Jpn. J. Cancer Res. **89**, 696–702, July 1998

Significant Correlation of Nitric Oxide Synthase Activity and *p53* Gene Mutation in Stage I Lung Adenocarcinoma

Hisao Fujimoto,[1] Ji-ichiro Sasaki,[1, 2] Mitsuhiro Matsumoto,[1, 5] Moritaka Suga,[1] Yukio Ando,[1] Richard Iggo,[3] Mitsuhiro Tada,[4] Hideyuki Saya[2] and Masayuki Ando[1]

[1]First Department of Internal Medicine, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-0811, [2]Departments of Tumor Genetics and Biology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, [3]Oncogene Group, Swiss Institute for Experimental Cancer Research (ISREC), CH-1066 Epalings, Switzerland and [4]Department of Neurosurgery, Hokkaido University School of Medicine, North-15 West-7, Kita-ku, Sapporo 060-0815

Nitric oxide (NO) and its derivatives can directly cause DNA damage and mutation *in vitro* and may play a role in the multistage carcinogenic process. It has been reported that NO induces mutation in the *p53* tumor suppressor gene; we therefore analyzed the relationship between NO synthase 9 activity and *p53* gene status in early-stage lung adenocarcinoma. Surgical samples were classified into two categories: 14 lung adenocarcinomas with high NOS activity (>25 pmol/min/g tissue, category A), and 16 with low NOS activity (<25 pmol/min/g tissue, category B). A yeast functional assay for *p53* mutations disclosed a red colony that corresponded to a mutation in the *p53* gene in 8 cases (57.1%) in category A and 3 cases (18.8%) in category B, the frequency being significantly higher in the former (P<0.05). A *p53* DNA sequence analysis revealed that 5 of the 8 *p53* mutation-positive samples in category A had a G:C-to-T:A transversion, which is reported to be a major target of NO. The mechanism of carcinogenesis of adenocarcinoma is not fully understood, but these results suggest that an excess of endogenously formed NO may induce a *p53* gene mutation containing mainly G:C-to-T:A transversion in the early stage of lung adenocarcinoma. Our results suggest that NO has potential mutagenic and carcinogenic activity, and may play important roles in human lung adenocarcinoma.

Key words: Nitric oxide — Nitric oxide synthase — *p53* — Lung adenocarcinoma — Yeast functional assay

Lung cancer arises through exposure to carcinogens which cause initiating events (mutations), and so-called tumor promotion, resulting in the outgrowth of cells containing mutation.1) The most important source of carcinogens and tumor promoters for lung cancer is, of course, cigarette smoking. Adenocarcinoma is the most common lung cancer in Japan, and the number of patients with this disease is increasing in some other countries.2) Etiologically, lung adenocarcinoma has less association with cigarette smoking than either squamous cell carcinoma or small cell carcinoma, and its mechanism of carcinogenesis is not well understood. Carcinogenesis is a multistep process, and thus an accumulation of several independent mutations is necessary for the progression of lung cancer.

Chronic infection and inflammation are well recognized as risk factors for a variety of human cancers.3) It has been proposed that reactive oxygen and nitrogen species, both formed in infected and inflamed tissues, play roles in the multistage carcinogenic process, each by a different mechanism. Nitric oxide (NO), a potentially toxic gas with free radical properties, is generated from L-arginine by constitutive or inducible NO synthase (NOS).4) It has been reported that different isoforms of NOS are expressed in not only human tumor cell lines,5–7) but also solid tumor tissues.8–10) We recently reported that the total NOS activity in lung adenocarcinoma samples was significantly higher than that in other types of lung cancers and normal lung samples.11) These results suggested that NO might play an important role in the metabolism and behavior of lung adenocarcinoma. However, the role of NO in solid tumor biology is not yet fully understood. NO is a highly reactive free radical that reacts with other free radicals to form cytotoxic compounds, such as peroxynitrite, which may directly cause DNA damage and mutation *in vitro*.12, 13) Oxyradicals can enhance the rate of deamination reaction, and the production of NO produced by NOS could thus contribute to the endogenous mechanism of mutagenesis.

