Renal (Perfusion) 腎・循環系
Andrew D. Baines (Univ. of Toronto)
急性腎傷害では，creatinine値は余り信頼性が無い．重要な初期マーカーとして，尿量，N-acetyl-beta-D-glucosaminidase (NAG), Urinary neutrophil gelatinase-associated lipocalin (NAGL), IL-18, Kidney injury molecule (KIM-1) などがある．

Gastrointestinal 消化器系
Mitchell P. Fink (Logical Therapeutics)
急性膵臓炎 (Lipase, amylase上昇)，肝細胞傷害 (AST, ALT上昇)，食道蠕動への影響

Cardiac 心臓系
David C. Warltier (Medical College of Wisconsin)
リコンビナントヘモグロビン: rHb1.1の投与により，HR低下，MAP上昇，LVEDP上昇．HBOCsの投与による心筋損傷は，Burhopらが初めて発表した．CPK上昇，壊死など．

Central Nervous System 中枢神経系
Raymond F. Regan (Thomas Jefferson Univ.)
神経系細胞培養系にHBOCsを添加した実験では，毒性は無いことを確認している．しかしMP-4 (Sangart社製) の添加ではLDHの上昇が認められた．metHb生成が速いためか，臨床試験ではHBOCsの脳神経系への影響は良くわかっていない．

Shock Mechanism ショックの機序
Joseph E. Parrillo (Univ. of Medicine and Dentistry of New Jersey)
ショックの分類，病因，敗血症の際の心筋異常について概説．最近出版されたNEJM誌を参照[6]．

Pulmonary 肺系
Mark Gladwin (Natuional Heart, Lung and Blood Institute)
肺に関連するHBOCsの副作用として，肺高血圧症，心拍停止，肺炎，呼吸停止，ARDS (急性呼吸促迫［窮迫］症候群)，MOF，血栓症などがある．

Session IV Finding a Way Forward: What is the Best Way Forward Scientifically and Ethically?
Moderator: George P. Brio (Univ. of Ottawa and Univ. of Toronto)

Biochemical Approaches and Mitigation Strategies for HBOCs

The Way Forward: Can Nitrite Modulate HBOC Toxicity?
Mark Gladwin (National Heart, Lung and Blood Institute)
亜硝酸イオン (NO_2^-) がdeoxyHbと反応して，NOを産生する反応の機序と，生理学的な役割，更には投与の効果について概説[7]．また，HBOCs投与後の血圧亢進を，NO吸入によって低減させる事に成功したZapolらの論文を紹介[8]．同様にNO_2^-を投与してやれば，HBOCsと反応してNOを産生し，血管収縮作用が低減出来るのではないか．

Strategies for Engineering Safer, More Efficient and More Stable Recombinant Hemoglobins for Use as O_2 Delivery Pharmaceuticals
John S. Olson (Rice University)
遺伝子組換えHbの分子設計において，P_{50}値の制御による酸素輸送量の調節，NO結合速度の制御による血管収縮の抑制が可能となっている (Somatogen-Baxter社の例)．また，自動酸化の低減，ヘム遊離の低減，などが検討されている．大量製造も可能ではあるが，実際には，費用の問題，E. coliからの産生であるので，LPSの除去を効率よく行うことが課題である．また，分子量を大きくするための工夫 (化学修飾，カプセル化) も検討する必要がある．

Role of Microvascular Reactions in the Design of Hb Based Oxygen Carrying Plasma Expanders
Marcos Intaglietta (La Jolla Bioengineering Institute, University of California, San Diego)
各種HBOCsを投与した後の微小循環系の観察から明らかになったことは，血漿中のHb濃度は，血管収縮を誘発しうる重要因子である．血漿層の粘度調節は，血管収縮を回避するために重要．各種分子状HBOCsは，それぞれ異なった血管活性を示す．HBOCsによるNO運搬 (Stamlerらの説) は不明．全てのHBOCsはNOを捕捉する．血漿層の粘度を増大することでNO産生を増加できる．日本のHb小胞体のほか，PEG-Hb, Polymerized Hbなど，様々なHBOCsを検討して明らかになったことは，分子設計にあたって，血漿増量のため膠質浸透圧 (50 mmHg) は充分である．P_{50}値は12-16 mmHgにすることで，抵抗血管よりも下流の血管での酸素放出を可能とする．全血中のHb濃度は，Transfusion Triggerである7 g/dL以上に維持しつつ，血漿中のHb濃度は2 g/dL程度が良い．血液粘度は2-4 cP程度を目安とし，平滑筋に対するmechanotransduction効果を維持する．

Endogenous Hb Scavengers and HBOC Toxicity
Dominik J. Schaer (University of Zurich, Switzerland)
血中の遊離Hbの排泄には，ハプトグロビン (Hp) およびレセプターCD163が関与している．これらがどのようにHbの毒性を低減しているか．Hp, CD163とHBOCsの相互作用の強さは，その構造に依存し，修飾度が高いほど相互作用は低減する．マクロファージCD-163と相互作用をすると，HO-1が誘導され，酸化的傷害を低減する．HpはHbの血圧亢進の効果を低減させる．

Utility of Animal Models in HBOC Evaluation
Joy Cavagnaro (AccessBio)
前臨床試験について，動物実験では臨床の結果を100％予見することはできないし，たとえ臨床試験を行ってもその後，予想しないことも起こりうる．病態の動物モデルを用いた実験，種差の検討が必要である．毒性の許容範囲を明らかにしてそれを見るための動物実験モデルを作製することが重要．

Alternative Focused Clinical Designs
Jeffrey L. Carson
(University of Medicine and Dentistry of New Jersey)
臨床試験のエンドポイントは，臨床的な結果とすべきであり，「同種血輸血の低減」とするべきではない．

Panel Discussion
Jerry L. Carson (University of Medicine and Dentistry of New Jersey)
Joy Cavagnaro (AccessBio)
Ezekinel Emanuel (National Institute of Health)
臨床試験の倫理的問題について概説したJAMA誌の論文を配布[9]．
Thomas R. Flemng (University of Washington)
Marcos Intaglietta (La Jolla Bioengineering Institute, University of California, San Diego)
John S. Olson (Rice University)
Dominik J. Schaer (University of Zurich, Switzerland)
Gus J. Vlahakas (Harvard Medical School)
David C. Warltier (Medical College of Wisconsin)

Closing Remarks
Jay S. Epstein (Office of Blood Research Review)
二日間の討論の中で，全ての問題点に対して十分な答えを得る事は出来なかったかもしれないが，多くの情報を得ることが出来た．また，大まかな意見の一致が見られたのではないか．それは，重要なUnmet medical needsがあること，HBOCsの研究開発を先に進めるべきこと，そして，新しい戦略が引続き必要であること．組織委員会および，Dr. J. Goldsmithに謝意を表して，本会を閉会する．

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ORIGINAL ARTICLE

Syndrome of inappropriate secretion of antidiuretic hormone after chemotherapy with vinorelbine

Hiroaki Kuroda · Masafumi Kawamura · Tai Hato · Kazunori Kamiya · Masahiro Kawakubo · Yotaro Izumi · Masazumi Watanabe · Hirohisa Horinouchi · Koichi Kobayashi

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Abstract
Purpose To describe a case of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) after administration of vinorelbine (VNB) for recurrence of lung cancer.
Case A 76-year-old man underwent bronchial arterial infusion (BAI) of VNB for postoperative recurrence of lung cancer. Seven days later, hyponatremia and natriuresis developed. Based on his clinical and laboratory findings, we diagnosed him with SIADH. He improved within a couple of days with fluid restriction only.
Conclusions Administration of VNB may potentially cause SIADH. This is the second report of the SIADH caused by VNB. It is important to monitor the serum sodium level and clinical findings after chemotherapy with VNB.

Keywords Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) · Vinorelbine (VNB) · Non-small cell lung cancer · Chemotherapy

Introduction

We present a case of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) in a 76-year-old man after administration of vinorelbine (VNB). He underwent bronchial arterial infusion (BAI) of VNB for postoperative recurrence of lung cancer. Seven days later, hyponatremia and natriuresis developed.

