Original Article

Clinicopathological study of fractional exhaled nitric oxide dynamics and intratumoral inducible nitric oxide synthase expression in primary lung cancer patients

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Background: Inducible nitric oxide synthase (iNOS) is expressed in non-small cell lung cancer (NSCLC) tumor cells and contributes to tumorigenesis. Nitric oxide, an indicator of airway inflammation, is concurrently produced in the airway epithelium. However, the interrelationships and predictive importance of iNOS remain unclear. This study aimed to investigate whether iNOS could serve as a novel biomarker for NSCLC.

Methods: Immunohistochemical analysis of iNOS expression in the tumor cells of 101 consecutive patients with NSCLC undergoing lung resection was conducted. The fractional exhaled nitric oxide (FeNO) levels were evaluated pre- and postoperatively using a clinically applied respiratory function testing device. iNOS expression was assessed by immunochemical staining for expression within tumor cells.

Results: iNOS expression in the tumor cells was significantly associated with squamous cell carcinoma ($P<0.01$). No significant correlation between the FeNO levels and iNOS expression scores existed; however, the FeNO levels in positive cases of squamous cell carcinoma were significantly higher than those in negative cases ($P<0.01$). The FeNO levels did not decrease in the iNOS-negative cases after tumor resection in the squamous cell carcinoma group but were significantly lower in the positive cases ($P=0.03$).

Conclusions: iNOS expression in tumor cells showed a characteristic tendency toward squamous cell carcinoma, suggesting its potential for FeNO-mediated localization and diagnosing lung cancer.

Keywords: Lung cancer; inducible nitric oxide synthase (iNOS); fractional exhaled nitric oxide (FeNO); squamous carcinoma

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Introduction

Background

Non-small cell lung cancer (NSCLC) is a major cause of cancer-related deaths worldwide (1). Surgical, chemotherapeutic, and radiation therapies have improved the prognosis of NSCLC (2,3). Moreover, recent advances in the development of new therapies, such as molecular-targeted therapy and immunotherapy, have led to further improvements (4,5). Because of the availability of multiple treatment options, diagnosing recurrence and managing lung cancer treatment is essential.

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Existing biomarkers, such as blood tests and tissue diagnostics, are invasive and may not be suitable for repeated applications. Using alternative methods to identify simpler and more practical biomarkers is necessary.

Inducible nitric oxide synthase (iNOS) is expressed in the tumor microenvironment and has been associated with the prognosis of specific solid cancers (6). While its involvement in NSCLC pathogenesis has been suggested, its clinical significance remains uncertain.

Furthermore, iNOS is renowned for its distinct role in generating nitric oxide in the airway epithelium, playing a vital physiological function. The concentration of fractional exhaled nitric oxide (FeNO), measured through respiratory function testing equipment, is a common clinical indicator of airway inflammation (7,8). Although nitric oxide is an important common factor in the development of these diseases, a direct relationship has not been proven.

Rationale and knowledge gap

Previous comprehensive studies demonstrated that FeNO levels in patients with lung cancer were slightly higher compared with healthy individuals (9,10). Our research on airway inflammation also demonstrated that FeNO concentrations were higher in patients with lung cancer (10,11). However, no reports have examined the relationship with iNOS expressed within lung cancer tissues.

Highlight box

Key findings

- The findings of our study suggest that inducible nitric oxide synthase (iNOS) expression in tumor cells showed a characteristic tendency toward squamous cell carcinoma, suggesting its potential in fractional exhaled nitric oxide (FeNO)-mediated localization and diagnosing lung cancer.

What is known and what is new?

- Non-small cell lung cancer (NSCLC) is a major cause of cancer-related deaths worldwide. Recent advances have led to treatment improvements. However, existing biomarkers, such as blood tests and tissue diagnostics, are invasive and may not be suitable for repeated applications.
- This is the first study to address the importance of the association between FeNO levels and the expression of iNOS in the tumor cells of patients with NSCLC.

What is the implication, and what should change now?

- FeNO may be a potential biomarker for predicting tumor progression in squamous cell carcinoma. Our findings can catalyze a broader discussion on optimizing patient care strategies and clinical outcomes by paving the way for medical practitioners to make evidence-based decisions when treating patients with NSCLC.

Objective

This study aimed to explore the interplay of these factors and their impact on NSCLC by analyzing FeNO and iNOS expression in patients with NSCLC. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-178/rc).