It is widely accepted that the mutation of the *p53* tumor suppressor gene plays a critical role somewhere in the multistage process of the carcinogenesis of lung cancers. As previously reported, benzo(a)pyrene, which is present in cigarettes, induced G:C-to-T:A transversions in the *p53* gene *in vitro*,14) and this transversion is the most frequently observed in the *p53* gene in lung cancers.15) However, the mutagens of *p53* in lung adenocarcinomas,

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[5] To whom correspondence should be addressed.

which are known not to correlate with cigarette smoking, have not yet been identified. Murata *et al.* recently reported that NO and its derivatives induced G:C-to-T:A transversions in a *p53* gene study *in vitro*.16) The above results indicate that NO, which is produced by NOS, may be one of the mutagens of the *p53* gene in lung adenocarcinomas.

In the present study, we analyzed NOS activity and assessed *p53* mutations in a yeast *p53* functional assay17) and performed a DNA sequence analysis in lung adenocarcinoma samples. We also investigated the possibility of a relationship between NO and *p53* gene mutation in the early stage (stage I) of lung adenocarcinoma.

MATERIALS AND METHODS

Clinical samples Thirty lung adenocarcinoma tissue samples and corresponding normal lung samples at a distance from the cancer were obtained from patients who were treated surgically at Kumamoto Chuo Hospital and Kumamoto University School of Medicine between 1994 and 1997. The clinicopathological features of the cases are summarized in Table I. All of the cases were pathologically categorized as T1N0M0 or T2N0M0 (stage I) according to the guidelines of the American Joint Committee on Cancer Staging.18) Patients with peripherally located tumors, accurate pathological staging and potentially curative resection were selected as the subjects. The ages of the patients ranged from 45 to 76 years (mean age 63.8 years). Fourteen were men and 16 were women. Clinical data were available for all of the patients, including age, sex, history of smoking, T factor, and degree of differentiation. None of the patients involved in this study had received chemotherapy or radiation before surgery. The degree of histologic differentiation was evaluated as poor, moderate or well according to the World Health Organization (WHO) classification of lung tumors (1982).19) Samples were frozen immediately in liquid nitrogen after resection and stored at −80°C until the assay of NOS activity and the *p53* mutational studies.

Assay of NOS activity The NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline with the modified method of Bredt and Snyder.20) The frozen tissues were homogenized at 4°C in 4 volumes (relative to the sample weight) of buffer containing 0.1 m*M* EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 100 μ*M* *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, 1 m*M* dithiothreitol, 0.32 *M* sucrose, and 15 m*M* HEPES, pH 7.6. The homogenates were centrifuged at 100,000*g* at 4°C for 1 h. The supernatants were used for NOS assay. The NOS activity in these supernatants was measured by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline, using the sodium form of a resin column which absorbs L-[U-^{14}C]arginine. L-[U-^{14}C]Citrulline was eluted and its radioactivity was measured to determine the NOS activity. Blank values were determined in the absence of added supernatants. Samples (100 μl) were added to 25 U/ml calmodulin, 0.1 m*M* $CaCl_2$, 1.0 m*M* NADPH, 20 m*M* flavin adenine dinucleotide, 20 m*M* flavin mononucleotide, 5.0 m*M* L-[U-^{14}C]arginine and 5.0 m*M* HEPES (total volume: 200 μl) and incubated for 2 h at 37°C. The background activity was determined as the radioactivity in the absence of 1 m*M* NADPH. To determine the constitutive NOS (c-NOS) activity, trifluoperazine was added to the assay mixture to give a concentration of 0.1 m*M*. The c-NOS activity was calculated by subtracting the activity of the trifluoperazine-containing medium from the total NOS activity.

Table I. Clinicopathological Characteristics of the Study Patients with Stage I Lung Adenocarcinoma

Total no. of patients	30
Sex	
Male	14
Female	16
Age	
Mean	63.8
Minimum	45
Maximum	76
Smoking history	
Positive	14
Negative	16
Degree of differentiation	
Well	16
Moderate	9
Poor	5
T factor	
T1	20
T2	10