Based on his clinical and laboratory findings, we diagnosed him with SIADH. He improved within a couple of days with fluid restriction only. This is the second report of the SIADH caused by VNB therapy for lung carcinoma.

Case report

A 76-year-old man underwent left upper lobectomy for lung adenocarcinoma (pathological staging T3N1M0, stage IIIA) and 14 months later, chest computed tomography (CT) showed bilateral pulmonary metastases. Five courses of chemotherapy with docetaxel (DOC) 100 mg and gemcitabine (GEM) 1,400 mg resulted in stable disease (SD) and he was begun on modified chemotherapy with vinorelbine (VNB 40 mg). After three courses of VNB, he was admitted to hospital, complaining of left chest pain and cough.

Chest X-ray and CT on admission showed the tumor occluding in the left main bronchus and complete atelectasis of the remaining left lower lobe (Fig. 1a, b).

Transbronchial interventions, such as tumor resection, injection of ethanol and YAG laser ablation, were performed repeatedly, after which the chest X-ray showed gradual restoration in his remaining left lobe. Irradiation (total 50 Gy) of the recurrent tumor was performed for 5 weeks and then chemotherapy with VNB 40 mg (BAI 20 mg + intravenous 20 mg) was repeated.

Seven days after administration of 40 mg VNB, he complained of anorexia, nausea and lethargy. His consciousness was clear. His physical and neurogenic examinations were almost intact. His plasma sodium concentration was 113.1 mEq/l, serum osmolality was 242 mOsm/kg lower

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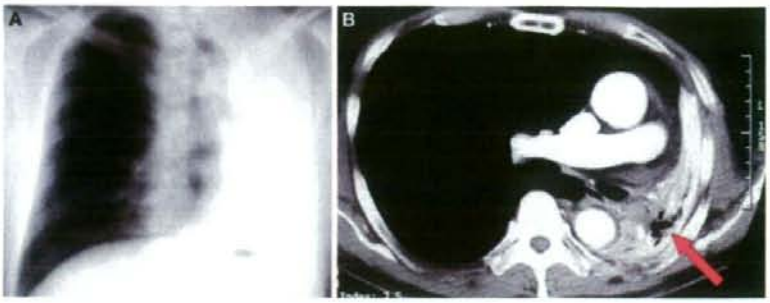

Fig. 1 **a** Chest X-ray on admission shows complete atelectasis of remaining left lobe. **b** Chest computed tomography scan shows the tumor occluding the left main bronchus (*arrow*)

than normal limit of 270 mOsm/kg and urine osmolality was 309 mOsm/kg higher than normal limit of 300 mOsm/kg. Urine sodium value was 20.2 mEq/l higher than normal limit of 20 mEq/l. His cortisol concentration value was normal. The plasma arginine vasopressin (AVP) concentration was 0.59 pg/ml, which was within normal limits, as were other parameters. Clinically, there were no symptoms related to adrenal or anterior pituitary dysfunction. The patient was euvolemic and renal function tests were within normal limits. VNB is considered to be strongly associated with SIADH, so he was diagnosed as having SIADH because his clinical features and laboratory data satisfied all standard criteria.

He was treated with water restriction, oral intake plus drip infusion into vein (DIV, total 750 ml/day). Within 2 days, his serum sodium concentration rose gradually and was restored to 130.3 mEq/l (Fig. 2). His mentation and appetite recovered in accordance with the increasing serum sodium concentration without central pontine myelinolysis. There was a possibility of developing SIADH again with a fifth cycle of VNB, so the chemotherapy agent was changed. He has been free of SIADH and has lived with lung cancer for 1 year.

Discussion

Schwartz et al. first reported SIADH in patients with lung cancer in 1957 [1]. Standard criteria include (1) hyponatremia, (2) plasma hypo-osmolality and urine hyperosmolality, (3) continuous secretion of sodium in urine, (4) normal renal function without hydration, (5) no adrenal gland dysfunction and (6) hyponatremia and hyposmotic pressure that recover with water restriction therapy without change in blood pressure [2]. The present patient fulfilled these criteria.

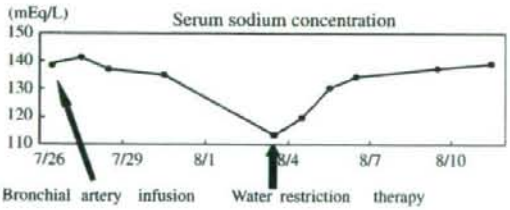

Fig. 2 Clinical course of serum sodium concentration 7 days after administration of vinorelbine. The patient complained of anorexia, nausea and lethargy and the sodium concentration was 113.1 mEq/l. He was treated with oral water restriction only and 2 days later, the serum sodium concentration was restored to 130.3 mEq/l

Various causes of SIADH have been reported, such as disorders of the cerebral nervous system, malignant tumors, diseases of the thoracic cavity, medicinal side-effects and idiopathic [3]. Approximately 75% of tumor-associated cases of SIADH are related to small-cell lung cancer (SCLC) [4]. The occurrence of SIADH with non-small-cell carcinoma (NSCLC) has been only rarely reported [5]. It has been reported that SIADH is caused by the tumor invading the vagus nerve or releasing ADH-like product. In the present study, imaging showed no evidence of invasion of the vagus nerve and we identified that the serum level of AVP was normal. We do not consider that its upregulation or secretion of ADH-like material occurred because the patient never experienced other electrolytic abnormalities and his serum sodium concentration was restored rapidly by water restriction therapy alone. There was no evidence of a relationship between progression of lung carcinoma and the onset of SIADH in this case.

Another possibility is that when the extension receptors of the left atrium detect hyperthoracic pressure and abnormal hemodynamics, they decrease the suppressor signal level and induce continuous release of ADH from the posterior lobe of the pituitary. But in the present case, bronchoscopic interventions were performed to target the local recurrence and restore his remaining left lobe. Syndrome of inappropriate secretion of antidiuretic hormone developed after 5 weeks of irradiation therapy following these interventional procedures. Even if there was a change in the respiratory circulation with release of the atelectasis, it is unlikely because of the time delay.

Enhancing release of AVP, potentiating the renal action of AVP and unknown mechanisms are reported as the main causes of SIADH by drugs [3]. Syndrome of inappropriate secretion of antidiuretic hormone associated with vinca

alkaloids, especially vincristine (VCR) and vinblastine (VBL), has been reported [6–8]. Garrett and Simpson first reported that SIADH occurred after a single treatment of VNB for breast cancer [9]. In addition, they reported that there was a slight structural difference between VNB and other vinca alkaloids, however, the precise mechanism is unclear and they may possess common neural or renal adverse effect profiles. In the present case, we firmly concluded that SIADH was induced by chemotherapy with VNB because concomitant medication was steroids only. In addition, SIADH occurred after four courses and not with earlier exposures of VNB. Although we think that repeated administration or retention of VNB may have been associated with SIADH, the precise mechanism of this SIADH was unclear.

In the report by Garrett and Simpson, SIADH from VNB did not respond to fluid restriction and patient had to be give 3% NS. But in our case, patient recovered with fluid restriction only. This difference with these two mechanisms was unclear. Furthermore, they also reported that prophylactic use of demeclocycline, which interacts with ADH at the renal collecting duct, might usefully prevent recurrence of SIADH associated with continuous treatment with VNB [9]. Stuart and Cuaso reported that SIADH was prevented by rigorous water restriction [10]. For our patients, we choose an alternative because of the high possibility of SIADH caused by VNB. If chemotherapy with VNB results in complete response or partial response, we would choose to readminister VNB with restriction of water, or use demeclocycline and monitor the sodium concentration.

We consider that VNB should be regarded as very likely to cause SIADH, but as this is only the second report of SIADH associated with use of VNB alone, it is a rare occurrence.

Conclusion

It is known that lung cancer can give rise to SIADH as a paraneoplastic syndrome and there are some anticancer agents that can potentially cause SIADH. It is important to monitor the serum sodium level and clinical findings after chemotherapy for lung cancer.