Methods

Patient samples

Tumor tissue samples were obtained from 109 consecutive patients with stage I–IV NSCLC who underwent surgical resection at the Shiga University of Medical Science Hospital between November 2017 and March 2019. Patients with *in-situ* adenocarcinoma were excluded from the study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board at the Shiga University of Medical Science Hospital (approval No. 29-175), and written informed consent was obtained from all individual participants.

Immunohistochemistry

Tissue sections of 4 µm thickness were prepared from formalin-fixed paraffin-embedded tissue at the maximum tumor surface. Primary rabbit polyclonal ab115819 antibody (Abcam, Cambridge, UK) was used and incubated at a dilution of 1/100 for the detection of iNOS. The tissue sections were deparaffinized with xylene and rehydrated with a graded ethanol solution to stain for iNOS. Heat-induced epitope retrieval was performed in a microwave using the Target Retrieval Solution (pH 9.0; DAKO, Glostrup, Denmark). The sections were deactivated with 3% hydrogen peroxide solution and rinsed with phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with EnVision Flex Peroxidase Block (DAKO) for 15 min at room temperature. Subsequently, the sections were incubated with the primary antibody at room temperature

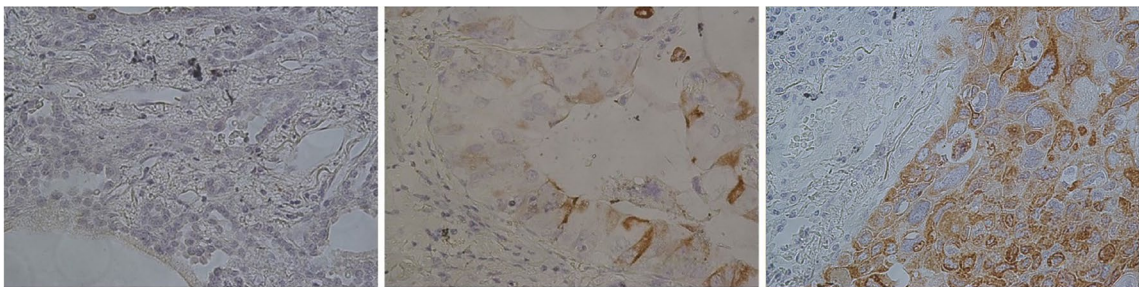

Figure 1 Atlas of iNOS expression in human NSCLC tumors by IHC. Atlas of finding pictures of 0, 1, and 2 IHC scores for iNOS expression in NSCLC tumor cells. iNOS positivity evaluated by immunohistochemical staining (400× magnification). iNOS, inducible nitric oxide synthase; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

for 1 h and rinsed with PBS. The sections were incubated with a secondary antibody (EnVision Flex/HRP; DAKO) for 30 min and rinsed with PBS again. Lastly, the slides were treated with substrate/chromogen (DAKO) for 5 min and counterstained with hematoxylin. Sections without primary antibodies were included as negative controls during staining. The expression of iNOS was evaluated by randomly selecting ten fields of view at 400× magnification, taking into account the potential heterogeneity in one section of each section. All evaluators were supervised by a histopathologist. The expression of iNOS was scored for each case using the mean value of each field of view and classified into three categories (*Figure 1*): score 0, negative; score 1, positive in 1–9% of cancer cells; and score 2, positive in more than 10% of tumor cells.

FeNO evaluation

The preoperative airway inflammation was measured as FeNO using NIOX VERO® (NOV, Aerocrine, Solna, Sweden) according to the American Thoracic Society and European Respiratory Society recommendations (12). The patients provided written informed consent for routine preoperative blood evaluation, physiological function tests, and imaging tests. FeNO levels are affected by foods, beverages, and passive smoking; therefore, the measurements were performed in a relaxed state during hospitalization while avoiding the interval after meals. The FeNO levels were measured in the same environment postoperatively. The obtained data were not disclosed to other physicians. Postoperative FeNO measurements were taken within 5 days after surgery while examining the patient's condition.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software (version 25.0; IBM, Inc., Armonk, NY, USA). Continuous values are expressed as mean ± standard deviation (SD). The Chi-square test and *t*-test were used to compare categorical and continuous variables between the two groups, respectively. Pearson's correlation analysis was performed to assess the relationships between continuous variables. Statistical significance was set at $P<0.05$.