Reverse transcription-polymerase chain reaction (RT-PCR) analysis Poly(A)+mRNA was extracted using a Micro Fast Track kit according to the instructions of the manufacturer (Invitrogen, San Diego, CA). mRNA was extracted from 10–20 6 μm frozen sections, and the proportions of tumor cells and normal cells in each area were assessed by histological examination of sequential sections stained with hematoxylin and eosin. First-strand cDNA was synthesized from mRNA with 1.25 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Tokyo) and random primers (Gibco-BRL). PCR was performed with a Perkin-Elmer Cetus Gene Amp PCR system 2400 (Norwalk, CT) for 35 cycles of 30 s at 94°C, 60 s at 65°C, and 80 s at 78°C, as previously described.21) For the amplification of *p53* cDNA, we used 100 ng of primers (P3: 5′-ATTTGATGCTGTC-

CCCGGACGATATTGAAC-3′, P4: 5′-ACCCTTTTTG-GACTTCAGGTGGCTGGAGTG-3′), 1.25 units of Pfu DNA polymerase (Stratagene, La Jolla, CA), 10% (vol/vol) dimethyl sulfoxide, and 50 μM dNTPs (Perkin-Elmer, Foster City, CA) in 25 μl of Pfu buffer (Stratagene). After the PCR, 5 μl of the PCR products was checked by 1% agarose gel electrophoresis and 5 μl was directly subjected to yeast functional assay.

Yeast functional asssay for *p53* mutation The yeast functional assay was performed according to the method modified by Tada *et al.*[21] The yeast expression vector pSS16[22] and the yeast reporter strain yIG397[17] were used in this study. The strain yIG397 contains an integrated plasmid with the *ADE2* (adenine biosynthesis 2; encoding phosphoribosyl-aminoimidazole carboxylase; EC 4.1.1.21) open reading frame under the control of a *p53*-responsive promoter. When the strain is transformed with a plasmid encoding mutant *p53*, the cells fail to express *ADE2* and form red colonies because of the accumulation of intermediates of adenine metabolism. Yeast was cultured in 150 ml of YPD medium supplemented with 200 μg/ml of adenine at 30°C, until the A_{600} value reached 0.8. The cells were pelleted, then washed twice in 50 ml of distilled water and once in 10 ml of a LiOAc solution containing 0.1 M lithium acetate, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA2Na, and pelleted again. Yeast cells were resuspended in 1 ml of the LiAc solution, and 50 μl of the yeast suspension was used for each transformation. Yeast was co-transformed with 5 μl of *p53* PCR product, 50–100 ng of linearized vector plasmid, 5 μl of sonicated single-stranded salmon sperm DNA (10 mg/ml) and 300 μl of LiOAc containing 40% polyethylene glycol 4000 (Kanto Chemical, Tokyo). The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. The yeast was then pelleted and resuspended in 150 μl of synthetic dextrose (SD) medium and plated on SD agar minus leucine plus adenine (5 μg/ml). Transformed yeast on the plate was incubated at 30°C for 48–60 h to generate colonies and stored at 4°C for 4–8 h to develop color. More than 100 colonies were examined in this assay.

Recovery of *p53* plasmids from transformed yeast The *p53* expression plasmids were recovered from transformed yeast according to the method modified by Ward.[23] More than 4 red yeast colonies on a plate were selected, and each was cultured in 5 ml of SD medium minus leucine plus adenine for 24–36 h at 30°C. The yeast was pelleted, washed in 1 ml of distilled water and then resuspended in 400 μl of 2.5 M LiCl, 50 mM Tris-HCl (pH 8.0), 4% Triton X-100, 62.5 mM EDTA2Na, and to this was added an equal volume of phenol/chloroform (1:1(w/v)) and 0.2 g of glass beads (0.45–0.50 mm). The mixture was vigorously vortexed for 2 min and centrifuged at 12,000g for 15 min. The upper phase was collected, extracted twice with phenol/chloroform, and precipitated with ethanol. Each plasmid was dried and dissolved in 30 μl of Tris-EDTA (TE) buffer.

PCR-DNA sequence analysis For the DNA sequencing, *p53* fragments were amplified from plasmids by means of the PCR procedure using *rTth* DNA polymerase (Perkin-Elmer). PCR was performed for 35 cycles using the same primers as in the yeast assay (P3 and P4) in a 25 μl reaction volume. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 60 s. The PCR products were electrophoresed on a 1% agarose gel and sequenced with a Dye Terminator kit (Perkin-Elmer) on an ABI 373S automated sequencer (Perkin-Elmer). The conditions used for the sequencing were according to the manufacturer's protocol, and the same primers as above (P3 and P4) were used. If a given mutation was found in more than 50% of the clones analyzed, it was defined as a functional mutation.

