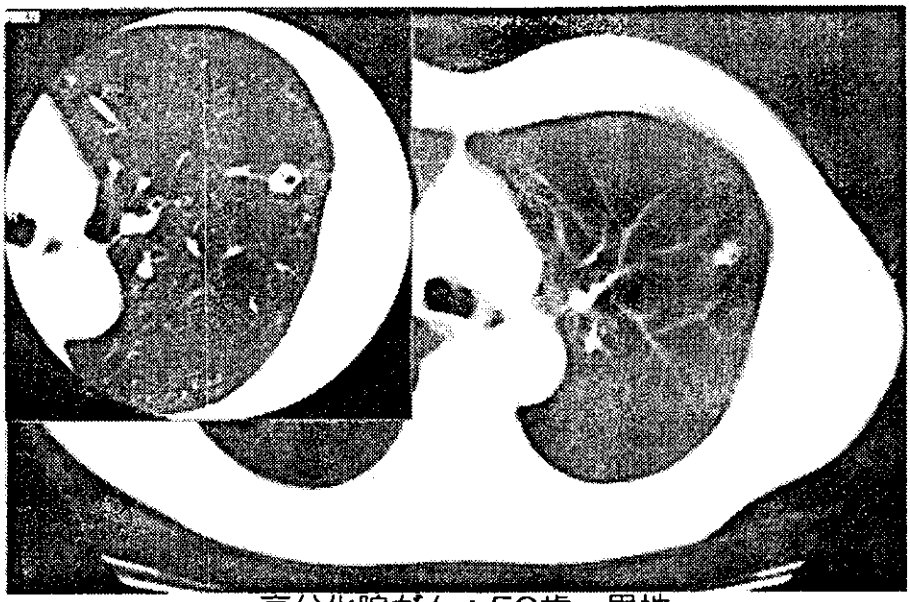
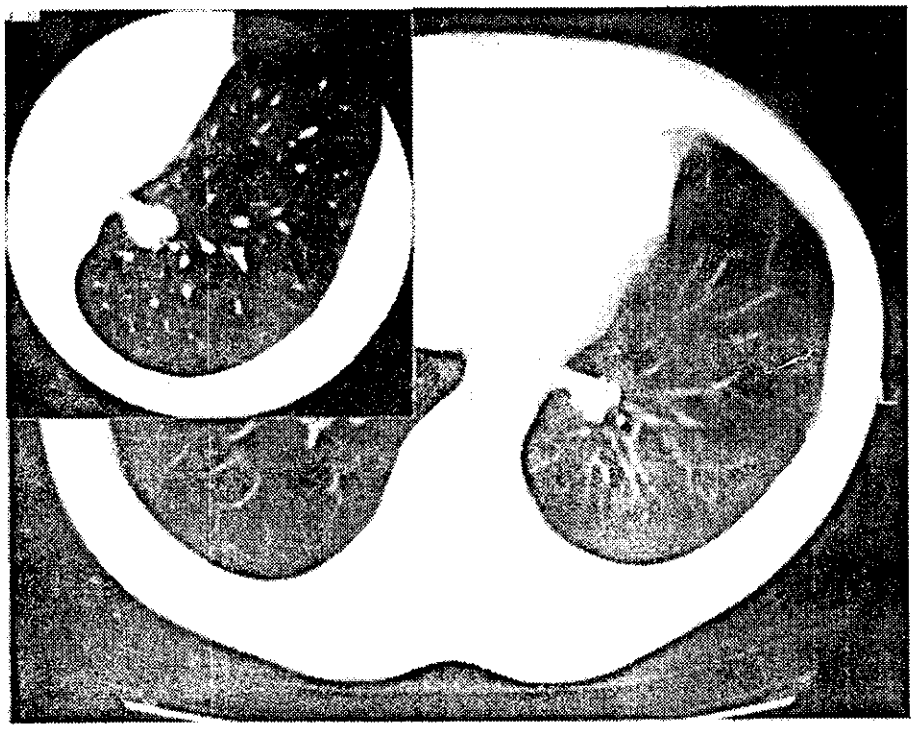
図１０：初回検診で発見された進行肺がん



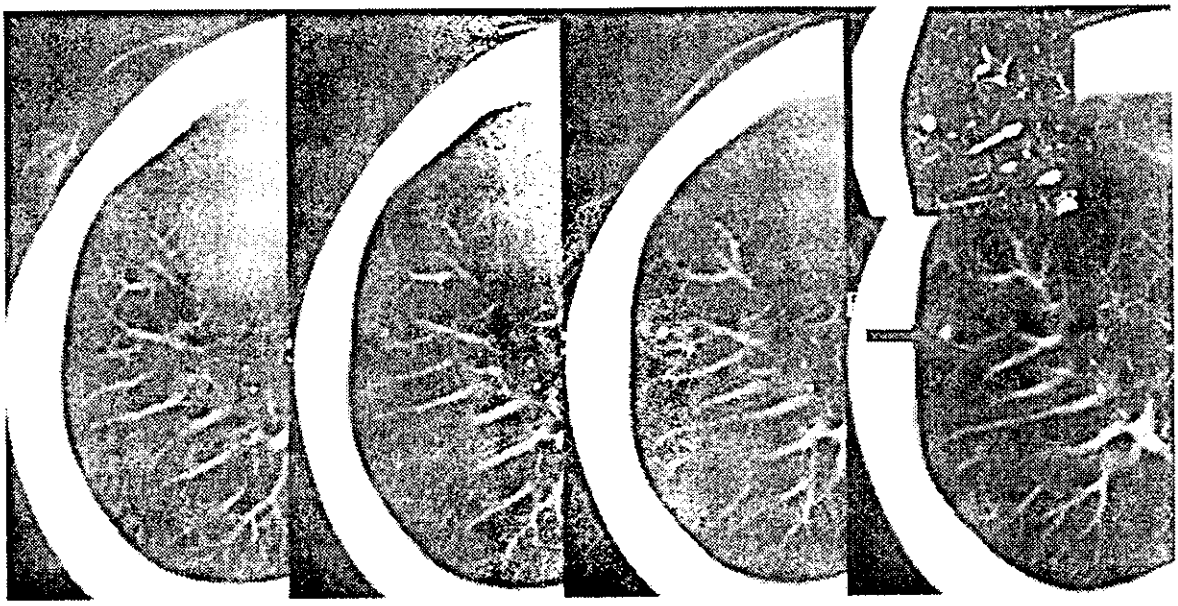
高分化腺がん：50歳・男性



中分化腺がん：50歳・男性

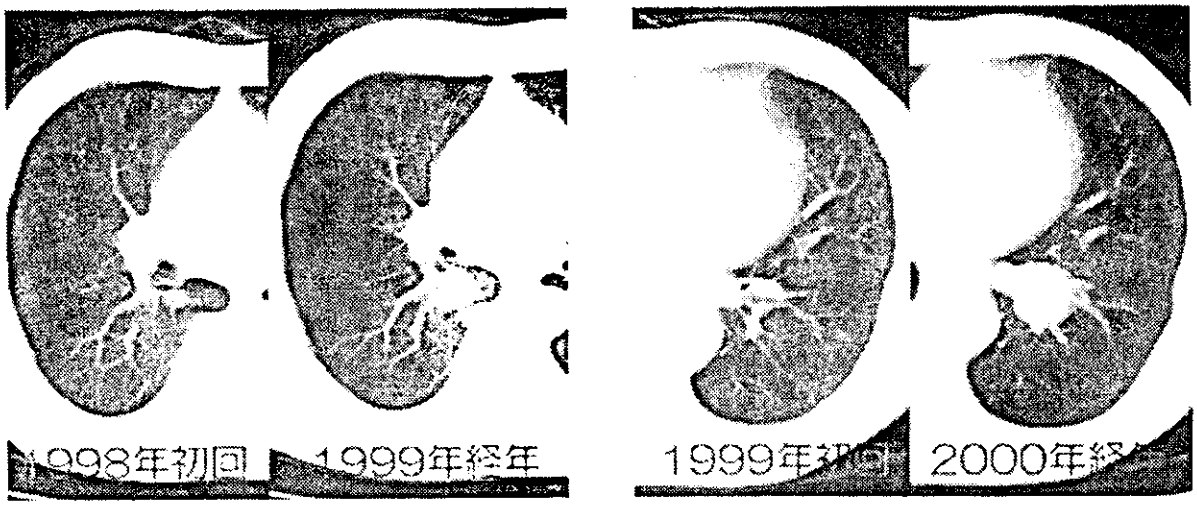
残念ながら，リンパ節転移が診断された進行肺がん症例です．肺がんは直径 10mm 程で非常に小さいサイズで発見されたにも関わらず，転移が証明された症例です．どちらも 50 歳で初めて CT 検診を受診された方です．もう少し年齢を下げて検診を実施するほうがよいのかどうか議論の分かれるところです．