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Surgical outcomes for pulmonary metastases from hepatocellular carcinoma

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Abstract

Background: Although favourable prognosis following aggressive treatment of extrahepatic metastases from hepatocellular carcinoma (HCC) has been reported, surgical outcomes for pulmonary metastases are unclear. **Methods and materials:** Sixty-one patients (2.6%) of 2297 registered with the Metastatic Lung Tumor Study Group of Japan between 1990 and 2006, who underwent surgery for pulmonary metastases from HCC, were retrospectively reviewed from the registry. **Results:** The overall 5-year survival rate was 32.2%. The prognosis was significantly better for ≤ 2 lesions than for ≥ 3 lesions ($p = 0.046$), for ≤ 3 lesions than for ≥ 4 lesions ($p = 0.0070$), and for ≤ 4 lesions than for ≥ 5 lesions ($p = 0.029$). No other factors that influence outcomes were identified. A stepwise regression analysis showed three or less pulmonary metastases to be an independent factor for better prognosis ($p = 0.048$). **Conclusion:** With careful patient selection, comparatively good outcomes can be expected following surgical resection of pulmonary HCC metastases. Among them, patients with multiple metastases, if number of metastases is small such as four or less, can be expected to survive long after surgery.

Keywords: Hepatocellular carcinoma; Pulmonary metastasis; Extrahepatic metastasis; Surgery; Metastasectomy

1. Introduction

Advances in localised treatments for hepatocellular carcinoma (HCC) have seen better local control achieved in recent years. The prognosis remains poor for extrahepatic recurrence however, although some studies have reported improved outcomes following aggressive treatment of extrahepatic metastases [1]. Pulmonary metastases account for over 50% of extrahepatic HCC metastases [2,3]. The primary lesion in more than 80% of HCC with extrahepatic HCC metastases is advanced, stage III or IVa, so there are few opportunities for aggressive surgical treatment of extrahepatic metastases [3]. Surgical resection is contraindicated in most cases of pulmonary HCC metastases due to the number of lesions. There have accordingly been few studies of the results of surgical treatment of pulmonary metastases. In this study we analysed the results of surgical procedures on pulmonary HCC metastases in 61 patients to determine prognostic factors.

2. Methods and materials

From 2297 patients registered with the Metastatic Lung Tumor Study Group of Japan from January in 1990 to May in 2006 who underwent resection of metastatic lung tumours, the subjects of this study were the 61 patients (2.6%) who underwent surgery for pulmonary HCC metastases. Information regarding subject gender, date of birth, date and time of hepatic surgery, date of detection of pulmonary metastases, number of lesions on right and left, maximum tumour diameter, date and time of resection of pulmonary metastases, and the type of procedure performed, were recorded in the registration form. Detection of pulmonary metastases was performed by CT scan. Multi-detector CT scan

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has become available since late 1990s. Video-assisted thoracic surgery (VATS) has been undergone since 1996. Choice of surgical procedure was left to each surgeon. Although the patients were required to have sufficient liver function for pulmonary resection, information of their liver condition was not mandatory for registration because of chest surgeons' registration. Patients were followed-up basically with chest CT scan twice a year for the recurrence of pulmonary metastases. Outcome surveys were subsequently conducted at 1-year intervals. In general, we followed the indications for surgical resection of pulmonary HCC metastases proposed by Thomfold et al. [4], that the primary lesion is under control or is planned to be under control, there are no metastases to other organs, and the patient's general condition is good enough to withstand surgery [4]; we also included patients with bilateral disease. We counted two stage procedures for bilateral pulmonary metastases as a single procedure, and we defined re-do surgery for recurrent pulmonary metastases as surgery for pulmonary metastases newly detected after the initial procedure. The disease-free interval (DFI) was defined as the time between the day of hepatic surgery and the day of detection of pulmonary metastases, giving a DFI of 0 if pulmonary metastases were discovered prior to or at the time of the initial hepatic surgery. Cumulative survival rates were calculated using the Kaplan–Meier method, and comparisons among the survival curves were made using the log-rank test. Multivariate analysis for prognostic factors was assessed using a stepwise regression. A p value <0.05 were considered as a statistically significant difference. Statistical analysis was performed using the SPSS Base 11.0J software package (SPSS Inc., IL, USA).

3. Results

There were 46 male and 15 female subjects, ages ranging from 27 to 80 (mean 60 years). Patient background characteristics are shown in Table 1. There were no operation related deaths. Preoperative evaluation of hilar and mediastinal lymph node metastasis was recorded in 44 cases. Among them actual lymphatic metastases were proven histologically in four cases. Pulmonary metastases were already present at the time of diagnosis of HCC in six patients, of whom pulmonary surgery was performed first in one patient. The cumulative 1-year survival rate after the initial pulmonary surgery was 69.8%, the 3-year survival rate 46.9%, and the 5-year survival 32.2%. The cumulative 1-year survival rate after hepatic surgery was 93.2%, the 3-year survival rate 74.0%, the 5-year survival 50.3%, and the 8-year survival 33.3% (Fig. 1). Of the eight subjects who survived at least 5 years following pulmonary surgery, three survived as cancer bearers.

We examined outcomes according to each parameter: gender, age (<60 vs ≥60), DFI (≤12 m vs ≥13 m, ≤24 m vs ≥25 m), number of pulmonary metastases at time detection (solitary vs multiple, $n = 1–2$ vs ≥3, $n = 1–3$ vs ≥4, $n = 1–4$ vs ≥5), maximum tumour diameter (<2 cm vs ≥2 cm), procedure (wedge resection vs segment resection/lobectomy), and number of pulmonary procedures (single vs multiple). As shown in Table 2, no significant difference was seen between

Table 1
Clinical characteristics of subjects with pulmonary metastases from hepatocellular carcinoma

Gender	
Male	46
Female	15
Age (range 27–80, average 60)	
Age <60	28
Age ≥60	31 + (unknown 2)
Disease-free interval (DFI) (range 0–180, average 28.7 m)	
0	6
0 < DFI ≤ 12	15
12 < DFI ≤ 24	16
24 < DFI	24
Number of pulmonary metastatic lesions (range 1–20, average 2.2)	
1	32
2	15
3	7
4	2
5~	5
Laterality of pulmonary metastases	
Right	30
Left	18
Bilateral	13
Mode of resection	
Wedge resection	47
Segmentectomy or lobectomy	14
Maximum diameter of pulmonary metastasis (MD) (range 0.4–4.8 cm, average 2.3 cm)	
MD < 1.0 cm	5
1.0 cm ≤ MD < 2.0 cm	23
2.0 cm ≤ MD < 3.0 cm	10
3.0 cm ≤ MD	18 + (unknown 5)
Number of pulmonary operations	
One	49
Two	9
Three	2
Four	1

solitary metastasis and multiple lesions ($p = 0.203$), whereas the prognosis was significantly worse for ≥3 lesions than for ≤2 lesions ($p = 0.046$), for ≥4 lesions than for ≤3 lesions ($p = 0.007$) (Fig. 2) and for ≥5 lesions than for ≤4 lesions ($p = 0.029$). No other factors that influence outcomes were identified. A stepwise regression analysis showed three or

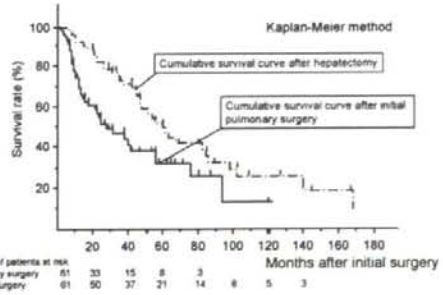

Fig. 1. Cumulative survival curves after hepatectomy and after initial pulmonary resection. Five-year survival rate after initial pulmonary metastasectomy is 32.2% and 5-year survival rate after hepatectomy is 50.3%.

