Clinical Significance of NADPH Oxidase 5 in Human Colon Cancer

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Abstract. *Background/Aim:* Recent studies have reported the involvement of NADPH oxidases (NOXs) in tumor progression. However, the role of NOX5 in colon cancer is unclear. We examined the clinical significance of NOX5 expression in colon cancer. *Patients and Methods:* NOX5 expression was evaluated by immunohistochemistry in 119 patients with stage II or III colon cancer, and the relationship between NOX5 expression and clinicopathological data was analyzed. *Results:* Of all tissues, 39.5% were negative and 60.5% were positive for NOX5 expression. Positive expression was significantly associated with undifferentiated histology ($p=0.037$) and lymph node metastasis ($p=0.023$). The 5-year progression-free survival rate of NOX5-positive patients was significantly worse than that of NOX5-negative patients ($p=0.046$). The rates of local recurrence observed in NOX5-positive patients were higher than that in NOX5-negative patients. *Conclusion:* NOX5 expression may be related to poor prognostic factors and could be useful as a prognostic biomarker.

The NADPH oxidase (NOX) family comprises seven transmembrane proteins, NOX1 through NOX5 and DUOX1 and 2, which affect reactive oxygen species (ROS) generation by transporting electrons across biological membranes to transform oxygen into superoxide. ROS are oxygen-derived chemical compounds that have been implicated in many human diseases such as atherosclerosis, diabetes, neurodegeneration, aging, and cancer (1, 2). ROS can damage DNA, RNA, lipids, and proteins, leading to increased mutations and altered enzyme and protein functions. Thus, XROS contribute to the multistage carcinogenesis process via

the induction of tumor growth by facilitating mutagenesis, tumor initiation, and tumor promotion (3, 4). Additionally, many studies have reported that ROS may regulate signaling pathways, including the platelet derived growth factor/epidermal growth factor receptors, the JAK-STAT pathway, and the ERK-MAPK pathway (5-8).

NOXs are activated in chronic inflammatory diseases such as inflammatory bowel disease and pulmonary fibrosis (9, 10). Further, NOXs are involved in the regulation of cell proliferation and tumorigenesis in various cancers, including colon, prostate, and pancreatic cancers (11-15). NOX1 expression is significantly higher in colon cancer tissues than in adjacent nonmalignant tissues. Recent studies have suggested that NOX1 plays a role in the regulation of colonic cell growth and apoptosis, tumor angiogenesis, and Ras oncogene transformation (16-18). Increased expression of NOX5 has been demonstrated in a number of cancers, including prostate cancer (19), malignant melanoma (20), Barrett's esophageal adenocarcinoma (21), breast cancer (22), and hairy cell leukemia (23). Previous research has suggested that NOX5 has a role in increasing proliferation, reducing apoptosis, and damaging DNA (24). According to a multi-tumor tissue microarray, NOX5 is highly expressed in colon cancer, as well as in melanoma, lymphoma, breast cancer, and prostate cancer (25). However, the significance of NOX5 expression in human colon cancer is still unclear. Therefore, in this study, we examined the levels of NOX5 expression via immunohistochemistry and investigated their association with the clinicopathological features and prognosis of human colon cancer.

Patients and Methods

Patients and clinical specimens. A total of 119 patients who underwent a curative operation for stage II or III colon cancer at the University of Yamanashi Hospital from January 2007 to December 2013 were enrolled in this study. The clinicopathological data of all patients were collected from hospital records and analyzed. Staging was based on the International Union Against Cancer/TNM classification of Malignant Tumors (8th edition) (26). The cecum/

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ascending/transverse colon was defined as the right-sided colon and the descending/sigmoid colon was defined as the left-sided colon. Regarding the recurrence pattern, recurrences at the peritoneum and retroperitoneum were included as local recurrence. The median follow-up period was 1833 days (range=1,507-2,058 days). This study was approved by the Ethics Committee of the University of Yamanashi Hospital (approval number of the institution: 1940), and written informed consent was obtained from all patients.