図１１：経年検診で発見された興味深い早期肺がん




2001年検診を契機に発見された中分化腺がん症例です．過去にさかのぼって2000年，1999年の検診画像でも微小な結節が確認できますが，1998 年でははっきりしません．経過観察ではじめて肺がんが疑われる興味深い症例でした．

図１２：経年検診で発見された急速増大した肺がん




中分化腺がん　67歳・男性　　中分化扁平上皮がん　66歳・男性

経過観察中に急激に体積増大した症例 2 例ですが，経年検診群から発見された多くの肺がんはこれらほど急激に変化はしないことが私たちの経験からわかりました．

まとめ

① 胸部 CT 検診では腺がんなどの肺野型肺がんの早期検出は容易であるが，肺門型肺がんを検出しておらず検討すべき大きな課題である．

② 経年受診における経過観察で肺野孤立性結節の質的診断が可能であるが，腫瘍直径増大を確認するための比較読影システム構築が必須の要件である．

③ 孤立性肺結節の質的診断のための適切な観察間隔についてはさらに検討を加えていく必要がある．

④ サイズ増大を示す経年発見肺がんは男性・喫煙者に多く，中分化腺がんの割合が初回群と比べて高かった．

今後 CT 検診展開へ向けての提言

◆ CT 検診は repeat screening が重要

repeat screening で発見される肺がんに overdiagnosis は含まれない．それは体積増大を診断根拠としているためだからです．repeat screening を誰に対して行なうべきか？これは検診の効率を考える上でも重要です．私たちは，50 歳以上・男性・喫煙者の方には毎年胸部 CT 検診を推奨しています．受診間隔はどうするか？これも検診の効率に密接に関係する重要な要因です．現在は総合健康診断にあわせて実施しているため一年に一回としているが，6ヵ月・3ヵ月で正確肺がんの診断が可能であれば，短い受診間隔で検査することができます．どのような CT 所見の病変に対して経過を追うか？まだはっきりとしたコンセンサスが得られていないのが現状です．5mm 未満の充実性結節や 10mm 未満の pure GGO をどう取り扱うかは解決すべき大きな課題です．

◆ CT 検診を導入する際 repeat screening をいかに効率的に運用するか熟考が必要

何度も繰り返すように胸部 CT 検診は repeat screening が非常に重要ですが，初回時の対応しか準備しないとあとあと後悔することになります．二年目以降比較読影のためにどのように過去データを手間をかけずに取り出せるようにするか，読影はフィルムでおこなうのか，それともモニタでおこなうのかなど，検診導入時から検討しておかれることをお勧めします．

◆ リスクに応じ適切な CT 検診システムをデザインすることが必要

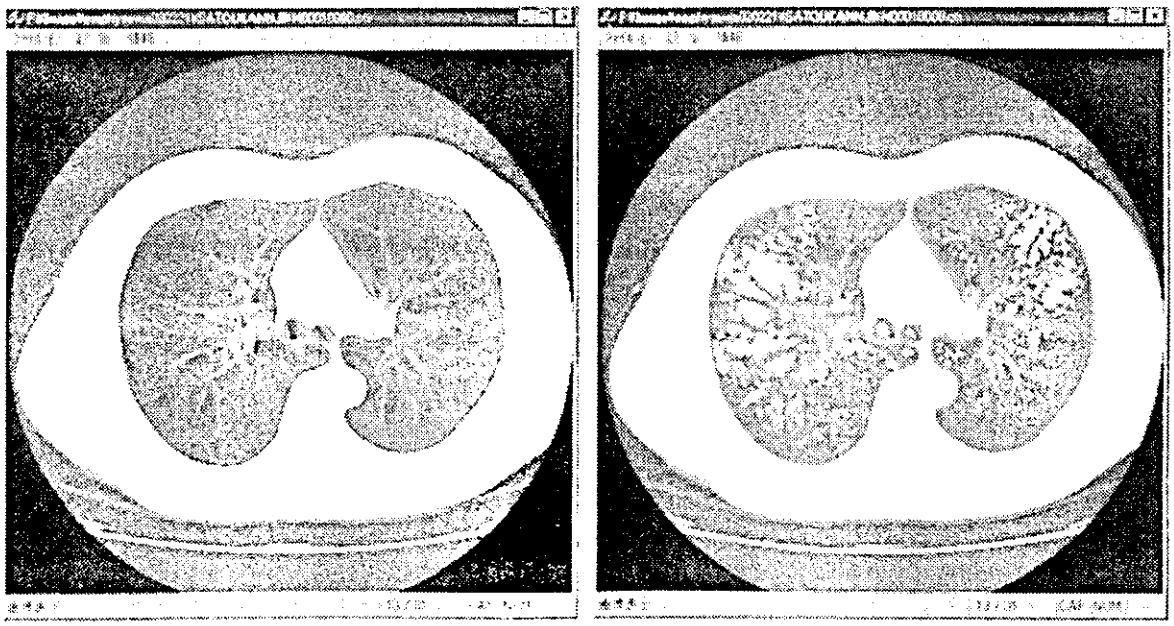
CT 検診は肺野型肺がんを早期に的確に検出できます．一番効率のよい対象群は実は女性なのです．女性の肺がんは 9 割が肺野末梢から発生する腺がんで，もっとも胸部 CT 検査が早期発見を得意とするのがこの腺がんなのです．しかも，私たちの経験では，一回の CT 検診で女性の腺がんは検出できるので，毎年繰り返し検査を行なわずとも良いと考えております．医療経済学的にみても非常に効率良い対象群です．また非喫煙者であれば repeat screening の必要はなさそうです．これからの検診は個人個人のがんにかかりやすいかどうかのリスクを勘案しながらデザインされるべきです．疫学

や分子生物学，遺伝子工学など知識を融合することで，いよいよ個別のオーダーメイド検診の時代に突入していくと思います．

◆ 検診を行う一方で現場で禁煙支援(一次予防)を推進することは重要

折角，自身の健康に向き合おうと検診に足を運ばれておられるのですから，効果的な広報を通じて正確で役に立つ情報を提供すべきでしょう．特に喫煙がおもな原因である肺がんだけでなく，慢性閉塞性肺疾患（COPD）の問題も今後顕在化するでしょうから，検診現場で禁煙支援（一次予防）を推進していくことには大変意義があります．私たちは，たばこが原因で肺が破壊された状態の受診者にはご自身のCT画像をお見せして，たばこが原因であること，このままたばこを吸いつづけると更に悪化すること，たばこをやめれば破壊の速度が緩やかになることを説明し，禁煙を側面から応援しています．(図１３)この説明を聞かれただけで約 3 割の方が禁煙を実行されています．これまでにこれほど強力な禁煙説得ツールを経験したことがありません．やはりCTで割ってみる画像のすばらしい効用のひとつであると思います．これまでは医療従事者が診断のためだけにCT画像を独占して利用してきましたが，一般の受診者にも十分理解可能な画像ですのでこれを活用しない手はありません．