Results

Study cohort

Figure 2 summarizes the flow of patients who participated in the study (n=101). The two patients referred had already undergone wedge resection and no tumor specimens were available for tissue analysis. The clinicopathological characteristics of all patients are presented in *Table 1*. Patients with active asthma or rhinitis with severe clinical symptoms were not included. Two-thirds of the patients were men. The average age was 69.5±9.8 years, and the average FeNO level was 25.0±17.1 ppb. Approximately two-thirds were former smokers, and all patients had quit smoking at least 1 month before admission. The average FeNO level in never smokers was 23.8±17.2 ppb, and the average FeNO level in former smokers was 25.7±17.0 ppb. There was no significant difference between these two

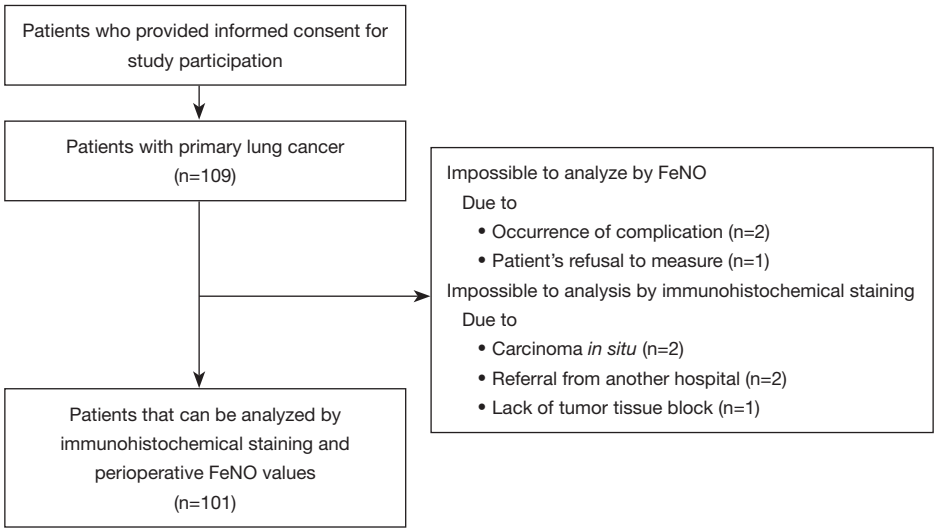

Figure 2 Overview of the study population. FeNO, fractional exhaled nitric oxide.

Table 1 The clinicopathological characteristics of the 101 patients included this study

Characteristics	N	%
Sex		
Female	37	36.6
Male	64	63.4
Age (years)		
≥69	61	60.4
<69	40	39.6
Preoperative FeNO (ppb)		
≥25	37	36.6
>25	64	63.4
Smoking		
Never	35	34.7
Former	66	65.3
TNM stage		
I	79	78.2
II	11	10.9
III	7	6.9
IV	4	4.0
Pathological type		
Adenocarcinoma	82	81.2
Squamous cell carcinoma	13	12.9
Other	6	5.9
iNOS expression		
Negative	72	71.3
Positive	29	28.7

FeNO, fractional exhaled nitric oxide; TNM, tumor-node-metastasis; iNOS, inducible nitric oxide synthase.

groups (P=0.70). The pathological stages, classified according to the 8th edition of tumor-node-metastasis (TNM), were as follows: 28 cases of IA1, 27 cases of IA2, 10 cases of IA3, fourteen cases of IB, four cases of IIA, seven cases of IIB, seven cases of IIIA, three cases of IVA, and one case of IVC. Adenocarcinoma was the most common histological type. iNOS expression was detected in 29 cases; 23 cases had a score of 1, and six cases had a score of 2.

iNOS expression status and positive rate in each histopathological type

As shown in *Table 1*, iNOS expression was detected in 29 patients. We analyzed this status according to the histopathological type (*Table 2*). The positive rate of iNOS expression was lower in the adenocarcinoma (19.5%) and higher in the squamous cell carcinoma (84.6%). In this study, six cases were classified as score 2, all of which were squamous cell carcinoma. We also investigated whether there were statistical differences in iNOS expression by histopathological type (*Table 2*). iNOS expression was significantly positive in squamous cell carcinoma compared with the rest of the histopathological types (P<0.001). Conversely, in adenocarcinoma, iNOS expression was significantly negative compared with the rest of the histopathological types (P<0.001). No differences were observed between the rest of the histopathological types (P=0.79).

