

# Presence of heat shock protein 47-positive fibroblasts in cancer stroma is associated with increased risk of postoperative recurrence in patients with lung cancer

**Takuto Miyamura**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Noriho Sakamoto** (✉ [nsakamot@nagasaki-u.ac.jp](mailto:nsakamot@nagasaki-u.ac.jp))

Nagasaki University Graduate School of Biomedical Sciences <https://orcid.org/0000-0002-7577-476X>

**Kaori Ishida**

Kansai Ika Daigaku

**Tomoyuki Kakugawa**

Yamaguchi Daigaku Igakubu Daigakuin Igakukei Kenkyuka

**Hirokazu Taniguchi**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Daisuke Okuno**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Atsuko Hara**

Nagasaki Daigaku Byoin

**Takashi Kido**

Nagasaki Daigaku Byoin

**Hiroshi Ishimoto**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Takuro Miyazaki**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Keitaro Matsumoto**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Tomoshi Tsuchiya**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Hiroyuki Yamaguchi**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Yasushi Obase**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Yuji Ishimatsu**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Takeshi Nagayasu**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

**Hiroshi Mukae**

Nagasaki Daigaku Igakubu Daigakuin Ishiyakugaku Sogo Kenkyuka

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## Research

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# Abstract

## Background

Heat shock protein 47 (HSP47), a collagen-binding protein, has a specific role in the intracellular processing of procollagen production. HSP47 expression is associated with cancer growth and metastasis in several types of cancers. However, none of the studies have assessed whether HSP47 expression is associated with the risk of postoperative recurrence of lung cancer until now. Therefore, we aimed to assess this association.

## Methods

The study population consisted of a cohort of consecutive patients who underwent surgery for lung cancer at Nagasaki University Hospital, Nagasaki, Japan, from January 2009 to December 2010. Patient characteristics, survival and disease-free survival (DFS), and laboratory findings were compared between patients who tested positive and negative for HSP47 expression in lung cancer cells and between those who showed high and low numbers of HSP47-positive fibroblasts in cancer stroma.

## Results

A total of 133 patients underwent surgery for lung cancer. Sixty-seven patients (48.5%) showed HSP47-positive cancer cells, and 83 patients (62.4%) showed a higher number of HSP47-positive fibroblasts. The patients with a high number of HSP47-positive fibroblasts had a shorter DFS than those with a low number of HSP47-positive fibroblasts. Multivariate analysis identified only the presence of a high number of HSP47-positive fibroblasts as an independent risk factor for recurrence of lung cancer after surgery (odds ratio, 4.086; 95% confidence interval, 1.116–19.68;  $P = 0.033$ )

## Conclusion

The present study demonstrated that the presence of a high number of HSP47-positive fibroblasts in the cancer stroma was a risk factor for recurrence of lung cancer after surgery.

# Introduction

Heat shock protein 47 (HSP47) is a collagen-binding, stress-inducible protein localized in the endoplasmic reticulum, and it plays a specific role in the intracellular processing of procollagen production as a collagen-specific molecular chaperone.[1] HSP47 expression is reported to be correlated with collagen expression. Therefore, HSP47 is strongly associated with collagen-related fibrotic diseases, including liver, heart, kidney and pulmonary fibrosis.[2] We have reported that HSP47 expression is upregulated and associated with progression of pulmonary fibrosis and pulmonary fibrotic disorders using animal models of the same in humans.[3-7]

HSP47 has also been reported to be associated with several types of cancers, including cervical, breast, pancreatic, gastric, and colon cancer.[8-11] It is encoded by the SERPINH1 gene located on chromosome 11q13.5, and this region is one of the most frequently amplified in human cancer.[12] Several types of cancers are associated with abnormal protein folding, and HSP47 has been described as an important chaperone in the control and maintenance of cellular protein homeostasis.[13] Furthermore, HSP47 expression promotes cancer progression in part by enhancing the deposition of extracellular matrix (ECM) proteins, including collagens.