Immunohistochemistry staining analysis. Paraffin sections (4- μ m thickness) of the tumor tissues were subjected to immunohistochemical staining for the NOX5 protein using the avidin-biotin complex method. Briefly, paraffin sections were dewaxed in xylene and dehydrated through a graded series of alcohols. Antigen retrieval was performed by heating samples in Dako Target Retrieval Solution (Agilent Technologies, Santa Clara, CA, USA) for 10 min at 98°C. Endogenous peroxidases were suppressed by incubating the sections for 10 min in Dako REAL Peroxidase-Blocking Solution (Agilent). The signals caused by endogenous avidin and biotin were decreased by incubating the sections in Dako Biotin-Blocking System (Agilent). The sections were incubated in normal serum, and then incubated overnight at 4°C with NOX5 antibody (1:100; Biorbyt, Cambridge, UK), which was diluted in Dako Antibody Diluent with Background (Agilent). The avidin-biotin-peroxidase complex system (VECTASTAIN Elite ABC Rabbit IgG kit; Vector Laboratories, Burlingame, CA, USA) was used with diaminobenzene tetrahydrochloride for color development. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated through a graded series of alcohols, cleared in xylene, and mounted.

Assessment of NOX5 expression levels. The levels of NOX5 expression in the immunohistochemically stained samples were graded according to the strongest staining intensity. Staining intensity was scored as 1 (weakly reactive), 2 (moderately reactive), and 3 (strongly reactive) (Figure 1A-C). Samples with a score of 1 were defined as negative for NOX5 expression, and those with a score of 2 or 3 were defined as positive for NOX5 expression.

Statistical analysis. The data were analyzed using JMP statistical software (version 14.2; SAS Institute, Cary, NC, USA). The chi-square test or Fisher's exact test were used to assess differences between proportions, and Student's *t*-test was used to assess continuous variables. The Kaplan-Meier method was utilized to calculate survival curves, and differences in survival were evaluated using the log-rank test. The Cox proportional hazards regression model was applied to analyze the independent prognostic factors, and a *p*-value less than 0.05 was considered to indicate statistical significance.

Results

The immunohistochemistry staining for NOX5 revealed that the number of tumor tissues with scores of 1, 2, and 3 were 47 (39.5%), 55 (46.2%), and 17 (14.3%), respectively. Of the 119 patients, 72 (60.5%) were NOX5 positive (score of 2 or 3), and a strong staining pattern of NOX5 was particularly observed around areas of mucus (Figure 1D).

Table I shows that female sex ($p=0.014$), poorer differentiated histology ($p=0.037$), and lymph node metastasis ($p=0.023$) are significantly associated with

Table I. Correlation between clinicopathological features and NOX5 expression in 119 colon cancer patients.

Variable	NOX5 expression		<i>p</i> -Value
	Negative (n=47)	Positive (n=72)	
Gender			
Male	32 (68.1)	32 (44.4)	0.014
Female	15 (31.9)	40 (55.6)	
Age, years	67.1 $\pm$ 17.2	67.4 $\pm$ 15.1	0.927
Location of the tumor			
Cecum	15 (31.9)	19 (26.4)	0.455
Ascending	3 (6.4)	13 (18.0)	
Transverse	2 (4.3)	2 (2.8)	
Descending	19 (40.4)	26 (36.1)	
Sigmoid	8 (17.0)	12 (16.7)	
Tumor size, mm	52.8 $\pm$ 20.8	48.5 $\pm$ 19.2	0.251
Histological type			
Differentiated	42 (89.3)	52 (72.2)	0.037
Undifferentiated	5 (10.6)	20 (27.7)	
Depth of tumor			
T2	1 (2.0)	2 (2.7)	0.249
T3	33 (70.2)	50 (69.4)	
T4a	6 (12.7)	16 (22.2)	
T4b	7 (14.8)	4 (5.5)	
Lymph node metastasis			
Negative	33 (70.2)	35 (48.6)	0.023
Positive	14 (29.7)	37 (51.3)	
Lymphatic invasion			
Negative	15 (31.9)	23 (31.9)	1
Positive	32 (68.1)	49 (68.1)	
Venous invasion			
Negative	14 (29.8)	23 (31.9)	0.842
Positive	33 (70.2)	49 (68.1)	
Adjuvant chemotherapy			
Negative	15 (31.9)	25 (34.7)	0.844
Positive	32 (68.1)	47 (65.2)	

Values are n (%) or mean $\pm$ SD unless otherwise indicated.

positive NOX5 expression. However, tumor location, tumor depth, and lymphovascular invasion are not significantly correlated with the expression levels of NOX5.