Statistical analysis The results were evaluated by means of the χ^2 test, and a two-tailed $P<0.05$ was considered significant.

RESULTS

Total NOS activity in lung adenocarcinoma samples The total NOS activity ranged from 1.7 to 116.5 pmol/min/g tissue in the 30 adenocarcinoma samples (Fig. 1). A trifluoperazine inhibition assay revealed that most of the total NOS activity in the samples was due to c-NOS, though some of the samples predominantly contained inducible NOS (i-NOS). The presence and localization of NOS subtypes in adenocarcinoma samples was also examined by immunohistochemical analysis using monoclonal anti-brain NOS (b-NOS), anti-endothelial NOS (ec-NOS) and anti-i-NOS antibodies. NOS was predominantly expressed in the tumor cells, and the adenocarcinoma samples predominantly contained b-NOS, as we previously reported.[11] The total NOS activity in all of the corresponding normal lung samples (Fig. 1) was <25 pmol/min/g tissue, in agreement with our previous report.[11] Thus, the surgical samples were classified into two categories: 14 lung adenocarcinomas with high NOS activity (>25 pmol/min/g tissue, category A), and 16 with low NOS activity (<25 pmol/min/g tissue, category B). No significant relationship was demonstrated between NOS activity and T factor, the degree of histological differentiation, or smoking history (Table II).

***p53* Mutation analysis by yeast functional assay** A *p53* functional assay performed on clinical samples containing only wild-type *p53* typically gave 5 to 10% red colonies.[17] These background red colonies were due mainly to cDNA fragmentation or PCR mutation. In the present study, we examined 50 non-cancerous lung tissues using yeast functional assay. They gave less than 15% of red colonies (data not shown), so the samples with more than

15% red colonies were considered to be positive for *p53* mutation. Among the 30 samples tested, the yeast assay gave more than 15% red colonies in 11 cases (36.7%), which is similar to the previously reported frequency of *p53* mutation in stage I lung adenocarcinoma.[24)] The proportion of samples giving over 15% red colonies ranged from 29.6 to 87.4% (Table III). The relationship between *p53* gene mutation and clinicopathological characteristics is presented in Table II. The incidence of the cases positive for *p53* mutation tended to increase with the T factor values. With regard to the degree of histological differentiation, the incidence of *p53* mutation tended to be higher in cases of poorly differentiated adenocarcinoma than in those of well- or moderately differentiated adenocarcinoma. No significant relationship was demonstrated between *p53* mutation and the patients' age, sex, or smoking history.

***p53* DNA sequence analysis** Samples with more than 15% red colonies were further tested by DNA sequencing. We detected 12 clonal mutations, which included 11 missense mutations in 10 cases and an in-frame deletion in 1 case (Table III). One case had double missense mutations. The G:C-to-T:A transversion was the most frequent mutational event among the 11 missense mutations and was observed only in high NOS activity samples (5/8; 62.5%). Three of these 5 samples with a G:C-to-T:A transversion of *p53* gene were from nonsmokers (Table III). The G:C-

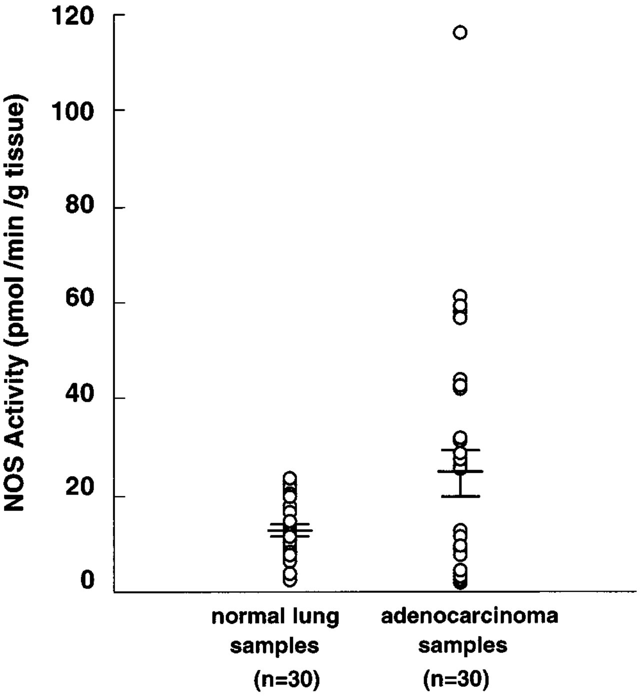

Fig. 1. Total NOS activity in control and adenocarcinoma samples.