In lung cancers, expression of HSP47 in squamous cell carcinomas is higher than that in normal human bronchial epithelium cells.[14] However, no previous study has discussed the clinical significance of HSP47 expression in lung cancer. Therefore, this study aimed to reveal the association between the expression of HSP47 and lung cancer.

## Methods

### Study population and covariates

The study protocol was approved by the Institutional Review Board at Nagasaki University Hospital (approval number 17112009) and conducted in accordance with the Declaration of Helsinki. Informed consent was not required in view of the retrospective study design and the anonymity of the patient records reviewed, pursuant to the ethical guidelines of the Japanese Ministry of Health, Labor, and Welfare. The study population consisted of a cohort of consecutive patients who underwent surgery for lung cancer at Nagasaki University Hospital, Nagasaki, Japan, from January 2009 to December 2010. Patient characteristics, overall survival (OS), disease-free survival (DFS), and laboratory findings obtained from the clinical records were compared between patients showing pathologically positive and negative HSP47 expression in lung cancer cells, and high and low numbers of fibroblasts in the cancer stroma. The observation period was defined from surgery to December 2018.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (thickness, 3  $\mu$ m) were prepared from surgical specimens of patients with lung cancer. After deparaffinization and dehydration, the sections were placed in pH 9.0 ethylenediaminetetraacetic acid buffer and autoclaved at 95 °C for 30 min for epitope retrieval. The sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After incubation in blocking solutions, the sections were washed twice in tris-buffered saline (TBS) and incubated with anti-HSP47 antibodies (diluted 1:15000; M16.10A1, ADI-SPA-470; Enzo life sciences) at room temperature for 60 min. Sections were washed in TBS two times and incubated with peroxidase-labeled anti-rabbit or anti-mouse antibodies (Histofine Simplestain Max PO; Nichirei) for 30 min at room temperature. Peroxidase activity was detected with diaminobenzidine (Sigma-Aldrich). Sections were counterstained with hematoxylin and dehydrated.

# HSP47 expression scores in lung cancer cells

Each slide was observed by scanning the entire tissue specimen. One pathologist (KI) and one respiratory physician (TM) without prior knowledge of the patient's clinical characteristics evaluated the HSP47 immunoreactivity of lung cancer cells. Staining intensity was scored as follows; no staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3 (Figure 1 a-d). The extent of HSP47 staining was scored according to the percentage of HSP47-expressing lung cancer cells. HSP47 expression in lung cancer cells was categorized as positive or negative on the basis of the intensity and extent score (Table 1). The evaluators used a distal microscope (Nano Zoomer Distal Pathology. view 2).

## Quantitation of HSP47-positive fibroblasts in cancer stroma

In cancer stroma, the number of fibroblasts that reacted with the anti-HSP47 antibodies were counted using a distal microscope (Nano Zoomer Distal Pathology. view 2), and images of cells in a 1-mm<sup>2</sup> square field were acquired at 400× magnification.

## Lung cancer staging

Lung cancer stage was based on the IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of TNM classification for lung cancer.[15, 16] All TNM classifications and stages were determined on the basis of pathological findings.

## Statistical analysis

Categorical variables are presented as frequency and quantitative variables are presented as the median and interquartile range (IQR). Univariate tests (Wilcoxon rank-sum and chi-squared tests) and univariate and multivariate logistic regression analyses were performed to identify differences between patients who showed positive and negative HSP47 expression in lung cancer, high and low numbers of HSP47-positive fibroblasts in cancer stroma, and possible risk factors for primary disease death and recurrence. Receiver operating characteristic (ROC) curves were generated to determine cutoff values of risk factors for recurrence. The Kaplan-Meier method was used to determine the cumulative probability of OS and DFS, and the differences were evaluated using log-rank tests. Firth penalized logistic regression was used in the analyses of possible risk factors for primary disease-related death of male patients and of those with M classification (M1) because of quasi-complete separation. All P-values were two-sided and considered statistically significant when they were less than 0.05. All the statistical analyses were performed using the JMP Pro software program (version 14.0.0; SAS Institute, Inc., Cary, NC, USA).