We also assessed whether NOX5 expression is a prognostic factor for human colon cancer. The 5-year progression-free survival (PFS) rate of the NOX5-positive group (71.1%) was significantly lower than that of the NOX5-negative group (88.9%) ($p=0.046$) (Figure 2). Specifically, the 5-year PFS of patients with scores of 1, 2, and 3 were 88.9%, 73.2%, and 62.2%, respectively. The 5-year PFS rate of the three groups appeared to be related to NOX5 intensity, and a significant difference was found between patients with scores of 1 and 3 ($p=0.032$) (Figure 3). Conversely, the 5-year disease-specific survival rates of the NOX5-negative (88.7%) and NOX5-positive (90.5%)

Figure 1. Immunohistochemical staining of primary human colon cancer samples with NOX5 antibody. A: Score 1. B: Score 2. C: Score 3. D: Strong expression around mucus.

groups did not significantly differ ($p=0.832$). The univariate analysis showed that the presence of deeper tumor invasion ($p=0.001$), lymph node metastasis ($p=0.001$), and venous invasion ($p=0.033$) were also correlated with a poorer 5-year PFS (Table II). Further, the multivariate analysis showed that the presence of deeper tumor invasion (hazard ratio [HR]=3.740, $p=0.002$), lymph node metastasis (HR=3.142, $p=0.016$), and venous invasion (HR=4.321, $p=0.019$) were independent prognostic factors (Table III). NOX5 expression levels were not selected as an independent prognostic factor (HR=2.021, $p=0.188$).

The number of patients with an initial recurrence at local, distant, and both sites were 0, 5 (100%), and 0 in the NOX5-negative group and 5 (26.3%), 13 (68.4%), and 1 (5.3%) in the NOX5-positive group, respectively. No significant difference was found between the groups in terms of the recurrence pattern ($p=0.640$).

Discussion

In the present study, we examined the clinical significance of NOX5 expression in human colon cancer tissue by immunohistochemical staining. To our knowledge, this is the first report to describe the relationship between NOX5 expression and clinicopathological features, including patients' prognosis, in locally advanced colon cancer patients.

The NOX family comprises seven members, and many studies have reported that NOX family members modulate ROS generation and consequently promote tumorigenesis by activating cell signaling in various cancers (11, 14, 21, 24, 27, 28). Several studies on colon cancer have reported that NOX1 is highly expressed in colon cancer tissue and promotes colon cancer cell growth, migration, invasion, and the activation of signaling pathways by modulating ROS generation (10, 12, 13, 29). Juhasz *et al.* reported that the

Table II. Univariate analysis for prognostic factors associated with 5-year progression-free survival.

Variable	Number n=119	5-year PFS [†] rate (%)	p-Value
Gender			
Male	64	75.2	0.298
Female	55	81.7	
Age, years			
<70	55	75.5	0.537
70≤	64	80.8	
Location			
Right side	70	83.1	0.244
Left side	49	71.9	
Tumor size, mm			
<50	58	79.4	0.772
≥50	61	77.2	
Histological type			
Differentiated	94	77.4	0.606
Undifferentiated	25	81.8	
Depth of tumor			
T2, T3	86	85.3	0.001
T4	33	58.2	
Lymph node metastasis			
Negative	68	88.9	0.001
Positive	51	64.0	
Lymphatic invasion			
Negative	38	85.7	0.200
Positive	81	74.9	
Venous invasion			
Negative	37	90.9	0.033
Positive	82	72.8	
Adjuvant chemotherapy			
Negative	40	74.5	0.537
Positive	79	80.3	
NOX5 [‡] expression			
Negative	47	88.9	0.046
Positive	72	71.1	

[†]PFS: Progression-free survival; [‡]NOX5: NADPH oxidases 5.

depletion of NOX1 expression resulted in decreased tumor growth, blood vessel density, and hypoxia-inducible factor 1α expression in a colon cancer xenograft model (12).