図１３：CT肺気腫自動検出システム

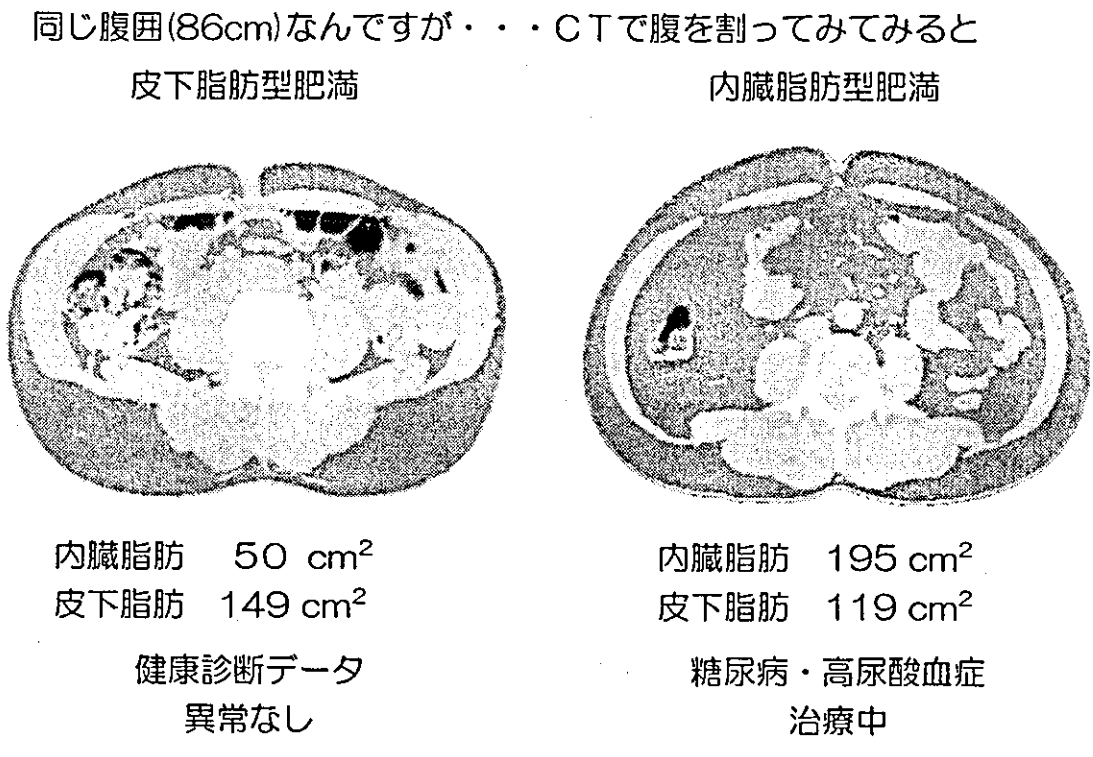


左画面にCT肺気腫部分が色づけされて表示，同部はたばこにより破壊されて蜂の巣のように空洞化した部位です．このシステムは自動的に肺全体の体積に占めるCT肺気腫の割合を表示し，視覚的評価から定量化できるようになりました．

胸部CT検査に引き続き，おへその部位を一段面だけ撮影すると，おなかの脂肪の分布が

簡単にわかります．私たちは，腹腔内の脂肪と皮下の脂肪を自動的に認識し，面積を測定するソフトを開発しました．（図１４）2003年9月より胸部CT検診受診者のうち希望者にはこの内臓脂肪CT検診を実施しております．受診者の評判も上々で，2004年4月からは，内臓脂肪CT検診のみの受診も可能となるように準備しております．

図１４：内臓脂肪自動検出システム




最後に

肺がんにしてもCT肺気腫や内臓脂肪にしても，CTで割ってみるということは，“わかりやすい”の一言に尽きます．技術革新に伴って得られた利益は医療従事者が診断に利用するためだけでなく積極的に医療消費者に還元するべきです．CT画像は非常にわかりやすい，専門家以外でも理解が出来る，興味が持てる画像ですから積極的に様々な場面で利用すべきなのです．

がんや心筋こうそく，糖尿病など生活習慣と密接に関係して発症する病気にならないように生活習慣へ改善することが大切です．禁煙やダイエットのモチベーションを維持することに，いままで活用されてこなかったCT画像が役立つことを期待しています．

今後も受診者の利益・不利益を見極め，大きな利益が受診者に得られる画像検査であれば積極的に展開していきたいと思っております．

最後までお付き合いいただき誠にありがとうございました．

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役に立つ胸部CT検診関連ウエブサイト

① 胸部CT検診研究会：国内の胸部CT検診に関する発表の中心にある研究会
http://www.thoracic-ct-screening.org

② 日本肺癌学会：内科・外科・放射線科・病理科など肺癌に関わる情報が集約される学会
http://www.haigan.gr.jp

③ International Collabolation to Screen for Lung Cancer：Early Lung Cancer Action Project(ELCAP)の情報が入手できるサイト
http://icscreen.med.cornell.edu

④ コンピュータ支援画像診断学会：CT画像のCADの研究発表が増加すると予想される
http://www.toriwaki.nuie.nagoya-u.ac.jp/~cadm/japanese/

⑤ Natinal Lung Screening Traial：米国国立がん研究所の肺がんCT検診の無作為化比較試験の情報が入手できるサイト
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# Identification of Epigenetic Aberrant Promoter Methylation in Serum DNA Is Useful for Early Detection of Lung Cancer

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## ABSTRACT

***Purpose:*** **The purpose of this study is to evaluate the usefulness of serum DNA methylation of five tumor suppressor genes for early detection of lung cancer.**

***Experimental Design:*** **Methylation status in serum DNA from 200 patients undergoing bronchofiberscopic examination for abnormal findings on chest radiograph detected by lung cancer screening or surveillance was examined using methylation-specific PCR.**

***Results:*** **Ninety-one patients were given a pathologic diagnosis of lung cancer, 9 other malignant diseases, and 100 nonmalignant pulmonary diseases. In patients with lung cancer, methylation was detected in 18.7% for *MGMT*, 15.4% for $p16^{INK4a}$, 12.1% for *RASSF1A*, 11.0% for *DAPK*, and 6.6% for *RAR-β*, which was higher compared with that in patients with nonmalignant diseases. Age and smoking status seemed to associate with methylation status. Sensitivity, specificity, and predictive value of methylation in at least one gene for diagnosis of lung cancer were 49.5%, 85.0%, and 75.0%, respectively. Adjusted odds ratio (95% confidence interval) for having lung cancer was 5.28 (2.39-11.7) for patients with methylation in one gene and 5.89 (1.53-22.7) for those with methylation in two or more genes. It is of note that methylation was identified in 50.9% of stage I lung cancer patients, whereas serum protein tumor markers were positive in 11.3% of them.**

***Conclusions:*** **These results suggest that identification of promoter methylation of tumor suppressor genes in serum DNA could be useful for early detection of lung cancer.**

## INTRODUCTION

Despite intensive treatment, the prognosis of patients with lung cancer is poor. The 5-year survival rate of patients with clinical stage I disease is ~60%, but in those with clinical stage II to IV diseases, the 5-year survival rate ranges from 40% to <5% (1). Thus, the prognosis of lung cancer is strongly correlated to its clinical stage. Over two thirds of lung cancer patients have an advanced disease at the time of initial presentation (2), and lack of efficient diagnostic methods for early detection is considered to be the major reasons for the poor prognosis of lung cancer.

Although lung cancer screening with annual chest radiograph and sputum cytology is currently conducted in many municipalities in Japan (3), the usefulness of mass screening is yet to be fully confirmed. The previous screening trials sponsored by the National Cancer Institute failed to show that screening with sputum cytology and chest radiography reduced mortality from lung cancer (4–7). However, because one of these trials indicated more favorable survival rates associated with the diagnosis of resectable tumors, the American Cancer Society maintains that physicians and patients may decide to have these screening tests on an individual basis (8). Therefore, the development of more useful method in addition to the chest radiograph and sputum cytology for lung cancer screening is urgently required.

Aberrant methylation of CpG islands, which are in or near the promoter region of various genes, is a common feature in various neoplasms and is associated with the transcriptional silencing of tumor suppressor genes (9–11). In addition, this alteration has been described to occur in the very early stage of carcinogenesis (12). Recent advances in techniques simplified the methods for identification of promoter methylation, among which methylation-specific PCR (MSP) is a simple, sensitive, and specific method to determine the methylation status of any CpG-rich region (13).

Several studies have shown that several genes, including tumor suppressor genes, such as retinoic acid receptor β (*RARβ*; ref. 14) and $p16^{INK4a}$ (15, 16), apoptosis-associated genes, such as death-associated protein kinase (*DAPK*; ref. 17) and *ras* association domain family 1A (*RASSF1A*; refs. 18, 19), and the DNA repair gene $O^6$-methylguanine DNA methyltransferase (*MGMT*; refs. 20, 21), were frequently methylated in lung cancer cells. Zochbauer-Muller et al. showed that 82% of the non–small cell lung cancer tissues had methylation of at least one gene from eight genes and rarely identified methylation of these genes in nonmalignant lung tissue (20). These findings suggest the potential use of DNA methylation as a marker for lung cancer.

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It has been shown that cancer patients have increased levels of free DNA in their sera, which is thought to be released from cancer cells (22–24). Many investigators have reported that microsatellite alterations and p53 and/or *ras* gene mutations could be identified in the serum and/or plasma DNA of patients with various cancers (25–28). Thus, circulating tumor-derived DNA might be used as a source for tumor detection by PCR analysis, including MSP.

