup>[9]</sup>, and generate more proangiogenic, anti-apoptotic products, eicosanoids; leading to an increase tumor cells growth. The absence of a statistically significant correlation between 5-Lox overexpression and more aggressive tumor behavior such as lymph node metastasis, confirmed the observation by Ohd *et al*<sup>[19]</sup> that positive 5-Lox expression was not a strong predictor of survival in a study of 61 colorectal cancer patients<sup>[19]</sup>.

In contrast to Cox-2, little is known about the mechanisms by which lipoxygenases contribute to tumor growth and progression; but, emerging evidence from

several experimental studies suggest that these enzymes and their products, similarly to Cox-2 and PGs, may act on tumor cells through inhibition of apoptosis, increase of cell proliferation and stimulation of angiogenesis<sup>[23-25]</sup>. Recently, Hoque *et al*<sup>[25]</sup> using esophageal cancer cell lines, demonstrated that 5-Lox inhibitors caused a dose- and time-dependant reduction in cell viability and induced apoptosis, which was associated with the level of 5-Lox expression and LTB4 production in these cell lines<sup>[25]</sup>. According to Romano *et al*<sup>[24]</sup>, the regulation of vascular endothelial growth factor (VEGF) release and mRNA levels by 5-Lox activity in malignant mesothelial cells was a crucial mechanism of 5-Lox actions on proliferation and apoptosis<sup>[24]</sup>. Similarly, Ye *et al*<sup>[34]</sup> in a mice inflammation-associated colon cancer formation model study, found that 5-Lox expression was accompanied by an up-regulation of matrix metalloproteinase (MMP)-2 activity and VEGF expression, both of which are key angiogenic factors for tumorigenesis<sup>[34]</sup>.

Regarding other Lox family enzymes, little is known about 12-Lox and, a great controversy exists in the literature as to the involvement of 15-Lox in human carcinogenesis. Ikawa *et al*<sup>[5]</sup> reported that 15-Lox-1 was highly expressed in human colorectal tumor compared with adjacent normal mucosa<sup>[5]</sup>. On the contrary, other investigators have extensively demonstrated not only a down-regulation, but also potential tumor-suppressor functions of 15-Lox-1/-2 in colorectal<sup>[27,28]</sup> and prostate<sup>[29]</sup> tumors.

Nevertheless, many questions still remain unanswered, including the exact contribution of individual enzymes as well as their interactions. Further studies are warranted. Finally, it is worthy to notice that several studies have consistently indicated that pharmacologic agents that specifically inhibit the Lox and/or Cox metabolic pathways, exhibit a dramatic anti-neoplastic activity in addition to their classic anti-inflammatory effect<sup>[7,12-15,35]</sup>. And, despite the recent withdrawal of Vioxx (rofecoxib) from the

market and warning about other Cox-2 inhibitors over risk of cardiovascular side effects<sup>[36]</sup>, these pathways still remain an exciting and promising research area for cancer chemoprevention and treatment. Particularly the class of dual 5-Lox/Cox inhibitors such as licofelone (previously named ML3000) has emerged as an effective and well-tolerated therapy that could offer safety advantages over Cox inhibition alone<sup>[4,37,38]</sup>.

In conclusion, our data suggest that 5-Lox over-expression may influence the development of colorectal cancer. Therefore, inhibition of this metabolic pathway might provide an effective therapeutic option for colorectal cancer prevention and treatment.

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