## Results:

## Subject characteristics

The study population consisted of 133 patients who underwent surgery for lung cancer. The characteristics of the patients are shown in Table 2. Median patient age was 70 years (male patients, 87 (65.4%)). Seventy-six patients had a history of smoking (61.8%), and 99 patients showed adenocarcinoma (74.4%). The percentages of patients showing each pathological stage of lung cancer were as follows: stage I, 77.4%; stage II, 14.5%; stage III, 7.6%; and stage IV, 0.9%.

## Comparison of patient characteristics between patients with HSP47-positive and HSP47-negative lung cancer cells

Sixty-seven patients (48.5%) showed HSP47-positive cancer cells (Table 3). Patients with HSP47-positive cancer cells showed a greater frequency of smoking history (48.4% vs. 27.9%), higher percentage of diffusing capacity for carbon monoxide (97.6% vs. 80.9%), and a greater frequency of adenocarcinomas (89.6% vs. 59.1%) than those with HSP47-negative cancer cells. In contrast, serum KL-6 levels (193 U/mL vs. 262.5 U/mL) and the number of HSP47-positive fibroblasts in the cancer stroma (71 vs. 124.8) were lower in the HSP47-positive group than in the HSP47-negative group.

## Comparison of patient characteristics between patients with high and low number of HSP47-positive fibroblasts in the cancer stroma

The optimum cutoff level for discriminating between high and low numbers of HSP47-positive fibroblasts according to the ROC curve analysis was 73. Thus, 83 patients (62.4%) showed a high number of HSP47-positive fibroblasts (Table 3). The group with a high number of HSP47-positive fibroblasts showed a greater proportion of male patients (74.7% vs 50.0%), patients with a history of smoking (71.4% vs 45.7%), patients with pT3 or pT4 classification (9.6% vs 0.0%), patients with pN1 or pN2 classification (24.1% vs 6.0%), and patients who showed recurrence (31.3% vs 6.0%) and higher scores on the Brinkman index (945 vs. 710) than those in the group with a low number of HSP47-positive fibroblasts. However, the proportion of patients showing adenocarcinoma was lower in the group with a high number of HSP47-positive fibroblasts (59.0% vs 100.0%) than in the group with a low number of HSP47-positive fibroblasts.

## Survival curve and risk factors for recurrence and death

Figure 2 shows the DFS curves according to the presence or absence of HSP47 expression in cancer cells, and high or low numbers of HSP47-positive fibroblasts in the cancer stroma. The HSP47-positive and HSP47-negative groups showed no significant differences ( $p = 0.372$ , log-rank test). However, patients with a high number of HSP47-positive fibroblasts had a shorter DFS than those with few HSP47-positive fibroblasts ( $p = 0.001$ , log-rank test). Logistic regression analysis identified male sex (odds ratio [OR] 6.109, 95% confidence interval [CI] 1.987–26.75,  $P < 0.001$ ), history of smoking (OR 4.664, 95% CI 1.642–

16.82,  $P = 0.003$ ), pT3 or pT4 classification (OR 7.014, 95% CI 1.611–36.14,  $P = 0.010$ ), pN1 or pN2 classification (OR 4.685, 95% CI 1.754–14.40,  $P = 0.002$ ), and a high number of HSP47-positive fibroblasts in the cancer stroma (OR 7.146, 95% CI 2.329–31.27,  $P < 0.001$ ) as significant risk factors for the recurrence of lung cancer after surgery (Table 4). On the contrary, logistic regression analysis revealed adenocarcinoma to be inversely associated with recurrence (OR 0.362, 95% CI 0.149–0.886,  $P = 0.027$ ). However, multivariate analysis identified only a high number of HSP47-positive fibroblasts in the cancer stroma as an independent risk factor for recurrence of lung cancer after surgery (OR 4.086, 95% CI 1.116–19.68,  $P = 0.033$ ) (Table 4).