Table 2
Cumulative overall survival results using log-rank analysis (Kaplan–Meier method)

Parameter	No. of patients	5-year survival rate	Significance (p)
Male vs female	46 vs 15	24.0% vs 51.4%	0.15
Age			
Age <60 vs Age ≥60	28 vs 31	26.1% vs 35.6%	0.46
DFI			
DFI ≥ 13 m vs DFI ≤ 12 m	40 vs 21	35.5% vs 21.0%	0.17
DFI ≥ 25 m vs DFI ≤ 24 m	24 vs 37	34.8% vs 28.1%	0.96
Number of pulmonary metastases			
Solitary vs multiple	32 vs 29	42.7% vs 20.0%	0.20
n = 1–2 vs $n \geq 3$	47 vs 14	37.2% vs 12.8%	0.046
n = 1–3 vs $n \geq 4$	54 vs 7	37.5% vs not reached	0.007
n = 1–4 vs $n \geq 5$	56 vs 5	36.0% vs not reached	0.029
Maximum diameter of pulmonary metastasis (MD)			
MD < 2 cm vs 2 cm ≤ MD	28 vs 28	37.2% vs 26.3%	0.41
Lung procedure			
Wedge vs segmentectomy or lobectomy	47 vs 14	34.5% vs 20.8%	0.98
Number of pulmonary lesions resected			
Single vs multiple	49 vs 12	35.7% vs 16.2%	0.98

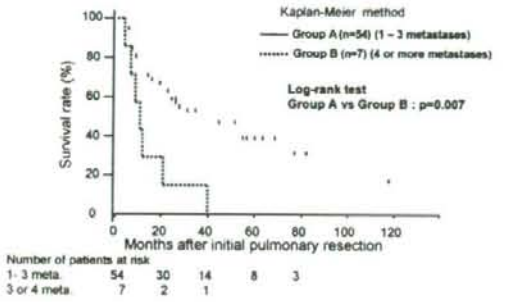

Fig. 2. Cumulative survival curves of patients with 1–3 metastases and with four or more metastases.

less pulmonary metastases to be an independent factor for better prognosis (p = 0.048) (Table 3).

4. Discussion

Treatment outcomes for HCC hepatic primary lesions have improved remarkably with recent advances in a variety of therapeutic modalities, including resection, hepatic artery injection sclerotherapy, ethanol injections and ablation therapy. This has led to consideration of more aggressive treatments for extrahepatic metastases. There have been some reports of prolonged life expectancy following aggressive therapy, including localised treatments, for extrahepatic metastases [1]. Pulmonary lesions are the most common extrahepatic HCC metastases, accounting for 50–60% of the total [2,3]. In most cases, surgery is contraindicated due to multiple lesions, so surgical treatment for pulmonary HCC metastases has yet to be fully evaluated.

Tomimaru et al. reported a series of 615 patients who underwent radical resection for HCC, 34 of whom developed pulmonary metastases during the postoperative follow-up period. Pulmonary metastases were resected in eight of the 14 patients with one or two pulmonary lesions, and outcomes were markedly better in the resection group than in the nonresection group [5]. This result indicates that surgical resection is effective in this highly selected group of patients with 1–2 pulmonary metastases, but at the same time leaves us with the suspicion that surgical resection may be indicated only in patients with no more than two pulmonary HCC metastases. Lam et al. reported a favourable 5-year survival rate of 67% in patients (n = 9) who underwent resection of solitary lesions [6]. Our analysis did not show any significant difference in the 5-year survival rate between subjects with solitary lesions (n = 32) and with multiple lesions (n = 29) (p = 0.203). On the other hand, there were significant differences in prognosis between ≥3 lesions and ≤2 lesions (p = 0.046), between ≥4 lesions and ≤3 lesions (p = 0.0070), and between ≥5 lesions and ≤4 lesions (p = 0.029). These data suggest that there is a relationship between metastatic number and prognosis and some patients with multiple metastases, if number of metastases is small such as four or less, can be expected to survive long after surgery. Actually about 90% of subjects had no more than three pulmonary metastases in this study. Multivariate

Table 3
Relationships of individual variables to patient prognosis (stepwise regression model)

Variable	Score	p
No. of metastases		
$n \leq 3$	3.908	0.048
Female	2.041	0.15
DFI ≤ 12 m	2.213	0.14
<60 (years)	0.806	0.67
Re-do operation	0.001	0.98
Segmentectomy or lobectomy	0.047	0.83
2 cm ≤ MD	0.535	0.77

DFI, disease-free interval; MD, Maximum diameter of pulmonary metastasis.

analysis also showed that three or less pulmonary metastases was an independent better prognostic factor for surgical treatment ($p = 0.027$).

In their studies of outcomes following surgical treatment of pulmonary HCC metastases, Koide et al. reported a 5-year survival rate (overall survival) of 26.8% for 14 subjects [7], and Nakajima et al. reported 23.8% for 20 subjects [8]. These are both single center therapeutic results. Our data, although collected from multiple institutions, yield a similar 5-year survival rate of 32%. All of the published studies to date have had insufficient patient numbers to properly assess the therapeutic effect of surgical resection for pulmonary HCC metastases. Koide and Nakajima both used roughly the same indications for surgery as we did, however, indicating that a 5-year survival rate of around 30% can be achieved with patients selected in this way.

Of the 12 subjects who underwent repeat surgery for recurrent pulmonary metastatic disease, only one survived for 5 years. This study does not demonstrate any efficacy for re-do surgery. There were no surgery-related deaths, however, and a number of studies have shown better outcomes for aggressive treatment of extrahepatic metastases [9–12], so there are no convincing reasons why repeat surgical resection should be contraindicated for recurrent pulmonary metastases.

5. Conclusions

With careful patient selection, comparatively good outcomes can be expected following surgical resection of pulmonary HCC metastases. Among them, patients with multiple metastases, if number of metastases is small such as four or less, can be expected to survive long after surgery.

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MODERN PATHOLOGY

Original Article

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Histopathological features and prognostic significance of the micropapillary pattern in lung adenocarcinoma

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Abstract

The micropapillary pattern is characterized by small papillary tufts with no fibrovascular core lying in spaces and has been reported as an aggressive variant of carcinoma in several organs. We investigated the histopathobiological properties of the micropapillary pattern with immunohistochemistry, serial sections, and electron microscopy in lung adenocarcinoma. We further analyzed its clinicopathological character and prognosis. The subjects included 383 adenocarcinoma cases, of which 184 (48%) were micropapillary pattern-positive and 199 (52%) were micropapillary pattern-negative. On histology, micropapillary tufts seemed to float in the alveolar space or spaces encased by connective tissues, whereas serial sections revealed that most tufts had continuity with other tufts and even with the main tumor. Positive staining for the adhesion molecules E-cadherin and β-catenin suggested the preservation of tight adhesion, and electron microscopy showed the existence of intercellular junctions. Negative staining for laminin and loss of basement membrane as determined by electron microscopy suggest a loss of cell–matrix contact. Positive staining for Ki-67 indicates that cells constituting micropapillary tufts retained their proliferation potency. There were no CD34-positive cells in micropapillary tufts, and the loss of the vascular core was confirmed. In micropapillary pattern-positive cases, lymphatic invasion was identified significantly more frequently than in micropapillary pattern-negative cases ($P<0.001$), even at stageIA (without lymph node metastasis, $N=197$) ($P<0.001$). The 5-year and 10-year overall survival rates of the micropapillary pattern-positive stageIA group were 77.6 and 67.6%, respectively, which were significantly less than those of the micropapillary pattern-negative stageIA group (98.1 and 98.1%) ($P=0.001$). In conclusion, cells constituting the micropapillary pattern are likely to have acquired anchorage-independent growth and a potential for high malignancy.

Keywords: lung adenocarcinoma, micropapillary pattern, histopathology, prognosis, immunohistochemistry, electron microscopy

Disease-Free Interval Length Correlates to Prognosis of Patients Who Underwent Metastasectomy for Esophageal Lung Metastases

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Background: Pulmonary metastasectomy is a standard method for treatment of selected pulmonary metastases cases. Nevertheless, because prognosis for patients with lung metastases from esophageal cancer who have undergone pulmonary metastasectomy is poor, candidates for this method of treatment are rare. Therefore, the efficacy of surgical treatment for pulmonary metastatic lesions from esophageal cancer has not been thoroughly examined.