In the present study, we attempted to identify methylated DNA in sera of patients with abnormal findings on their chest radiograph as detected by lung cancer screening or physician surveillance. Although there are some recent reports of DNA methylation analyses carried out with remote medium, including serum, plasma, sputum, and bronchoalveolar lavage fluid or brushing samples, this is the first report that has examined the methylation status of serum DNA on a population basis and showed the usefulness of the approach as a diagnostic tool for early detection of lung cancer.

## MATERIALS AND METHODS

**Sample Collection and DNA Extraction.** In this study, 200 patients undergoing fiberoptic bronchoscopy for abnormal findings on chest radiograph were investigated. The examinations were carried out as part of the lung cancer mass screening program in Okayama Prefecture (3) or through physician surveillance. Diagnosis of these patients was completely blinded to the laboratory researchers. Peripheral blood samples (6 mL) were collected to investigate methylation status of serum DNA with written informed consent. Serum (2 mL) was isolated after centrifugation at 3,000 rpm for 10 minutes and stored at −20°C until use. Serum DNA was extracted using QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We also examined methylation status of the tumor tissues. Of 200 patients evaluated in this study, 30 with lung cancer underwent surgical resection at Department of Cancer and Thoracic Surgery of Okayama University Hospital. Tumor tissues from these patients were investigated with written informed consent. Tumor DNA was extracted from formalin-fixed, paraffin-embedded lung cancer tissues using QIAamp DNA Mini kit (Qiagen) according to manufacturer's instructions.

**Methylation-Specific PCR.** Sample DNA was treated with sodium bisulfite using the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the manufacturer's instructions. All bisulfite-modified DNA was resuspended in TE buffer [10 mmol/L Tris-0.1 mmol/L EDTA (pH 7.5)] and used immediately or stored at −20°C until subsequent MSP. Primer sequences for the *RAR*β, *p16*$^{INK4a}$, *DAPK*, *RASSF1A*, and *MGMT* were as described elsewhere (18, 20). DNA from a small cell lung cancer cell line SBC-3 (29), which has promoter methylation of all tested genes, was used as a positive control for the methylated form and that from serum of normal volunteer for the unmethylated form. The PCR mixture contained 10× PCR buffer [100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L $MgCl_2$], deoxynucleotide triphosphates (each at 2.5 mmol/L), 0.5 μmol/L of each primer, 0.9 units Taq DNA polymerase (Takara Bio, Shiga, Japan), and 3 μL bisulfite-modified DNA in a final volume of 30 μL. Initial denaturation at 95°C for 5 minutes was followed by 50 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at each specific annealing temperature for 30 seconds, and an extension step at 72°C for 30 seconds and a final extension step at 72°C for 10 minutes was added. After amplification, each PCR product was electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. All tests were duplicated to confirm the result by blinded two researchers. Methylation-positive cases were defined as the cases whose serum DNA showed a visual band amplified with methylated-specific primers, even if it was faint. Representative results of methylation analysis by MSP are shown in Fig. 1.

**Measurement of Serum Protein Tumor Marker Levels.** A part of each blood sample was used for examination of conventional serum protein tumor markers, including carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide. The serum levels of carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide were determined by chemiluminescent immunoassay using commercial kits from Abbott Laboratories (Abbott Park, IL), electrochemiluminescent immunoassay from Roche Diagnostics (Basel, Switzerland), and ELISA from Fujirebio (Tokyo, Japan). The cutoff values of these markers were set at 5.0 ng/mL for carcinoembryonic antigen, 3.5 ng/mL for cytokeratin 19 fragment, and 46.0 pg/mL for progastrin releasing peptide according to the manufacturer's instructions.

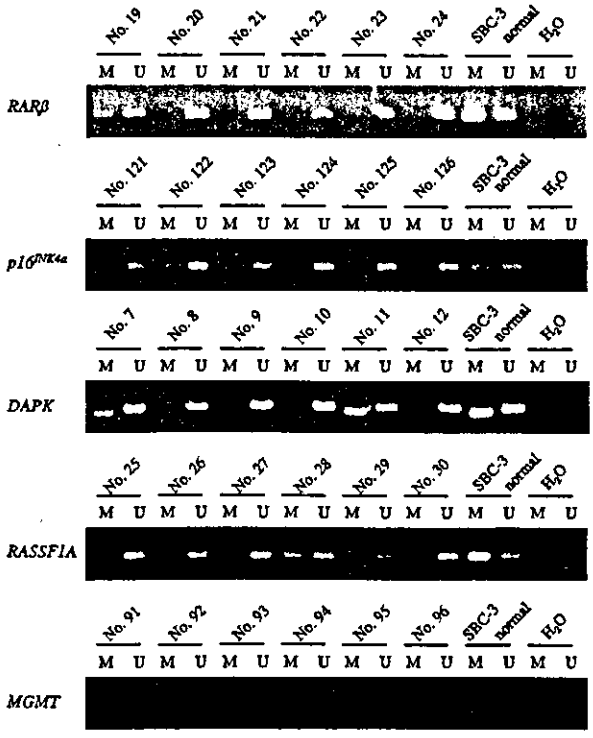


*Fig. 1* Representative results showing promoter methylation amplified by MSP. Lanes *M* and *U*, amplified product with primers recognizing methylated and unmethylated sequences, respectively; DNA from SBC-3 cell line and normal volunteer, positive control for methylated and unmethylated forms, respectively.

**Statistical Analysis.** The methylation status in five genes was scored in each patient as the total number of methylated genes. The methylation score of patients with lung cancer was compared with those with nonmalignant diseases using a *t* test with unequal variance. An unconditional logistic regression model was applied to estimate the odds ratios and its 95% confidence intervals for the occurrence of lung cancer. We used the patients without methylation in every five genes as a reference group and estimated the odds ratios for patients having one methylated gene and those having two or more methylated genes in five genes tested. Crude and multivariate models were examined. The factors adjusted in the multivariate model included age as a continuous variable divided by 10, sex, smoking status divided into quartiles (pack-years calculated by number of packs smoked per day multiplied by the number of years of smoking), and results of three tumor markers (carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide) in a binary variable. $\chi^2$ or Fisher's exact test were applied to examine the distribution in categorical variables. A *t* test was applied to test the continuous variables. The statistical significance was defined as $P < 0.05$. All the statistical analyses were implemented by Stata version 8 (College Station, TX).

## RESULTS

**Patients Characteristics.** Between January 2001 and December 2002, a total of 200 peripheral blood samples were collected from consecutive patients undergoing fiberoptic bronchoscopy. Of these patients, 91 were given a pathologic diagnosis of lung cancer (median age, 71 years; range, 45-92 years; male/female 61:30), 100 nonmalignant diseases (median age, 65.5 years; range, 26-89 years; male/female 64:36), and 9 other malignancies. The histologic subtypes of the lung cancers, based on WHO classification (30), were adenocarcinoma in 64 patients, squamous cell carcinoma in 21 patients, small cell carcinoma in 4 patients, and carcinoid in 2 patients. Clinical stage classifications, based on the International Staging System (1), were as follows: 53 patients had stage I disease, 7 patients stage II, 22 patients stage III, and 9 patients stage IV. Nonmalignant diseases mostly consisted of benign pulmonary diseases, such as tuberculosis, atypical mycobacteriosis, pneumoconiosis, interstitial pneumonia, bronchitis, organizing pneumonia, and bronchiectasis. Malignant diseases other than lung cancer included pulmonary metastasis of laryngeal cancer in 3 patients, invasive thymoma in 2 patients, and non-Hodgkin's lymphoma, thyroid cancer, breast cancer, and rectal cancer in 1 patient each. Elderly patients were more frequently represented among patients with lung cancer than those with nonmalignant diseases (median age, 71 versus 65.5 years; $P < 0.001$). Current smokers were more commonly represented among patients with lung cancer than those with nonmalignant diseases (67% versus 54%; $P = 0.046$).

**Methylation Status of Five Genes.** As shown in Table 1, serum methylated DNA was detected in 18.7% for *MGMT*, 15.4% for *p16*$^{INK4a}$, 12.1% for *RASSF1A*, 11.0% for *DAPK*, and 6.6% for *RAR*β in lung cancer patients. When analyzed individually, the proportions of patients with methylated serum DNA were higher in patients with lung cancer than those with nonmalignant diseases in every five genes. Difference is especially evident for *p16*$^{INK4a}$ ($P = 0.003$) and *MGMT* ($P < 0.001$). Of 91 lung cancer patients, 45 (49.5%) had methylation of at least one gene. When methylation of at least one gene was assessed as positive, specificity and predictive values of methylation were 85.0% and 75.0%, respectively. The total number of methylations in five genes per patient was 0.64 in patients with lung cancer, which was higher than that in patients with nonmalignant diseases (0.19; $P < 0.0001$; Table 2). Of 9 patients with malignant diseases other than lung cancer, 6 (66.7%) had at least one methylation in five genes (data not shown). These 9 cases were excluded from subsequent analyses.