The OS curves based on HSP47 expression in cancer cells and high or low numbers of HSP47-positive fibroblasts are shown in additional file 1. There was no significant difference between the HSP47-positive and HSP47-negative groups ( $p = 0.388$ , log-rank test). However, patients with a high number of HSP47-positive fibroblasts had a shorter OS than those with a low number of HSP47-positive fibroblasts ( $p = 0.002$ , log-rank test). Logistic regression analysis identified age (OR 1.055, 95% CI 1.007–1.109,  $P = 0.028$ ), male sex (OR 22.50, 95% CI 4.535–408.2,  $P < 0.001$ ), history of smoking (OR 11.70, 95% CI 3.243–75.17,  $P < 0.001$ ), pN1 or pN2 classification (OR 4.390, 95% CI 1.680–11.55,  $P = 0.028$ ), pM1 classification (OR 4.261, 95% CI 1.194–50.32,  $P = 0.002$ ), and a high number of HSP47-positive fibroblasts (OR 3.879, 95% CI 1.477–12.20,  $P = 0.049$ ) to be significant risk factors for death because of lung cancer after surgery. However, there was no independent risk factor for death because of lung cancer after surgery as identified by multivariate analysis (Additional file 2).

The DFS and OS curves based on HSP47 expression status in cancer cells and the number of HSP47-positive fibroblasts in only adenocarcinoma patients showed similar differences as those in all patients. Only in patients with squamous cell carcinoma, no significant differences were observed in the DFS or OS curves based on HSP47 expression status in cancer cells and the number of HSP47-positive fibroblasts (data not shown).

## Discussion:

The present study showed that patients with a higher number of HSP47-positive fibroblasts in the lung cancer stroma had a shorter DFS than those with few HSP47-positive fibroblasts. Logistic regression analysis identified the presence of a high number of HSP47-positive fibroblasts in the cancer stroma to be a significant risk factor for the recurrence of lung cancer after surgery, and multivariate analyses identified the presence of a higher number of HSP47-positive fibroblasts as the only independent risk factor for the recurrence of lung cancer after surgery. This is the first study demonstrating the association between HSP47 expression in lung cancer stroma and the recurrence of lung cancer after surgery.

Increased HSP47 expression in cancer cells promotes cancer progression in part by enhancing deposition of the ECM proteins,[9] and several types of cancers are correlated with HSP47 expression in cancer cells. [8–10] The present study demonstrated that HSP47-positive fibroblasts in the stroma, but not HSP47-positive cancer cells, were associated with recurrence of lung cancer after surgery. Fibroblasts in solid