Table 2 Histopathological type and iNOS expression in tumor cells

Histopathological type	N	iNOS expression					P value
		Score 0	Score 1	Score 2	Negative	Positive	
Adenocarcinoma	82	66	16	0	66	16	<0.01
Squamous cell carcinoma	13	2	5	6	2	11	<0.01
Other	6	4	2	0	4	2	0.79

iNOS, inducible nitric oxide synthase.

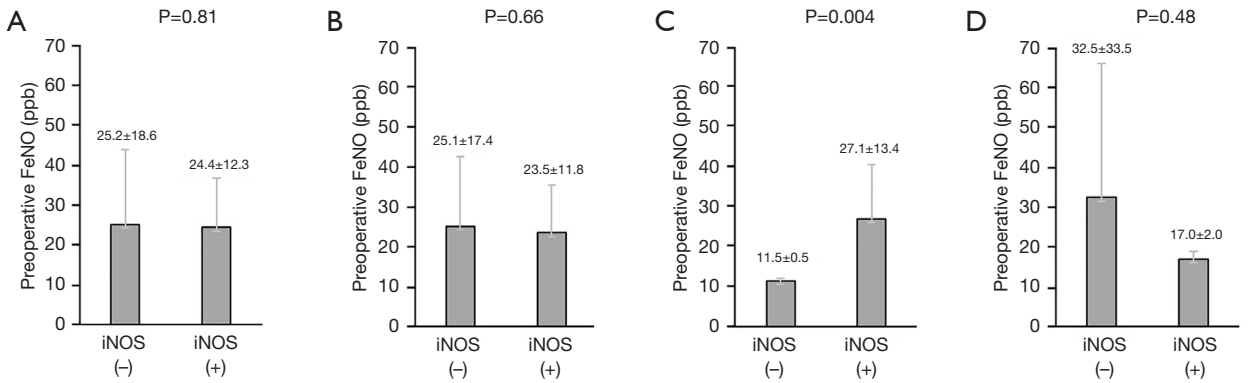

Figure 3 Boxplots for FeNO values and iNOS staining. (A) Preoperative FeNO levels in all patients based on iNOS expression. (B) Preoperative FeNO levels in patients with adenocarcinoma based on iNOS expression. (C) Preoperative FeNO levels in patients with squamous cell carcinoma based on iNOS expression. (D) Preoperative FeNO levels in patients with other histopathological type based on iNOS expression. FeNO, fractional exhaled nitric oxide; iNOS, inducible nitric oxide synthase.

Correlation between iNOS expression and FeNO level, and dynamics of perioperative FeNO level

We evaluated the relationship between iNOS expression scores and preoperative FeNO levels using correlation coefficients. First, across the patients, no significant correlation was observed (r=–0.03, P=0.71). Next, we analyzed the correlation by smoking status, and no significant correlation was observed for both never smokers and former smokers (r=–0.08, P=0.64 and r=–0.04, P=0.74).

Furthermore, a similar correlation analysis was performed for early stage (TNM stage I) and advanced stage (TNM stages II–IV). No significant correlation was observed for both early stage and advanced stage (r=–0.04, P=0.74 and r=–0.06, P=0.77).

Correlation analyses were also performed for adenocarcinoma, squamous cell carcinoma, and other histopathological types, and the results were r=–0.04 (P=0.73), r=0.05 (P=0.87), and r=–0.25 (P=0.62), respectively. No significant differences were noted.

Next, we analyzed whether there was a statistically significant difference in preoperative FeNO levels based on iNOS expression in all patients and in each histopathological type. As shown in *Figure 3* for squamous cell carcinoma, preoperative FeNO levels were significantly higher in iNOS-positive cases (11.5±0.5 *vs.* 27.1±13.4 ppb, P=0.004). In addition, we compared preoperative and postoperative FeNO levels in squamous cell carcinoma based on iNOS expression. The bar graph in *Figure 4* shows perioperative FeNO levels, i.e., preoperative and postoperative FeNO levels. In iNOS-negative cases, FeNO level changed from 11.5±0.5 to 13.5±3.5 ppb postoperatively (P=0.22). In iNOS-positive cases, FeNO level decreased significantly from 27.1±13.4 to 19.9±9.1 ppb postoperatively (P=0.03).