Twenty-three of 30 (77%) tumor tissues obtained from lung cancer patients showed methylation of at least one gene (Fig. 2). Sixteen of 18 (89%) tissues from patients with serum DNA methylation also had methylated genes. Methylation of *RASSF1A* gene was identified in one serum sample and *MGMT* gene in two samples, but they were not identified in the corresponding tumor.

**Association with Clinicopathologic Features.** We analyzed the correlations between methylation status in serum DNA and clinicopathologic variables of the patients. There was no correlation between methylation status and sex or histology in this study. In addition, frequency of serum DNA methylation between smokers and nonsmokers was not significantly different in both patients with lung cancer (48.3% in nonsmokers, 45.8% in <40 pack-years smokers, and 52.6% in ≥40 pack-years smokers; $P = 0.863$) and those with nonmalignant diseases (Table 3). These findings were also observed when analyzed in individual gene. Although serum DNA methylation in ≥40 pack-years smokers with nonmalignant diseases tended to be more frequent than that in <40 pack-years smokers, this trend was particularly obvious in *DAPK* and *RASSF1A* genes. In control group, we found significant correlation between methylation and age (Table 3).

**Methylation Status and Risk of Lung Cancer.** Table 4 shows the results of a crude and adjusted logistic regression analyses evaluating correlation between number of methylated genes and risk of lung cancer. In the crude model, the patients with one methylated gene showed 5.08 (95% confidence interval, 2.28-11.3) times higher probability of having lung cancer compared with patients without any methylated genes. The odds ratio was higher in patients with two or more methylated genes. To consider the imbalance in baseline characteristics, we conducted similar analysis adjusting for age,

*Table 1* Frequency of methylation in five genes

| | Patients, *n* (%) | | |
|---|---|---|---|
| | Lung cancer (*n* = 100) | Nonmalignancy (*n* = 100) | Total (*n* = 191) |
| *MGMT* | 17 (18.7) | 2 (2.0) | 19 (9.9) |
| *p16*$^{INK4a}$ | 14 (15.4) | 3 (3.0) | 17 (8.9) |
| *RASSF1A* | 11 (12.1) | 8 (8.0) | 19 (9.9) |
| *DAPK* | 10 (11.0) | 5 (5.0) | 15 (7.9) |
| *RAR*β | 6 (6.6) | 1 (1.0) | 7 (3.7) |

*Table 2* Comparison of methylation status between lung cancer and nonmalignant cases

| | Mean* | SE | SD | 95% Confidence interval | |
|---|---|---|---|---|---|
| Nonmalignancy | 0.19 | 0.0506 | 0.0506 | 0.09-0.29 | |
| Lung cancer | 0.64 | 0.0774 | 0.738 | 0.48-0.79 | $P < 0.0001$† |

*Total number of methylations in five genes per patient.
†*t* test with unequal variance.

sex, smoking status, and protein tumor marker results. The patients with methylation in at least one gene and two or more of five genes showed 5.28 (2.39-11.7; $P < 0.001$) and 5.89 (1.53-22.7; $P = 0.010$) times higher probability of having lung cancer, respectively.

**Frequencies of Methylation According to Clinical Stage of Lung Cancer.** We investigated the correlation between clinical stage and methylation status in five genes or conventional serum protein tumor markers (Table 5). Of 53 patients with stage I disease, 27 (50.9%) patients had methylated serum DNA in at least one gene, whereas only 6 (11.3%) patients showed elevation of at least one serum protein tumor marker. In patients with stage II, III, or IV diseases, the difference was not evident.

## DISCUSSION

This study shows that identification of serum DNA methylation is a potentially useful approach to detect lung cancer patients from subjects screened by chest radiograph. Serum DNA methylation was more frequently observed in patients with lung cancer than those with nonmalignant diseases. Although the sensitivity for the diagnosis of lung cancer was only 49.5% when analyzed by a combination of five genes, the relatively high specificity (85.0%) indicates the usefulness for subjects screened by with chest radiograph. The odds ratio for diagnosis of lung cancer was >5.0 in patients with at least one methylated gene even after statistical adjustment by other clinicopathologic risk factors, such as smoking, age, sex, and results of tumor marker tests. Of note, serum DNA methylation could be identified even in patients in the early stages of lung cancer, whereas conventional serum protein tumor markers were rarely elevated, indicating that this DNA-based method is more sensitive than protein-based method for diagnosis of lung cancer in early stage.

In former studies, methylation in tumor tissues was detected in 40% to 43% of non-small cell lung cancer patients for *RAR*β, 25% to 41% for $p16^{INK4a}$, 16% to 44% for *DAPK*, 30% to 40% for *RASSF1A*, and 16% to 27% for *MGMT* (31). These results were consistent with our data in 30 tumor tissue samples. In our experiment, the frequency of detecting methylated genes in serum was about half to two thirds compared with that in tumor tissues. However, when we consider that tumor-derived DNA in blood is generally detectable in less than half of cancer patients (32), the frequency of methylation in serum DNA in our study may be reasonable. Laird reviewed the studies examining methylation status of serum/plasma DNA in patients with various neoplasms and indicated that clinical sensitivity of DNA methylation was ~50% (33). Esteller et al. did methylation analysis in serum DNA from patients with non-small cell lung cancer for multiple genes and showed 33% to 80% of clinical sensitivity by combination analysis of these genes (34). Our results are consistent with the results of these studies, indicating that similar sensitivity is achievable even after mass screening.

Among various techniques used for methylation analysis, we adopted a simple method of qualitative MSP analysis. The specificity of the primers we used in this study had been verified using genomic sequencing and/or restriction analysis in previous reports (13, 35). Recently, several studies showed improved detection rates of methylation status using a nested PCR approach or a quantitative real-time PCR technique (36-38). Particularly, sensitivity of the Taqman method was reported to be 10-fold higher than conventional qualitative MSP (39). To apply DNA methylation as tumor marker for detection of lung cancer, the use of these improved methods is an attractive strategy.

Although promoter methylation was observed predominantly in lung cancer patients, 1% to 8% of patients with nonmalignant disease were methylation positive for each gene in this study. In addition, three lung cancer patients with serum DNA methylation did not show same alteration in the corresponding tumor tissues. We considered the following as possible explanation of these positive results. Firstly, the methylated serum DNA might be derived from undetected precancerous lesions in these cases. According to the previous reports, aberrant promoter methylation is detectable in precancerous lesions, such as dysplasia and nonmalignant lung tissues of patients with lung cancer (20, 37). Methylation-positive nonmalignant patients may develop malignant diseases in the near future. Secondly, aberrant methylation might be caused by environmental factors, such as smoking (12, 40). In

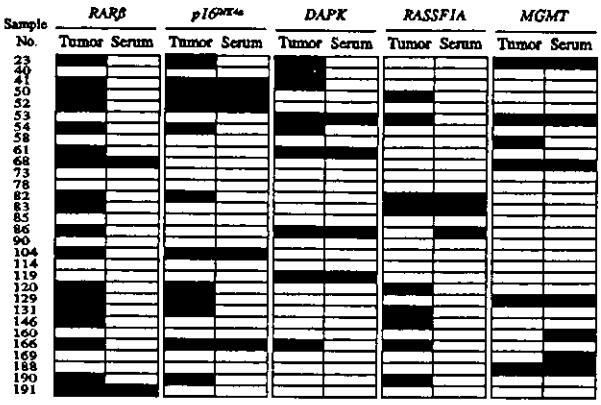


*Fig. 2* Summary of methylation of *RARβ*, $p16^{INK4a}$, *DAPK*, *RASSF1A*, and *MGMT* in 30 corresponding tissue and serum samples. *Black boxes*, methylated samples; *white boxes*, unmethylated samples.