tumors are known as carcinoma-associated fibroblasts (CAFs).[17, 18] These arise from bone marrow-derived precursors and/or tissue-resident stromal cells through cancer cell-induced reprogramming and can be phenotypically identified using markers such as alpha smooth muscle actin ( $\alpha$ SMA), which is the marker of differentiated and activated fibroblasts called myofibroblasts.[19] Based on paracrine and juxtacrine signals, these stromal cells modulate cancer progression both directly and indirectly.[18, 20] Furthermore, the production and deposition of excessive ECM, commonly known as fibrosis, can also foster tumor progression, even though the underlying mechanisms remain to be fully elucidated.[21] In non-small-cell lung carcinoma cells, pirfenidone, a pyridine compound with therapeutic potential for idiopathic pulmonary fibrosis, significantly inhibits fibrosis and decreases tumor growth when used in combination with carboplatin.[22] These results suggest that fibrosis plays a pivotal role in the development of lung cancer. Furthermore, the relationship between HSP47 expression and lung fibrosis has been reported previously. [3–7, 23–25] Furthermore, HSP47 expression was associated with  $\alpha$ SMA-positive fibroblasts in animal models of and human with pulmonary fibrosis.[3] [5] These results suggest that the HSP47-positive fibroblasts in the present study represent CAFs. Collagen is the major component of ECM and plays a critical role in the tumor microenvironment.[26] HSP47 is essential for correct folding and secretion of collagen. Xu et al. reported that HSP47-positive stromal cell levels in patients with obstructing colon cancers were higher than those in non-obstructing colon cancers.[27] Mori et al. reported that HSP47-positive spindle cells serve as independent biomarkers for DFS and OS of patients with colorectal cancer.[11] They also showed that tumor budding, which represents a highly invasive phenotype, was associated with HSP47-positive spindle cells.[11] [27] In addition, HSP47 is reported to be expressed in the tumor-associated stromal desmoplasia of patients with pancreatic carcinoma, but HSP47 expression was absent in the majority of nonneoplastic pancreas.[28] Hirai et al. examined the HSP47 expression intensity in gastric cancer tissues, and found that the intensity of fibroblast staining was stronger than that of cancer cells.[29] These reports and our present study suggest that HSP47-positive fibroblasts might induce cancer progression and metastasis as CAFs and are associated with recurrence of lung cancer after surgery.

HSP47 expression in lung cancer cells was not a risk factor for reoccurrence and survival in the present study. HSP47 is encoded by the SERPINH1 gene, which is located on chromosome 11q13.5, one of the most frequently amplified regions in human cancer.[12] Altered HSP47 expression levels have been reported in several types of cancer, and HSP47 expression in colorectal cancer cells was upregulated in patients with lymph node metastasis and in those with high HSP47 expression showed longer DFS.[11] However, another study showed that there were no differences in the number of HSP47-positive cancer cells between obstructing and non-obstructing colon cancers.[27] In patients with breast cancer, HSP47 expression was activated during breast cancer development and progression, and HSP47 promotes cancer progression by increasing cell proliferation and invasion.[9] One report stated that HSP47 was a common antigen expressed in pancreatic nonductal neoplasms.[30] In non-small lung cancer cells, especially in squamous cell carcinomas, HSP47 expression was higher than that in the normal bronchial epithelium,[14] although adenocarcinoma was the most common type of tumor in the present study.

These reports and our present results suggest that the expression and roles of HSP47 in cancer cells, especially in a clinical setting, might differ according to the type of cancers.

This study has limitations that should be considered when interpreting the results. The present study included all types of lung cancers such as adenocarcinoma and squamous cell carcinoma. Although similar results were shown in the adenocarcinoma group, the meaning of HSP47 expression in each type of lung cancer was not examined separately. In addition, the mechanism and role of HSP47 expression in lung cancer progression was not demonstrated by in vitro studies. Further studies are needed to clarify the mechanism and the role of HSP47 in lung cancer progression.

## Conclusions

The present study demonstrated that the presence of a high number of HSP47-positive fibroblasts in the cancer stroma was a risk factor for recurrence of lung cancer after surgery.

## Abbreviations

HSP47

heat shock protein 47

ECM

extracellular matrix

IPF

idiopathic pulmonary fibrosis

NSIP

nonspecific interstitial pneumonia

DAD

diffuse alveolar damage

AIP

acute interstitial pneumonia

IQR

interquartile range

ROC

receiver operating characteristic

OS

overall survival

DFS

disease free survival

OR

odds ratio

CI

confidence interval

# Declarations

## Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board at Nagasaki University Hospital (approval number 17112009), and the study was conducted in accordance with the Declaration of Helsinki. Informed consent was not required in view of the retrospective study design and the anonymity of the patient records reviewed, pursuant to the ethical guidelines of the Japanese Ministry of Health, Labor, and Welfare.