Table II. Relationship between Clinicopathological Characteristics, NOS Activity and *p53* Gene Mutation

Variable	High NOS activity samples (%)	*p53* Mutation analysis positive samples (%)
T factor		
T1 (*n*=20)	9 (45.0)	6 (30.0)
T2 (*n*=10)	5 (50.0)	5 (50.0)
Differentiation		
Well (*n*=16)	7 (43.8)	4 (25.0)
Moderate (*n*=9)	4 (44.4)	3 (33.3)
Poor (*n*=5)	3 (60.0)	4 (80.0)
Smoking history		
Smoker (*n*=14)	7 (50.0)	6 (42.9)
Nonsmoker (*n*=16)	7 (43.8)	5 (31.3)

Table III. Results of Yeast Functional Assay and DNA Sequence Analysis in *p53* Gene Mutation-positive Samples

Sample	Age	Sex	Smoking history	% Red colonies	Codon	Base change	Base change
1	50	M	positive	65.9	273	G:C→T:A	Arg→Leu
2	68	F	negative	45.3	158	G:C→C:G	Arg→Pro
3	49	F	positive	29.6	158	G:C→A:T	Arg→His
4	74	M	positive	85.7	244	G:C→T:A	Gly→Ser
5	50	M	negative	71.1	213	G:C→T:A	Arg→Leu
6	70	F	negative	87.4	270	T:A→G:C	Phe→Val
					273	G:C→T:A	Arg→Leu
7	78	F	positive	67.7	248	G:C→A:T	Arg→Gln
8	60	M	negative	67.3	249	G:C→T:A	Arg→Met
9	64	M	positive	68.6	113	T:A→G:C	Phe→Val
10	52	F	negative	60.2	in-frame deletion		
11	68	M	positive	64.0	255	T:A→C:G	Ile→Thr

Samples 1–8: high NOS activity group (category A). Samples 9–11: low NOS activity group (category B).

Table IV. Relationship between NOS Activity and *p53* Gene Mutation in Lung Adenocarcinoma

	p53 Mutation positive (%)	*p53* Mutation negative (%)	Total
High NOS activity group	8 (57.1)	6 (42.9)	14
Low NOS activity group	3 (18.8)	13 (81.2)	16
Total	11 (37.0)	19 (63.0)	30

to-A:T transition was detected in 2 cases in high NOS activity samples.

Relationship between NOS activity and *p53* mutation The data demonstrating a relationship between the NOS activity and *p53* mutation are presented in Table IV. Eight (57.1%) of the 14 high NOS activity samples were positive for *p53* mutation. In contrast, only three (18.8%) of the 16 low NOS activity samples were positive for *p53* mutation. Thus, the NOS activity was significantly correlated with *p53* mutation in stage I lung adenocarcinoma ($P<0.05$).

DISCUSSION

Our previous study revealed that the total NOS activity in lung adenocarcinoma samples was significantly higher than that in other types of lung cancers or normal lung samples. Nguyen *et al*. reported that NO and its metabolites produced in the inflammatory site may cause cell death, DNA damage and adduct formation, and may induce the activation of oncogenes or the inactivation of tumor suppressor genes.13) It has also been reported that NO concentrations are elevated in chronic hepatitis25) and ulcerative colitis,26) which are well known to increase cancer risk. These results suggest that the overproduction of NO by NOS activation may play a role in human carcinogenesis.

The present study demonstrated that NOS activity was closely associated with *p53* gene mutation in stage I lung adenocarcinoma. The frequency of *p53* mutation in stage I lung adenocarcinomas with low NOS activity (19%) in this study is similar to that observed in a previous study.24) However, stage I lung adenocarcinomas with high NOS activity in our study showed *p53* mutations at a higher frequency (57%) than that reported in the previous study.24) Mutations can be caused by endogenous mutagenic mechanisms or exogenous mutagenic agents and are archived in the spectrum of *p53* gene mutations found in human cancer. The most common exogenous mutagen for the *p53* gene is cigarette smoke, which contains benzo(a)pyrene and other carcinogenic agents.14, 27) However, lung adenocarcinomas etiologically have less association with cigarette smoking than either squamous cell carcinoma or small cell carcinoma. Our findings show that NO is an important factor inducing mutations in the *p53* gene in human lung adenocarcinoma.