Methods: Between March 1984 and May 2006, 57 patients underwent resection of pulmonary metastases from primary esophageal cancer. These cases were registered in the database developed by the Metastatic Lung Tumor Study Group of Japan and were retrospectively reviewed from the registry. After excluding eight cases because of missing information, we reviewed the remaining 49 cases and examined the prognostic factors for pulmonary metastasectomy for metastases from esophageal cancer.

Results: There were no perioperative deaths. After pulmonary metastasectomy, disease recurred in 16 (33%) of the 49 patients. The overall 5-year survival was 29.6%. Median survival time was 18 months. The survival of patients with a disease-free interval (DFI) less than 12 months was significantly lower than patients with a DFI greater than 12 months. Through multivariate analysis, we identified DFI as a clinical factor significantly related to overall survival ($p = 0.04$).

Conclusions: We identified that patients with a DFI less than 12 months who underwent pulmonary metastasectomy for metastases from esophageal cancer had a worse prognosis. Pulmonary metastasectomy for esophageal cancer should be considered for selected patients with a DFI ≥12 months.

Key Words: Esophageal cancer, Pulmonary metastasis, Metastasectomy.

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Pulmonary metastasectomy is a standard method of treatment for selected pulmonary metastases cases.[1] When patients are appropriately selected for this treatment, the overall 5-year survival after pulmonary metastasectomy is about 30 to 40%.[1,2] In general, because prognosis for patients who have undergone this method of treatment is poor with disease frequently recurring, pulmonary metastasectomy is not a frequently chosen method of treatment for lung metastases from esophageal cancer. Consequently, survival after surgery for pulmonary metastases from esophageal cancer has not been thoroughly examined. In Japan, the annual report by the Japanese Association for Thoracic Surgery does not document patients who underwent metastasectomy for metastasized esophageal cancer.[3] Because the outcome of pulmonary metastasectomy for metastases from esophageal cancer has not been thoroughly investigated, it is controversial whether surgery is an effective treatment for metastatic esophageal cancer. To identify prognostic factors of pulmonary metastasectomy for metastases from esophageal cancer, in the present study, we reviewed cases registered in the Metastatic Lung Tumor Study Group of Japan database of patients who underwent metastasectomy for metastasized esophageal cancer.

PATIENTS AND METHODS

The Metastatic Lung Tumor Study Group of Japan developed a database for registration of lung metastases cases. These patients all underwent surgical resection. The database documents the following parameters: gender; age; histology; status of the primary tumor; treatment for the primary tumor; date of primary surgery; kind of surgery; curability; date of metastasis; disease-free interval (DFI); side, size and numbers of resected metastases; date of metas-

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Disclosure: The authors declare no conflict of interest.

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tasectomy; and follow-up. Between March 1984 and May 2006, 57 patients underwent resection of pulmonary metastases from primary esophageal cancer. These cases were registered in the Metastatic Lung Tumor Study Group of Japan database and were retrospectively reviewed from the registry. Preoperative examination, surgical indication, and operative procedure were at the discretion of each institution.

After excluding eight cases because of missing information such as number of resected metastases, age, or DFI, we examined the remaining 49 cases (46 males and 3 females) in our study. Surgery alone for the primary tumor was performed in 26 cases (53%), surgery and chemoradiotherapy were performed in 7 cases (14%), surgery and radiotherapy were performed in 6 cases (12%), surgery and chemotherapy were performed in 3 cases (6%), radiotherapy alone was performed in 2 cases (4%), and treatment data were not available for 5 cases (10%). We examined the following variables (Table 1): age (≥70 or <70), number of resected metastases (solitary or multiple), resected side (unilateral or bilateral), tumor size (≥3 or <3cm), DFI (≥12 or <12 months), surgical procedure (partial resection, segmentectomy, or lobectomy), and curability (complete or incomplete).

The present study was analyzed using anonymized data that were collected in each institution. Therefore, informed consent was not specifically obtained and institutional review board approval was not necessary.

Statistical Analysis

Overall survival was analyzed by the Kaplan-Meier method, and differences in variables were calculated by the log-rank test. The date of pulmonary resection was defined as the starting point. Cox's proportional hazards model was used for multivariate analysis. The data were calculated using version 5.0 of the StatView software package (SAS Institute Inc, Cary, NC). A p value of less than 0.05 was defined as indicative of statistical significance.

TABLE 1. Survival of 49 Patients According to Clinical Factors of Pulmonary Metastases

Variables	n (%)	5-yr Survival (%)	p
Age (yr)			
≥70	13 (27)	32.9	0.928
<70	36 (73)	27.8	
Number			
Solitary	39 (80)	27.4	0.797
Multiple	10 (20)	42.9	
Resected side			
Unilateral	44 (90)	29.3	0.621
Bilateral	5 (10)	30.0	
Tumor size[a]			
≥3 cm	10 (21)	40.0	0.640
<3 cm	38 (79)	26.7	
DFI			
≥12 mo	28 (57)	39.2	0.048
<12 mo	21 (43)	15.7	
Surgical procedure			
Partial and segment	31 (63)	36.4	0.338
Lobectomy	18 (37)	22.9	
Curability			
Complete	45 (92)	31.4	0.990
Incomplete	4 (8)	25.0	

[a] No cases were available.
DFI, disease-free interval.

RESULTS

The median interval between treatment of esophageal cancer and diagnosis of pulmonary metastasis (disease-free interval) was 14 months (range: 0–124 months). There were no perioperative deaths. The median age of patients at the time of pulmonary metastasectomy was 65 years (range: 35–82). The median number of resected metastatic lesions per patient was one (range: 1–5). The metastases ranged in size from 0.4 to 5.5 cm, and the median size was 2.0 cm. The metastases were squamous cell carcinoma in 48 cases and adenocarcinoma in one case. The surgical procedure was wedge resection in 23 cases (47%), lobectomy in 16 cases (33%), segmentectomy in 8 cases (16%), and bilobectomy in 2 cases (4%). The median follow-up period after the first pulmonary resection was 18 months (range: 0–206 months). Recurrence developed in 16 (33%) of the 49 patients. Recurrences were as follows: lung, nine; lymph node, three; neck, one; distant metastasis, one; stomach, one; and unknown, two. The overall 5-year survival after pulmonary metastasectomy was 29.6% (Figure 1). Median survival time was 27 months. We investigated the relationships between prognostic factors and survival (Table 1). Patients with a DFI less than 12 months had a significantly worse prognosis, as assessed by survival rates, than patients with a DFI greater than 12 months (Figure 2). Multivariate analysis of these variables was performed using Cox's proportional hazards model for disease-specific survival. A DFI less than 12 months was shown to be an independent prognostic factor ($p = 0.04$) (Table 2). At the time of submission, 28 patients examined in our study have died. Although 23 patients died of esophageal cancer, 7 patients were not available for recurrent sites. Five patients have died of other diseases (two cases

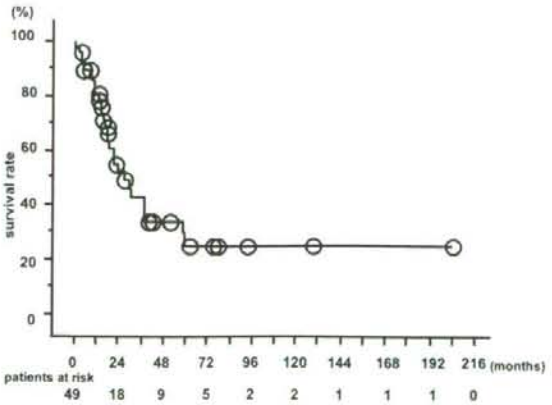

FIGURE 1. Overall survival of the 49 patients after pulmonary metastasectomy. The 5-year survival was 29.6%.