## Consent for publication

Not applicable

## Availability of data and materials

Not applicable

## Competing interests

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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## Author contributions

TM, TK, and NS were involved in drafting the article. TK made substantial contributions to study conception and design. KI interpreted the pathological sections. TM, KM, TT, and TN provided the dates of surgery. HT, DO, AH, TK, HI, YI, and HM were involved in critically revising the article for important intellectual content. All authors read and approved the final manuscript.

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## Tables

Due to technical limitations, Tables 1-4 are provided in the Supplementary Files section.

## Figures



Figure 1

Extent of HSP47 immunostaining in lung cancer a) score 0: no immunostaining; b) 1: weak staining; c) 2: moderate staining; and d) 3: strong staining. Fibroblasts in cancer stroma e) Hematoxylin-Eosin staining, f) HSP47 immunostaining.

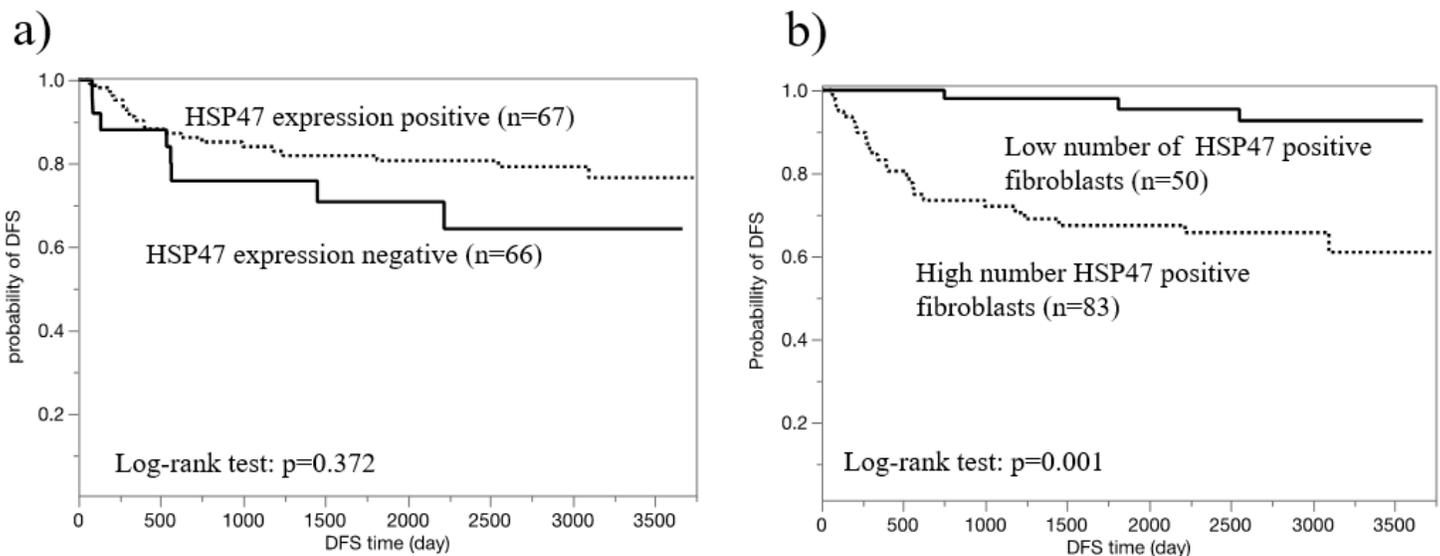


Figure 2

Survival curves of patients with lung cancer according to HSP47 expression in lung cancer cells or the number of HSP47-positive fibroblasts in the stroma. a) No significant difference in progression-free survival curves of patients showing HSP47-positive or HSP47-negative cancer cells ( $p = 0.372$ , log-rank test). b) Patients with a high number of HSP47-positive fibroblasts had significantly shorter disease-free survival ( $p = 0.001$ , log-rank test, cut-off value = 73)

## Supplementary Files

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