NOX5 was the last to be identified in the NOX family and is unique because it is regulated by Ca²⁺; its hyperactivation has been observed in cardiovascular and kidney diseases (30). Additionally, unlike NOX1 through NOX4, which are primarily located in cell membranes, NOX5 moves from perinuclear and endoplasmic reticulum regions to cell membranes upon activation by Ca²⁺, growth factors, vasoactive agents, and cytokines (30). Antony *et al.* examined a multi-tumor tissue microarray for NOX5 and reported that the expression levels of NOX5 were elevated in tumor tissues of various organs, including the prostate (NOX5 positive in 81%), ovary (70%), breast (61%), colon

Figure 2. The 5-year progression-free survival rates were 88.9% and 71.1% in the NOX5-negative and NOX5-positive groups, respectively ($p=0.046$).

Figure 3. The 5-year progression-free survival rates were 88.9%, 73.2%, and 62.2% in the Score 1, 2, and 3 groups, respectively. The p-values were 0.083 (Score 1 vs. 2), 0.046 (Score 1 vs. 3), and 0.428 (Score 2 vs. 3).

Table III. Multivariate analysis for prognostic factors associated with 5-year progression-free survival.

Variable	OR [†]	95%CI [‡]	p-Value
Depth of tumor			
T2, T3 versus T4	3.740	1.641-8.523	0.002
Lymph node metastasis			
Negative versus positive	3.142	1.238-7.964	0.016
Venous invasion			
Negative versus positive	4.321	1.276-14.770	0.019
NOX5 expression			
Negative versus positive	2.021	0.709-5.757	0.188

[†]OR: Odds ratio; [‡]CI: confidence interval.

(60%), and lung (56%) (25). However, the significance of NOX5 expression in human colon cancer is still unclear.

To our knowledge, this is the first study to examine the expression levels of NOX5 by immunohistochemical staining of human colon cancer tissues to assess the relationship between NOX5 expression and patients' clinicopathological outcomes. Our data showed a positive NOX5 expression rate of 60.5%, which is similar to the results of a previous study (60%) (25). Further, NOX5-positive expression was significantly correlated with poorer differentiated histology ($p=0.037$) (Table I), and NOX5 protein was strongly stained around areas of mucus in mucinous carcinomas (Figure 1D). Although Accetta *et al.* reported that NOX5-mediated ROS can induce differentiation of human oligodendrocytes (31), the relationship between NOX5 and cellular differentiation in cancer cells is unclear, and further research using an *in vitro* model are warranted.

The prognostic analyses revealed that the NOX5-positive group had a significantly worse 5-year PFS than the NOX5-negative group ($p=0.046$). Additionally, the 5-year PFS of patients with scores of 1, 2, and 3 were related to the NOX5 intensity, as shown in Figure 3. These results suggest that NOX5 might play an oncogenic role in colon cancer. Several studies have shown that NOX5 is also correlated with tumorigenesis in several kinds of cancers, including prostate cancer, Barrett's esophageal adenocarcinoma, hairy cell leukemia, breast cancer, and malignant melanoma (14, 15, 20-24). Further, Höll *et al.* reported that NOX5-derived ROS modulates intracellular signaling cascades, including protein kinase C zeta and c-Jun N-terminal kinase, and promotes proliferation and survival of prostate cancer cells (19). Dho *et al.* revealed that the expression of NOX5 long form (NOX5-L) is regulated by STAT5A, and that NOX5-L promotes proliferation and invasion in breast cancer cells (22). Moreover, in Barrett's esophagus, acid reflux increases NOX5 expression and ROS generation, which suggests that this increase in ROS generation might cause DNA damage, thereby contributing to the progression from Barrett's esophagus to esophageal adenocarcinoma (21, 24). However, no studies have clarified the oncogenic role of NOX5 in colon cancer; thus, further studies using molecular methods are warranted to clarify the role of NOX5 in the tumorigenesis of colon cancer.

Regarding the recurrence pattern, local recurrence was more common in NOX5-positive patients than in NOX5-negative patients. However, the number of recurrent cases was very small, and further research using a larger cohort is needed to confirm these findings. Additionally, the role of NOX5 in cellular movement needs to be clarified.

In conclusion, we demonstrated that NOX5 expression is related to poor prognostic factors and worse prognosis in locally advanced colon cancer patients. NOX5 may be a useful biomarker of tumor prognosis after curative surgical resection and could be used as a novel therapeutic target in colon cancer patients.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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Authors' Contributions

Naoki Ashizawa performed the majority of experiments and wrote the manuscript. Hiroki Shimizu designed the research and helped to draft the manuscript. All Authors reviewed the manuscript.

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