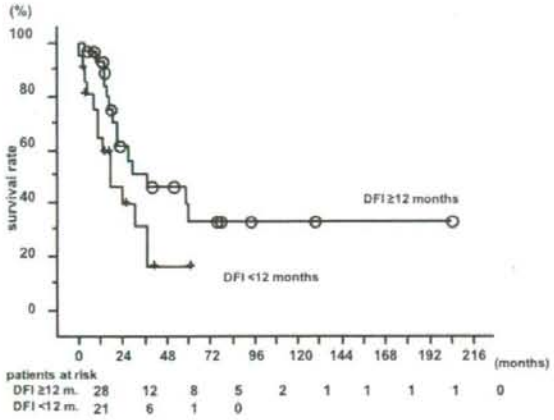

FIGURE 2. Overall survival after pulmonary metastasectomy according to DFI. Survival curves of patients with DFI <12 months and ≥12 months. DFI, disease-free interval.

TABLE 2. Relationships of Individual Variables to Survival (Cox's Proportional Hazards Model)

Variable	Risk Ratio	95% CI	*p*
≥70 yr	1.01	0.41–2.50	0.983
Multiple metastasis	1.67	0.30–9.19	0.557
Bilateral metastasis	1.19	0.19–7.53	0.853
Tumor size ≥3 cm	0.76	0.25–2.35	0.635
DFI <12 mo	2.30	1.04–5.09	0.040
Partial and segment	0.60	0.22–1.65	0.180
Incomplete resection	1.00	0.22–4.56	0.881

CI, confidence interval; DFI, disease-free interval.

were pneumonia, two cases were cerebral infarction, and one case was myocardial infarction).

DISCUSSION

Patients who are candidates for pulmonary metastasectomy for metastases from esophageal cancer are a minority. Analysis of the outcomes of surgery for pulmonary metastases from esophageal cancer has not been published. Quint et al.[4] showed that 29 of 147 (20%) patients with newly diagnosed metastasized esophageal cancer had lung metastasis. Although autopsy studies showed that the frequency of esophageal lung metastasis was 50%,[5] there was not a high percentage of esophageal cancer relapse after esophagectomy. Kyriazanos et al.[6] revealed that 12 of 151 (8%) patients who underwent a curative esophageal resection had lung metastases. Within our study the number of adenocarcinoma of the esophagus was very small. Because the frequency of adenocarcinoma of the esophagus is low in Japan, we do not speculate about the scarce incidence of lung metastasis from adenocarcinoma of the esophagus.

Matsubara et al. showed that 38 of 230 patients (17%) who underwent surgery for esophageal cancer with extended lymph node dissection had distant metastases and 14 (6%) patients had lung metastases. In their article, the outcomes after recurrence were dismal, and no patients were alive 5 years after detection of recurrence. Nevertheless, they showed that the 1-year survival of the patients who had recurrent lesions and were treated with resection and adjuvant therapy was 83%. They concluded that when recurrent lesions were localized macroscopically, surgical removal of the recurrent lesions was an effective treatment.[7] Through our analysis, we found a 5-year survival of 29.6% after pulmonary metastasectomy, which indicates that pulmonary metastasectomy is a promising treatment for metastases from esophageal cancer. Nevertheless, as it is not easy to differentiate esophageal metastases from primary lung squamous cell carcinomas, it is possible that our data might include primary lung squamous cell carcinoma. Survival after metastasectomy might be lower than what our data indicate. Virgo et al mentioned that genetic markers are needed to confidently distinguish between metastases and primary solitary nodules.[8] Further investigation is needed to clarify this matter.

An article from the international registry of lung metastases states that the 5-year survival was 37% after pulmonary metastasectomy. In addition, the article showed that among cases of complete resection, the 5-year survival was 33% for patients with a DFI of 0 to 11 months and 45% for those with a DFI of more than 36 months. Furthermore, the 5-year survival was 43% for single lesions and 27% for 4 or more lesions.[1] DFI and number of pulmonary metastases are significant prognostic factors. Because our present data show that the median DFI is 14 months, we categorized DFI as ≥12 or <12 months. Regarding the DFI, our study suggests that patients with a DFI less than 12 months have a poor prognosis. Osugi et al. showed that 83% of recurrences presented within 24 months after esophagectomy and that the chance of survival of patients whose disease recurred within 24 months after esophagectomy was better than that of patients who suffered recurrence within 24 months. Regarding follow-up studies after esophagectomy, meticulous care should be taken to detect hematogenous recurrence.[9]

In general, incomplete resection is a dismal prognostic factor in lung metastasectomy. We could not demonstrate whether surgical curability is a prognostic factor. McDonald et al. reported that incomplete resection appeared to have no influence on overall survival in metastatic breast cancer. They suggested that this could be due to the systemic nature of the disease at the time of thoracotomy with unsuspected occult metastasis in other areas.[10] Nevertheless, in our study, only four patients underwent incomplete resection. Because the report from The International Registry of Lung Metastases stated that cases with incomplete resection clearly had worse prognoses,[1] we speculate that patients with lung metastases from esophageal cancer have the same tendency.

Although our present study was multi-institutional, we could not analyze in detail all of the records for each patient. From this point of view, because our findings were based on a limited number of cases, pulmonary metastasectomy for lung metastases from esophageal cancer is still highly controversial. Nevertheless, we identified that patients with a DFI less than 12 months had a worse prognosis, as assessed by

survival rates, than patients with a DFI greater than 12 months.

Consequently, although metastases from esophageal cancer are a minority, we think that pulmonary metastasectomy for esophageal cancer should be considered for selected patients with a DFI ≥12 months. As this study is small, further clinical studies will be needed.

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An Alternative Method for Screening EGFR Mutation Using RFLP in Non-small Cell Lung Cancer Patients

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Introduction: Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors in non-small cell lung cancers (NSCLCs). Currently available methods of EGFR mutation detection rely on direct sequencing. Here, we describe the use of an alternative way to screen EGFR mutations.

Methods: A total of 109 frozen tumor specimens from NSCLC patients were obtained. For mutational analysis of EGFR exons 18, 19, and 21, reverse transcription-polymerase chain reaction was performed on the cDNA using original primers designed for restriction fragment length polymorphism (RFLP).

Results: EGFR mutations were detected in 37 patients (34%) by both RFLP and direct sequencing except one case in which it was detected only by RFLP. EGFR mutations were more frequently observed to be significant by multivariate analysis in patients with adenocarcinoma (OR = 5.56), no-smoking history (OR = 4.34), and 65-year-old or younger (OR = 2.64), but not in women (OR = 1.14). Among 37 patients, 18 were treated with gefitinib and 9 responded to the treatment. One patient without any mutation responded.

Conclusion: RFLP is a useful method for screening EGFR mutations and can also be applied to predicting the sensitivity of NSCLC patients to EGFR-tyrosine kinase inhibitors.

Key Words: EGFR mutation, Non-small cell lung cancer, RFLP.

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Lung cancer is the most common cause of death in both men and women worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of these cases.[1] Recently, two drugs, gefitinib (Iressa) and elrotinib (Tarceva), which target the epidermal growth factor receptor (EGFR) tyrosine kinase (TK), were approved in different countries to treat NSCLC.[2,3]

In 2004, three separate studies reported that mutations in the EGFR gene in lung carcinomas made the disease more responsive to treatment with TK inhibitors.[4–6] Since then, a multitude of data has emerged from different groups around the world.

Most EGFR somatic mutations were exclusively detected in adenocarcinomas, including bronchiolo-alveolar carcinomas. The mutations were detected in exons 18, 19, and 21, which encode the intracellular kinase domain. The mutations detected in exon 18 had substitution of the amino acid G719 in the P-loop, whereas those detected in exon 21 had substitution of an amino acid in the activation domain (L858 and L861). The mutations in exon 19 were in-frame deletions that may alter the structure of αC helices. All of the EGFR mutations affect amino acids near the ATP-binding pocket that is targeted by gefitinib. Functional assays revealed that the hotspot mutants of EGFR had a higher EGF-independent activation than did the wild-type EGFR.[4–7]

EGFR mutations are predominantly found in Asians, women, adenocarcinomas, and never-smokers, which explains the association between the clinical predictors and gefitinib sensitivity.[4–6,8–10]

Direct gene sequencing is a standard method for detecting gene mutations. However, it is not suitable for clinical pretherapeutic screening of patients because it is time-consuming, costly, and sometimes unreliable. Thus, an easy and reliable method for detecting EGFR mutations that can be used clinically is needed.