*Table 3* Correlation between clinical features and methylation of the different genes in nonmalignant cases

| | % of Cases | | | | | |
|---|---|---|---|---|---|---|
| | *RARβ* | *p16$^{INK4a}$* | *DAPK* | *RASSF1A* | *MGMT* | Total |
| Smoking status | | | | | | |
| Nonsmoker | 0 | 4.4 | 2.2 | 6.5 | 2.2 | 13.0 |
| Pack-years <40 | 0 | 0 | 4.8 | 4.8 | 0 | 9.5 |
| Pack-years ≥40 | 3 | 3 | 9.1 | 12.1 | 3.0 | 21.2 |
| *P* | 0.359 | 0.626 | 0.379 | 0.550 | 0.736 | 0.442 |
| Age | | | | | | |
| Quartile 1* | 3.5 | 0 | 6.9 | 0 | 0 | 10.3 |
| Quartile 2 | 0 | 0 | 0 | 5.6 | 0 | 5.6 |
| Quartile 3 | 0 | 5.0 | 5.0 | 20.0 | 5.0 | 25.0 |
| Quartile 4 | 0 | 13.3 | 13.3 | 13.3 | 6.7 | 33.3 |
| *P* | 0.480 | 0.051 | 0.230 | 0.063 | 0.271 | 0.036 |

*Age quartile was defined as follows: 1, <59; 2, ≥59 and <69; 3, ≥69 and <73; and 4, ≥73.

published series, controversial correlations between smoking and methylation have been reported (20, 41–44). We did not find any statistically significant correlation between smoking history and methylation; however, heavy smokers had at least one serum DNA methylation more frequently than mild to moderate smokers in patients with nonmalignant disease. The third explanation is possible occurrence of other occult malignancies. Indeed, we observed methylated serum DNA in 66.7% of the patients who were diagnosed as having another malignant disease. The final explanation is the possibility of detecting age-related methylation in control group.

To use this serum DNA methylation as a marker in lung cancer mass screening, several issues must be considered. The fairy good specificity even in patients screened by chest radiograph suggests the advantage of this approach. On the other hand, poor sensitivity may compromise the advantage of specificity. Improving sensitivity as a mass screening test might be achieved by two approaches. One is to increase sensitivity of DNA methylation itself by using large number of tested genes or applying a quantitative methylation assay. The other is to combine the methylation with highly sensitive screening method such as low-dose spiral computed tomography (45–47). Because one of the serious limitations of low-dose spiral computed tomography is its poor specificity (48), combination with the serum DNA methylation may overcome the limitation. Indeed, physicians often experience difficulty in sampling tumor specimen from small legions detected by computed tomography scans for pathologic diagnosis by invasive procedures, such as fiberoptic bronchoscopic examination or computed tomography–guided fine needle aspiration. Accordingly, in consideration of relatively higher frequency of serum DNA methylation in early-stage disease (50.9% in stage I) and serious complications after invasive procedures (49), serum DNA methylation may be a test to be conducted before invasive procedure. Although further evaluation is essential, the results in this study indicate the substantial usefulness for detection of lung cancer.

In conclusion, we examined the aberrant promoter methylation status in serum DNA and showed the usefulness of this approach as a tool for detection of lung cancer in patients screened by chest radiograph. Further studies are warranted to confirm the efficiency of the procedure and search for best combination of genes for methylation analysis. Moreover, it is important to investigate prospectively whether methylation-positive noncancer cases will have malignancies in the near future.

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*Table 4* Methylation status and risk of lung cancer

| | | | Model 1† | | | Model 2† | | |
|---|---|---|---|---|---|---|---|---|
| | Cases* | Controls* | Odds ratio | 95% Confidence interval | *P* | Odds ratio | 95% Confidence interval | *P* |
| No. methylations | | | | | | | | |
| 0 | 46 | 85 | 1.00 | | | 1.00 | | |
| 1 | 33 | 12 | 5.08 | 2.28-11.3 | <0.0001 | 5.28 | 2.39-11.7 | <0.001 |
| ≥2 | 12 | 3 | 7.39 | 1.87-29.2 | 0.0008 | 5.89 | 1.53-22.7 | 0.010 |
| Age (10-y increase) | | | — | | | 1.03 | 1.00-1.07 | 0.023 |
| Sex (male relative to female) | | | — | | | 1.49 | 0.61-3.64 | 0.386 |
| Pack-years (one level increase quartile) | | | — | | | 1.00 | 0.99-1.00 | 0.207 |
| Tumor marker positive | | | — | | | 3.08 | 1.32-7.17 | 0.009 |

*Cases, lung cancers; controls, nonmalignant diseases.

†Model 1 included number of methylation only. Model 2 included age, sex, pack-years of smoking, and tumor marker result in addition to number of methylation.

*Table 5* Frequency of DNA methylation and elevation of conventional serum protein tumor markers according to clinical stage

| | Patients, *n* (%) | | | |
|---|---|---|---|---|
| Clinical stage | I | II | III | IV |
| Methylation | 27 (50.9) | 3 (42.9) | 10 (45.5) | 5 (55.6) |
| *MGMT* | 10 (18.9) | 1 (14.3) | 4 (18.2) | 2 (22.2) |
| *p16$^{INK4a}$* | 7 (13.2) | 0 (0.0) | 5 (22.7) | 2 (22.2) |
| *RASSF1A* | 7 (13.2) | 1 (14.3) | 3 (13.6) | 0 (0.0) |
| *DAPK* | 5 (5.7) | 1 (14.3) | 4 (18.2) | 0 (0.0) |
| *RARβ* | 3 (5.7) | 0 (0.0) | 1 (4.5) | 2 (22.2) |
| Tumor marker | 6 (11.3) | 3 (42.9) | 10 (45.5) | 7 (77.8) |
| Carcinoembryonic antigen | 5 (9.4) | 1 (14.3) | 9 (40.9) | 5 (55.6) |
| Cytokeratin 19 fragment | 0 (0.0) | 0 (0.0) | 2 (9.1) | 2 (22.2) |
| Progastrin releasing peptide | 1 (1.9) | 2 (28.6) | 2 (9.1) | 1 (11.1) |
| Total ($n$ = 91) | 53 | 7 | 22 | 9 |

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# 腹水中 ADA 高値が診断に寄与した若年女性結核性腹膜炎の 1 例

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# 腹水中ADA高値が診断に寄与した若年女性結核性腹膜炎の1例

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（平成16年8月9日受理）

Key words：　*Mycobacterium tuberculosis*, peritonitis, ADA (adenosine deaminase)

## 序　文

結核性腹膜炎は，原因不明の腹水として発症することがあり，腹水の性状分析で結核菌の存在を証明できない場合，確定診断のため腹腔鏡あるいは開腹による生検がなされるのが一般的である．しかし，我々が過去5年間の結核性腹膜炎の報告症例[1)~32)]を検討したところ，病理学的確定診断を得た症例は少数であった(Table 1)．今回我々は，腹水ADA高値が診断に寄与した若年女性結核性腹膜炎を経験したので報告する．

## 症　例

患者：26歳　女性．化粧品販売員．

主訴：腹水貯留．

家族歴：母，母方祖父母，胃癌．父，肺結核．

現病歴：2003年7月ごろより生理不順であった．8月6日前医定期受診時に痛みを伴わない軽度の腹部膨隆を指摘されたが，本人自覚なくそのまま放置していた．その後次第に膨隆は増大し，軽度の発熱も伴ってきたため8月22日前医再受診したところ，腹部超音波にて大量の腹水を認めた．その後38.5℃の発熱も認め，8月29日腹水穿刺施行後，9月2日前医入院となった．腹水中ADA 73.1IU/*l*であったことから結核性腹膜炎も疑ったが，入院後抗菌薬の投与を行いつつ内科，婦人科にて全身精査を行った．診断に至らなかったため抗結核薬の投与を勧めたが，父親が抗結核薬投与中に腎障害を合併していたため拒否．9月9日診断的開腹術施行した．開腹では結核性腹膜炎を疑う所見であったが，病理所見からは乾酪性肉芽腫及び抗酸菌は証明できず，また，腹水4週培養陰性，PCR法にて結核菌遺伝子陰性，確定診断に至らなかったため，当院紹介入院となった．

入院時現症：身長156.6cm，体重48.7kg，腹囲80.5cm，BMI 20.0，脈拍72/min，血圧118/70 mmHg，体温35.7℃，顔面は蒼白，胸部は異常なし，腹部は臍部中心に膨隆あり，緊満感あり．波動を認める．腹部全体に軽度の圧痛あり．下腹部正中約5cmの手術痕あり．