Murata *et al*. reported that NO induces mutations which inactivate *p53* by inducing the deamination of guanine, leading to G:C-to-T:A transversions *in vitro*.16) In accordance with that report, we found that G:C-to-T:A transversions were present in 5 of 8 cases which had *p53* mutations with high NOS activity. The incidence (62.5%) of G:C-to-T:A transversions in stage I adenocarcinomas with high NOS activity in our study was much higher than that (23%) in total adenocarcinomas in the previous study.27) Denissenko *et al*. reported that benzo(a)pyrene formed DNA adducts and induced G:C-to-T:A transversions at hotspots of *p53* gene mutation.14) Although the number of cases analyzed in our study was limited and the results might thus be considered only preliminary, three of the 5 cases who showed G:C-to-T:A transversions in the *p53* gene had no smoking history. Tornaletti and Pfeifer reported that all cytosine in CpG sites in the *p53* coding region was methylated.28) Moreover, Lindahl noted in a review that NO induced deamination of methylcytosine and might lead to G:C-to-A:T transition *in vitro*.29) In our study, next to G:C-to-T:A transversions, G:C-to-A:T transitions were observed in CpG sites of *p53* gene in high NOS activity samples (2/8, 25%), but not in low NOS activity samples. These observations suggest that an excess amount of NO, which is endogenously produced by NOS, may induce *p53* gene mutations consisting mainly of G:C-to-T:A transversions and partly of G:C-to-A:T transitions early in the pathogenesis of lung adenocarcinoma.

It is well known that the major histologic subtypes of lung cancer differ not only in clinical behavior, but also in molecular pathogenesis in many respects, including *p53*.30) *p53* Mutations occur at different stages and possibly play a different biologic role in the multistage carcinogenesis pathway according to the type of lung cancer. It has been reported that positive *p53* immunostaining, like *p53* gene mutation, is significantly associated with lymph node and distant organ metastases and the pathological stage of the disease in adenocarcinoma, although such an association was not seen in squamous cell carcinoma or small cell carcinoma.24, 30) These results indicated that *p53* alterations might play an important role in the development of lung adenocarcinoma and might be a late event associated with progression, aggressive growth and metastatic potential. In fact, in other human cancers such as colon and brain tumors, *p53* abnormalities have been reported to be associated with malignant progression and to indicate the late stage of carcinogenesis.31–33) These results and our present findings suggest that increased NOS expression in some lung adenocarcinomas may play important roles in early developmental carcinogenesis, as well as in the process of malignant tumor progression. There is obviously cytologic, histologic and biologic heterogeneity of lung adenocarcinomas, even in the early stage. This heterogeneity is one of the unique characteristics of lung adenocarcinoma, and many cytologic subtypes have been proposed.31) A new classification method according to the NOS activity of lung adenocarcinoma may provide a new prognostic molecular marker for lung adenocarcinoma.

In summary, the present data point to a significant relationship between the NOS activity and *p53* gene mutation. Deamination of guanine leading to G:C-to-T:A transversions was the most common point mutation in early-stage lung adenocarcinoma with high NOS activity. If NO is produced for a long period of time at high concentrations in normal or chronically inflamed lung tissues, excessive NO could be one of the triggers of mutation which leads to lung adenocarcinoma. Although the histological precursor lesion of lung adenocarcinoma is not established, research on the interaction between NO and *p53* should shed new light on the molecular mechanism of the multistep carcinogenesis in human lung adenocarcinoma.

ACKNOWLEDGMENTS

We thank Drs. Teiso Kiyama and Noboru Fujino, Department of Respiratory Medicine, Kumamoto Chuo Hospital, for gifts of tissue materials, and Dr. Yoichiro Otsuka, Department of Clinical Pathology, Kumamoto Chuo Hospital, for his valuable advice on pathological examination. This work was partly supported by the Smoking Research Foundation (AM).

(Received April 13, 1998/Revised May 18, 1998/Accepted May 26, 1998)

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