The aim of this study was to establish an easy and reliable method with which to screen EGFR mutations. We studied a large series of consecutive NSCLC patients for EGFR mutations in exons 18, 19, and 21 using a comparative approach between 2 techniques: direct sequencing of polymerase chain reaction (PCR) products and restriction fragment length polymorphism (RFLP) analysis.

PATIENTS AND METHODS

Cell Lines and Plasmids Containing Wild-Type and Mutant EGFR Genes

Three NSCLC cell lines, SK-MES-1, H1650, and H1975, were purchased from American Type Culture Col-

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lection (Manassas, VA). SK-MES-1 has wild-type EGFR. H1650 contains an E746-A750 deletion mutation in exon 19. H1975 contains an L858R point mutation in exon 21 and a secondary T790M point mutation which is related to the resistance to gefitinib and erlotinib in exon 20.[11,12] pL420 plasmids containing wild-type, G719C and L858R mutant EGFR genes (generous gifts from Dr. Matthew Myerson, Dana-Farber Cancer Institute, Boston) were used to validate the RFLP assay for corresponding point mutations in exon 18 and exon 21, respectively.

Extraction of Nucleic Acids and Restriction Fragment Length Polymorphism for EGFR Mutants

Genomic DNA was isolated from tumors and lung cancer cell lines using the DNeasy Mini Kit (Qiagen, Munich, Germany), according to the manufacturer's protocol. Total RNA was also isolated from the same samples using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using an Omniscript Reverse Transcription kit (Qiagen).

We have designed original primers against cDNA for RFLP to detect mutations (Figure 1). The following primers containing appended M13 forward or reverse primer tails for direct sequencing were used for PCR amplification: exon 18 (forward, 5′-TGTAAAACGACGGCCAGTCCCTGGGGATC-GGCCTCTTCATGCGA-3′; reverse, 5′-CAGGAAACAGCT-ATGACCTATACACCGTGCCGAACGCACCGGGG-3′), exon 19 (forward, 5′-TGTAAAACGACGGCCAGTGATCA-AAGTGCTGGGCTCC-3′; reverse, 5′-CAGGAAACAGCT-ATGACCACGGTGGAGGTGAGGCAGAT-3′), exon 21 (forward, 5′-TGTAAAACGACGGCCAGTAACACCGCA-GCATGTCAAGAT-3′; reverse, 5′-CAGGAAACAGCTA-TGACCATTCCAATGCCATCCACTTGAT-3′), exon 20 (forward, 5′-TGTAAAACGACGGCCAGTCCTCGATGA-AGCCTACGTGATG-3′; reverse, 5′-CAGGAAACAGCTA-TGACCGGCAGCCGAAGGGTATGAGCTG-3′). The PCR reaction was performed on 1 μL of template cDNA, as prepared above, to which were added 10× buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 0.2 mM of both dNTP and 0.25 U Ampli*Taq* Gold, and 0.2 μM forward and reverse primers in a 50 μL reaction volume. The "hot start" PCR cycling parameters were: one cycle of 95°C for 15 minutes, 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by one cycle of 72°C for 3 minutes.

On the other hand, for the additional experiment to compare the sensitivity of the assay between cDNA and genomic DNA, we also performed RFLP against genomic DNA for exon 19 and exon 21. We chose external and nested primers designed by Paez JG[5] for PCR on genomic DNA. We use the following primers in external PCR: Exon 19, (forward, 5′-AAATAATCAGTGTGATTCGTGGAG-3′; reverse, 5′-GAGGCCAGTGCTGTCTCTAAGG-3′), Exon 21, (forward, 5′-GCAGCGGGTTACATCTTCTTTC-3′; reverse, 5′-CAGCTCTGGCTCACACTACCAG-3′). And we used in nested PCR: Exon 19, (forward, 5′-GTGCATCGCT-GGTAACATCC-3′; reverse, 5′-TGTGGAGATGAGCAG-GGTCT-3′), Exon 21, (forward, 5′-GCTCAGAGCCTGG-CATGAA-3′; reverse, 5′-CATCCTCCCCTGCATGTGT-3′). External-round PCR reaction was performed on 0.1 μg of genomic DNA with the same protocol as described above. For nested-round PCR reaction, 3 μL of the external-round PCR product was amplified in a second 50 μL reaction mixture using nested primers assembled as external-round PCR reaction, described above.

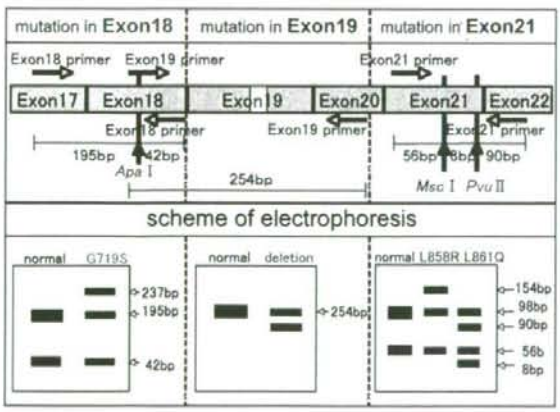

FIGURE 1. Scheme of digestion of PCR products and gel electrophoresis. PCR products were digested with corresponding enzymes (without digestion for exon 19), then were run on 2% agarose gel and the existence of mutations was assessed. Both *Apa*I and *Msc*I digest wild type EGFR allele, while *Pvu*II digests mutant EGFR allele.

Mutation Assay for G719X in Exon 18

The restriction enzyme *Apa*I digests the GGGCCC sequence in the amplicon of the wild-type allele. In contrast, the mutant allele was not digested because of the base substitution of G to X at the second base of GGGCCC. The PCR products after digestion were run on 2% agarose gel and the existence of the mutation was assessed (Figure 1).

Mutation Assay for Deletion in Exon 19

Because the range of exon 19 deletions containing commonly deleted codons 746 to 751 was reported to be from 9 to 18 bp, differences in the sizes of the PCR products enabled us to distinguish mutant from wild-type. The PCR products were run on 2% agarose gel and the existence of exon 19 mutations was assessed (Figure 1).

Mutation Assay for L858R and L861Q in Exon 21

The restriction enzyme *Msc*I was used to digest the TGGCCA sequence in the amplicon of the wild-type allele. In contrast, mutant type (L858R) was not digested because of the base substitution of T to G at the first base of TGGCCA. On the other hand, 2582T>G mutation creates a new *Pvu*II restriction site, CAGCTG, that can be used for a PCR-RFLP assay to distinguish L861Q mutant allele from wild-type. The PCR products were digested simultaneously with the restriction enzymes *Msc*I and *Pvu*II and run on 2% agarose gel, and the existence of these mutations was assessed (Figure 1).

Mutation Assay for T790M in Exon 20

The restriction enzyme *Nla*III was used to digest the CATG sequence in the amplicon of the mutant type (T790M) allele because of the base substitution of C to T at the third base of CACG. In contrast, wild-type allele was not digested. The PCR products after digestion were run on 2% agarose gel and the existence of the mutation was assessed (Figure 1).

EGFR Gene Sequencing

EGFR gene mutations in the cDNA samples were examined using PCR-based direct sequencing for exons 18, 19, and 21 to confirm the results of RFLP analysis. Sequencing was performed using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Corp., Foster City, CA) at the Central Research Center of Keio University Hospital.

Patients and Clinical Samples

Tumor samples from 109 patients diagnosed as having primary NSCLC by histopathological examination were obtained from Keio University (82 samples) and Kawasaki Municipal Hospital (27 samples). Ninety-one frozen tumor specimens were obtained either by surgery ($n = 48$), computed tomography-guided needle lung biopsy ($n = 32$) or ultrasonography-guided needle lung biopsy ($n = 5$), or TBLB ($n = 6$). Fourteen samples from pleural effusion, three samples from pericardial effusion, and one sputum sample were also obtained. Malignant effusion collected by pleurocentesis or cardiocentesis was centrifuged and the cell pellet was collected after removal of the supernatant. All samples were stored at −80°C until the DNA and RNA extraction procedures described above were performed.