入院時検査所見(Table 2)：血液一般，生化学，血清学的に非特異的な軽度の炎症所見を認め，CA-125 536.2IU/*l*と腫瘍マーカー高値であった．

胸部単純X線所見：異常なし．

腹部超音波所見：腹水貯留（最深部左側腹部8.5 cm）を認めた．

胸腹骨盤CT（Fig. 1）：多量の腹水と卵巣腫大を認めた．

腹水所見（Table 2）：浸出液であり，ADA 59.8 IU/*l*，CA-125 639.7U/mlと高値であった．

臨床経過（Fig. 2）：前医で抗菌薬の投与を行った結果，一時的ではあるが解熱，腹囲の縮小，CRPの低下を認めていること，抗菌薬中止より腹囲の増大を認めていることから，細菌性の腹膜炎も考えLVFX 400mg/日の内服を開始することとし

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Table 1　Analysis of tuberculous peritonitis reported in Japan during 5 years

| | ADA (IU/l)* | CA125 (U/ml)* | Acid-fast stain* | Culture* | PCR (tuberculosis DNA)* | Acid-fast stain** | Reference |
|---|---|---|---|---|---|---|---|
| 1 | | 1,435.7 | positive | | | | 1) |
| 2 | 31.4 | | negative | | negative | | 2) |
| 3 | 76.6 | | negative | | negative | | 2) |
| 4 | 73.1 | 119 | negative | negative | negative | | 3) |
| 5 | 112.3 | | positive | negative | | | 4) |
| 6 | 71.8 | 357.6 | negative | | positive | | 4) |
| 7 | | 670.9 | negative | negative | negative | | 5) |
| 8 | | 91 | negative | positive | | | 6) |
| 9 | 76.6 | | negative | negative | negative | | 6) |
| 10 | 119.8 | 1,300 | | positive | positive | | 7) |
| 11 | 99.5 | | | negative | negative | positive | 8) |
| 12 | | | negative | negative | | positive (culture) | 9) |
| 13 | 66.9 | | negative | negative | negative | | 10) |
| 14 | 62.2 | | | | | | 11) |
| 15 | 28 (blood) | | | | | | 12) |
| 16 | 59.7 | 858 | negative | negative | | positive | 13) |
| 17 | | | positive | | | | 14) |
| 18 | 50.6 | 531 | negative | negative | negative | negative | 15) |
| 19 | 82.4 | 1,400 | negative | negative | negative | negative | 16) |
| 20 | 184 | 305 | negative | negative | negative | | 17) |
| 21 | 125.1 | 1,464 | negative | | negative | positive | 18) |
| 22 | high value | | negative | negative | negative | | 19) |
| 23 | | 25 | | negative | | | 20) |
| 24 | | 30 | | | | negative | 20) |
| 25 | | 957 | | | | positive | 20) |
| 26 | 48.7 | | | negative | | | 21) |
| 27 | 92.5 | | | negative | | | 21) |
| 28 | 138.8 | | | negative | | | 21) |
| 29 | | 2.5 | | negative | negative | | 22) |
| 30 | 78 | 452 | negative | | negative | negative | 23) |
| 31 | | 449 | | negative | | | 24) |
| 32 | | | | negative | | positive (culture) | 25) |
| 33 | high value | 409 | | | negative | | 26) |
| 34 | | | negative | negative | positive | | 26) |
| 35 | | 406.4 | | | | negative | 27) |
| 36 | | | negative | positive | | | 28) |
| 37 | 34.8 | 110 | | negative | negative | | 29) |
| 38 | | 647 | | | | | 30) |
| 39 | 107 | | | positive | positive | | 31) |
| 40 | | 810 | | | | | 32) |

*…Specimen derived from Ascites
**…Specimen derived from Peritoneal Biopsy

た．卵巣の腫大を認め，卵巣の炎症および腫瘍性疾患を否定できないため，婦人科紹介したが，悪性所見を認めなかった．更に，腹水穿刺を施行するとともに，当院にて近医での病理標本の再精査を行った．その結果，Ziel-Neelsen 染色にて僅かであるが，多核巨細胞の胞体内に抗酸菌を認めた(Fig. 1)．そこで抗結核薬 RFP 450mg/日＋INH 200mg/日＋PZA 1,200mg/日＋SM 750mg/日の 4 剤併用療法にて加療開始した．途中聴力障害が出現したため SM を EB 750mg/日に変更した．腹囲は著明に縮小し，RFP＋INH＋EB にて継続加療中である．なお，前医での組織の抗酸菌培養は，最

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Table 2 Laboratory findings on admisson

| Blood chemistry | Analysis of Ascites |
|---|---|
| RBC 402万/μl, Hb 10.7g/dl, Hct 33.5%, MCV 83.3, Plt 33.7 × $10^4$/μl, WBC 4,500/μl (Neu 76.2%, Lym 13.4%, Mon 8.5% Eos 1.7% Bas 0.2%) | Appearance: Yellowish-brown, slightly corrupted |
| TP 8.03g/dl, Alb 4.22g/dl, T.bil 0.35mg/dl, D.bil 0.14mg/dl, AST33IU/l, ALT 16IU/l, ALP 174IU/l, γ-GTP 22IU/l, CHE 175IU/l, LDH 368IU/l, | TP 6.46g/dl, LDH 428IU/l, LDH isozyme LD1 6.5% LD2 14.6% LD3 23.5% LD4 27.9% LD5 27.5%, T.Cho 72mg/dl, Sugar 66mg/dl, ADA 59.8IU/l, IFN-γ 15.6IU/ml, |
| Na 141mEq/l, K 4.6mEq/l, Cl 100mEq/l, Ca 9.7mg/dl, BUN 11.5mg/dl, Cr 0.53mg/dl, UA4.8mg/dl, T.cho 100mg/dl, HDL-C 57mg/dl, LDL-C 30mg/dl, CRP 1.4mg/dl, ESR 21mm/hr, 53mm/2hrs | CEA 0.87ng/ml, CA-125 639.7U/ml, Cytology class II, Lymphocyte dominant |
| FBS 72mg/dl, HbA1c 4.7%, Free T3 2.76pg/dl, Free T 41.44ng/dl, TSH 1.79μU/ml, PT 13.7sec (88%INR1.09), APTT 36.3sec Fibg 299mg/dl, D-dimer 2.3μg/ml, CEA 1.27ng/ml CA19-9 5.5U/ml CA-125 536.2U/ml, RF 0.1IU/ml, ANA (−), $C_3$ 131.0mg/dl, $C_4$ 18.7mg/dl, $CH_{50}$ 56U/ml | specific PCR (*M. tuberculosis* or *M. avium* Complex DNA): (−) (specimen: Blood, Urine, Stool, Ascites) |

Bacterial body could not be find out by any staining method including Ziel-Neelsen stain in Ascites, Blood, Urine and Stool.

Fig. 1 I. II. CT scan of Abdomen and Pelvis revealed massive ascites and swollen ovary.
III. Pathologist confirmed the existence of bacterial bodies stained by acid-fast stain in giant cells. (Ziel-Neelsen stain, → : bacterial body)