Discussion

NSCLC is a disease consisting of inordinate growth in lung tissue; however, its mechanisms are not completely understood. Based on previous research, iNOS expression has been linked to the advancement of various tumor types

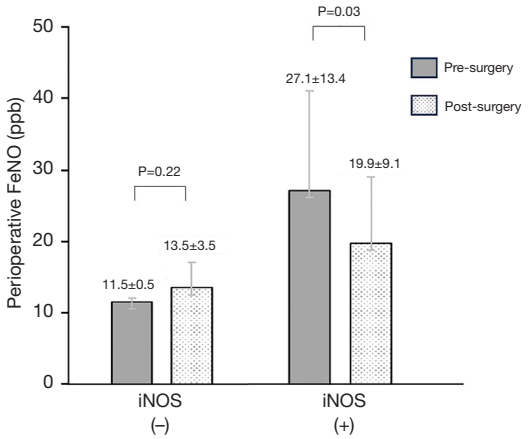

Figure 4 Boxplots for perioperative FeNO values and iNOS staining with squamous cell carcinoma. Changes in perioperative FeNO levels in patients with squamous cell carcinoma based on iNOS expression. FeNO, fractional exhaled nitric oxide; iNOS, inducible nitric oxide synthase.

(13,14), indicating that iNOS expression holds noteworthy significance in NSCLC. However, whether the expression of iNOS in tumor cells is a useful predictive biomarker is unclear. In the present study, we focused on exploring the significant role of iNOS expression beyond the tumor microenvironment, delving into its implications for the pathogenesis of respiratory-specific diseases.

FeNO is used as a clinical indicator of airway inflammatory diseases. The potential for further clinical applications in patients with lung cancer has long been of interest. Because FeNO levels are elevated in patients with lung cancer, we hypothesized that some of the nitric oxide produced within the tumor environment might be reflected in these levels. Therefore, we evaluated the relationship between the expression of iNOS in tumor cells and tissues in patients with NSCLC and FeNO. The average FeNO level of patients with lung cancer exceeded the average level of healthy Japanese adults (15).

To assess the expression levels of iNOS within tissues, we adopted the method of confirming iNOS expression within tumor cells using immunohistochemistry. As iNOS is also present in the normal airway epithelium, it is practically impossible to comprehensively observe and evaluate all aspects of its expression. Immunohistochemical analysis revealed that the expression of iNOS in tumor cells was significantly higher in patients with squamous cell carcinoma. Furthermore, it was discovered that this expression was significantly correlated with preoperative FeNO levels, and FeNO levels were significantly higher in the positive expression group. These results suggest that in some patients, FeNO may be useful for diagnosing the presence of lung cancer.

To investigate the cause of the elevated FeNO levels, we analyzed the postoperative FeNO levels and examined the impact of tumor resection due to surgery. According to our results, FeNO levels significantly decreased postoperatively in patients with squamous cell carcinoma, further supporting our hypothesis. Here, it is noteworthy that patients with squamous cell carcinoma often have elevated FeNO levels due to factors such as emphysema and smoking. This study focused on the expression of iNOS in tumor cells, and the expression of iNOS in lung tissues has not been confirmed. However, the analysis of the dynamics of perioperative FeNO revealed a significant numerical decrease. As a result, tumor removal was the most significant factor influencing this decrease.

One of the factors contributing to reducing airway inflammation is the use of immunosuppressive agents. In this regard, no patients received additional systemic or inhaled steroids before or after endotracheal intubation during the surgery or postoperatively. Fluctuations in the systemic immune response may also play a role; therefore, measuring the macrophage levels in the blood may be necessary. This issue should be addressed in future prospective studies. In addition, the timing of the measurements in this study was relatively early, within 7 days of surgery. Therefore, data from the remote period (several months postoperatively) were not reflected. If these values continue to decrease for several months postoperatively, our hypothesis may be further supported. This will be a topic for future research.

The present study has some limitations. First, the study cohort included a small, limited patient population from a single center. This limited the ability to perform multivariate analyses and predict factors. Second, the possibility of measurement bias in FeNO levels cannot be excluded. To control this factor, patients with severe allergic diseases were excluded from the study and a period of abstinence of more than 1 month was also ascertained. In addition, the FeNO levels of all patients were measured by the same physician, and every effort was made to match the measurement conditions.

Conclusions

In conclusion, this is the first report to address the

importance of the association between FeNO levels and the expression of iNOS in the tumor cells of patients with NSCLC. In particular, FeNO may serve as a potential biomarker in squamous cell carcinoma in human patients with NSCLC; however, further studies are needed to confirm this hypothesis.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-178/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-178/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-178/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-178/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board at the Shiga University of Medical Science Hospital (approval No. 29-175), and written informed consent was obtained from all individual participants.

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