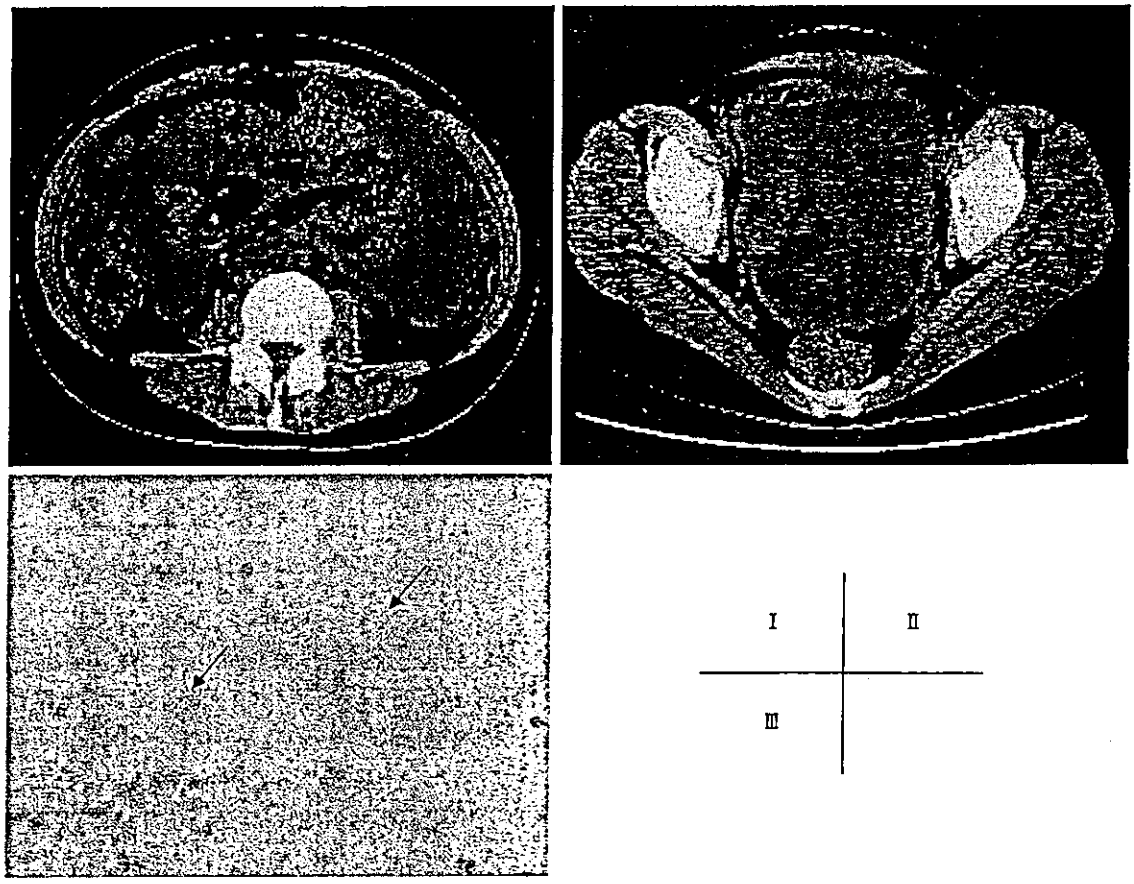
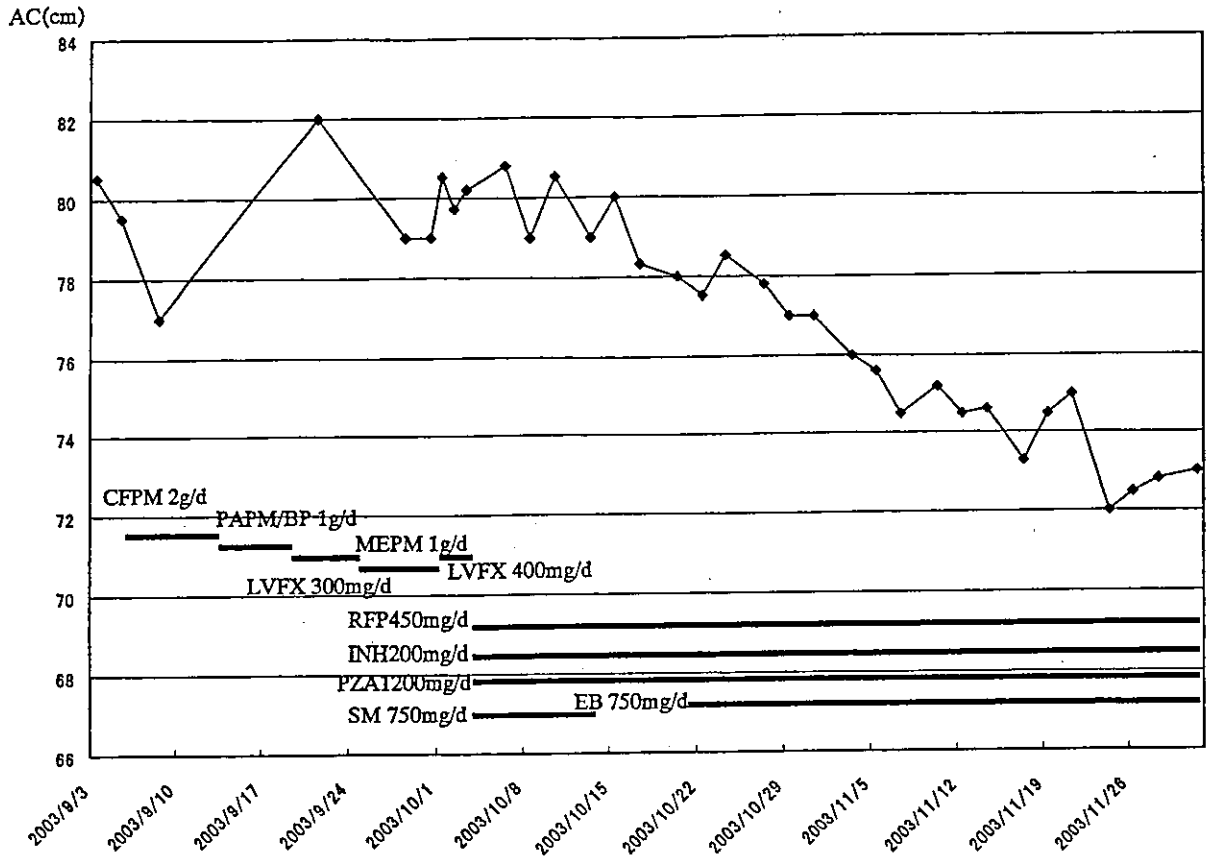


Fig. 2　Clinical course（AC：abdominal Circumference）




終的に 8 週で *Mycobacterium tuberculosis* 陽性となった．

## 考　察

結核性腹膜炎は極めて稀で，全結核に対する結核性腹膜炎は 0.55% 程度とされている[33]．好発年齢は 20～40 歳で，男女比は 1：2 である[34]．自他覚症状ともに多彩で，確定診断にいたる決定的要素に欠けることが多く，診断的治療を余儀なくされていることもしばしばである．本邦にて過去 5 年間に報告された症例を検討したところ，腹水抗酸菌染色にて陽性になったものは 21 例中 3 例，腹水培養にて結核菌陽性となったものは 25 例中 4 例，また PCR 法で陽性となったものは 20 例中 4 例，さらに開腹生検にて病理学的に菌体を検索しえたものは 9 例中 4 例であった(Table 1)．本症例では前医にて胸部から骨盤に至るまで CT を施行され，骨盤 MRI，上部，下部消化管内視鏡検査を施行されている．臨床的には結核性腹膜炎を疑ったが，患者の父親が抗結核薬による急性腎障害を発症していることから，診断的治療を拒否され，その結果，開腹し生検を行った．しかしながら，各種染色法，PCR 法を用いても確定診断に至らなかった．我々は，近年腹水中 ADA 値において，33U/*l* をカットオフ値とした場合，その感度は 100%，特異度は 96.6% であると報告されている[35]ことに注目し，本症例では ADA が高値であったため検体の再検を行った．このことにより，僅かな菌体の発見に至り，当院受診後に速やかな抗結核薬投与が開始可能となった．菌の検出による確定診断を得ることは臨床上の大原則ではあるが，腹水 ADA 高値は結核性腹膜炎の鑑別診断において有用と考えられる．一方 CA125 は，成人に微量

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にCA125の存在が証明されている卵管，子宮内膜，子宮頸管の上皮，腹膜に炎症が生じた場合高値になるものと考えられ[36]，過去5年間の報告における結核性腹膜炎での血中CA-125は，22例中21例で高値，腹水中CA-125値は，10例中9例で高値であった(Table 1)．本症例での血中CA-125は，8月22日：924.8U/ml，9月30日：536.2U/ml，10月1日：639.7U/ml，10月3日：340U/ml，11月5日196U/mlと加療に伴い減少しており，CA-125が経過観察としては有用であることが示唆された．

腹水貯留症例の原因検索については，卵巣癌をはじめとした骨盤腔内の悪性腫瘍，肝硬変をはじめとした肝疾患，結核をはじめとした感染症など，さまざまな鑑別診断があげられている．また，ADAは，アデノシンを加水分解し，イノシンとアンモニアを生成する酵素であり，その酵素活性はT細胞が活性化され分化を誘導されている際に高くなるとされている[37]．結核性腹膜炎では結核菌を抗原として活性化されたT細胞が遊走し，腹水中では有意にADA値が高値となると考えられ，その測定は，前述の如く感度特異度ともに高く，検査法として大変優れていると思われる．さらに，本症例のような若年の女性では，骨盤腔内の炎症を長引かせることは，そのまま不妊の原因にもなりうる[38]．したがって，結核性腹膜炎のより迅速な加療を開始するためには，腹水中ADA値が高値の場合，患者にADA値の感度，特異度および開腹での確定診断率を説明したうえで，診断的治療を一つの選択肢として挙げることも有用であると考えられる．

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