All patient samples were collected or tested with informed consent, as approved by our respective institutional review boards. Clinical parameters for the patients were obtained from their medical records.

Clinical information was available for all 109 patients and Table 1 summarizes the demographic and clinical data of the study cohorts.

TABLE 1. Clinicopathological Features of All Patients

Variables	Subset	No.	(%)
No. patients		109	
Age (yr)	Mean	64.9	
	Range	30–85	
Sex	Male	59	(54.1)
	Female	50	(45.9)
Stage	I	18	(16.5)
	II	7	(6.4)
	III	33	(30.3)
	IV	51	(46.8)
Smoking history	Never smoker	37	(34.0)
	Ever-smoker	72	(66.0)
Tumor type	Adenocarcinoma	79	(72.6)
	AWBF	1	(0.9)
	BAC	5	(4.6)
	Squamous cell carcinoma	13	(11.9)
	Large cell carcinoma	1	(0.9)
	LCNEC	1	(0.9)
	Adenosquamous carcinoma	1	(0.9)
	Pleomorphic	2	(1.8)
	Others	5	(5.5)
Tumor samples	Resected tumor	48	(44.0)
	CT-guided lung biopsy	32	(29.4)
	US-guided lung biopsy	5	(4.6)
	TBLB	6	(5.5)
	Pleural fluid	14	(12.8)
	Pericardial fluid	3	(2.8)
	Sputum	1	(0.9)
EGFR mutations		37	(33.9)
	Exon 18		1/37, 2.7%
	Exon 19	22	22/37, 59.5%
	Exon 21	14	14/37, 37.8%

AWBF, adenocarcinoma with bronchiolo-alveolar carcinoma features; BAC, bronchiolo-alveolar carcinoma; EGFR, epidermal growth factor receptor; LCNEC, large cell neuroendocrine carcinoma; TBLB, transbronchial lung biopsy.

Statistical Analyses

The association between EGFR mutational status and tumor response to gefitinib was assessed using the χ^2 test. Multivariate analysis using logistic regression models was performed to assess the associations among histologic subtypes, gender, smoking history, age, and mutational status. All analyses were performed using Stat View (version 5, SAS Institute Inc., Cary, NC) software on a Macintosh computer.

RESULTS

Patterns of PCR-RFLP for EGFR Mutations on Gel-Electrophoresis

Figure 1 presents the predicted gel-electrophoresis patterns for PCR-RFLP samples. We first confirmed the patterns of electrophoresis for EGFR mutations using vectors containing an EGFR exon 18 or 21 point mutation as well as wild-type or cell lines containing an exon 19 deletion mutation and wild-type genes. G719S and L858R mutant vectors were clearly distinguished from wild-type by PCR-RFLP (Figure 2*A* and Figure 2*C*, respectively). On the other hand, a shorter band from the deleted allele in exon 19 and a longer band from the wild-type allele were observed by reverse transcription-polymerase chain reaction from H1650 (Figure 2*B*).

Sensitivity of PCR-RFLP Analysis of EGFR Mutations in Exon 19 and Exon 21

We evaluated the sensitivity of our RFLP assay in exon 19 or in exon 21 by combining SK-MES-1 cells with H1975 or H1650, respectively, in different ratios (Figure 3*A*). In exon 19, a shorter band from the deleted allele was detected up to the level of 1×10^2-fold dilution. In exon 21, the mutant allele at the 154 bp band was also detected up to the level of 1×10^2-fold dilution. The band of 154 bp indicates digested mutant alleles, and the band of 98 bp indicates wild-type alleles.

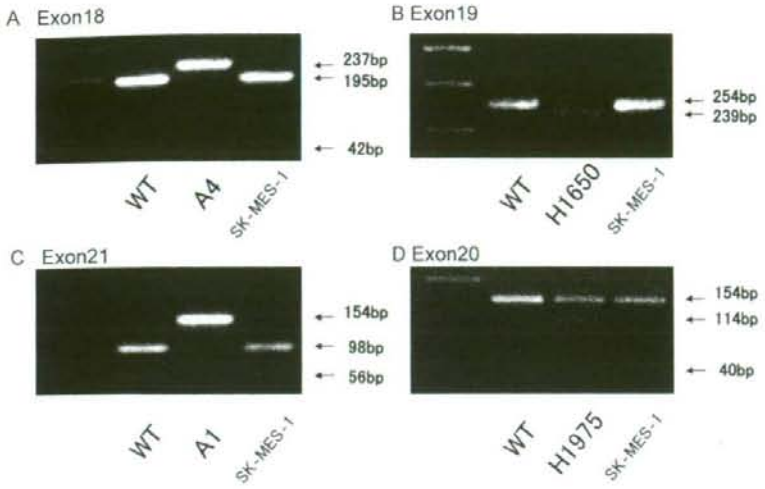

FIGURE 2. Demographic data of EGFR mutations in exon 18, 19, 21, and 20 by PCR-RFLP. *A*, The PCR products of exon18 treated with *Apa*I. *B*, The PCR products of exon19. *C*, The PCR products of the exon21 treated with *Msc*I and *Pvu*II, simultaneously. *D*, The PCR products of exon20 treated with *Nla*III. WT, EGFR (wild type) vector; A4, EGFR (G719S) vector; H1650, lung cancer cell line with EGFR mutation (del E746-A750); A1, EGFR (L858R) vector; H1975, lung cancer cell line with EGFR mutation (L858R and T790M); SK-MES-1, lung cancer cell line without EGFR mutation.

We also compared the sensitivity of cDNA and genomic DNA samples in pleural effusion from NSCLC patients for RFLP assay.

As malignant pleural effusion usually contains many hematopoietic cells such as macrophages and lymphocytes in addition to tumor cells, as a consequence, dilution of

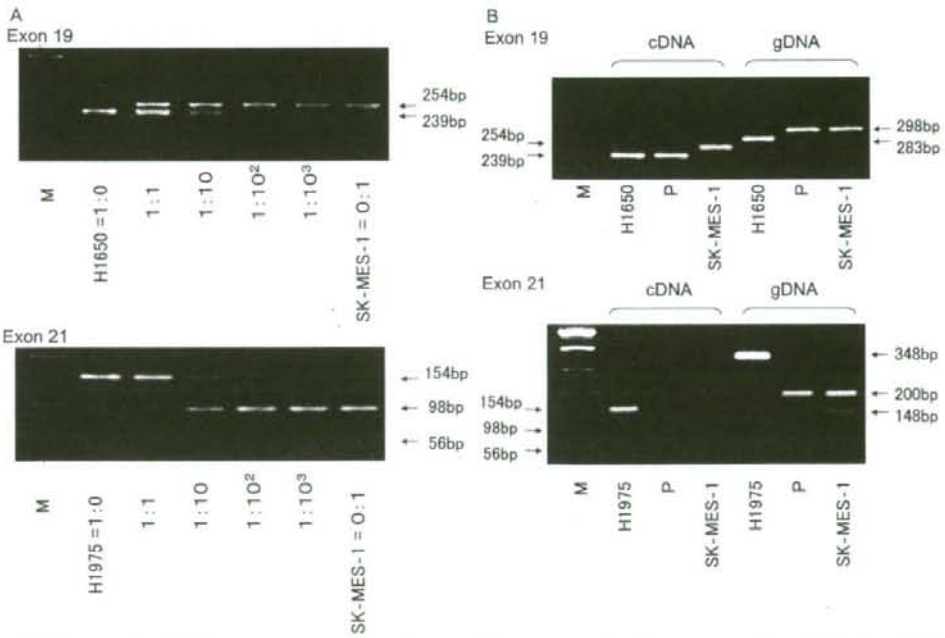

FIGURE 3. Sensitivity of PCR-RFLP analysis of EGFR mutations. *A*, In exon 19, H1650 was mixed with SK-MES-1 from 1- to 10^3-fold. A shorter band from the deleted allele was detected up to the level of 1×10^2-fold dilution. In exon 21, H1975 was mixed with SK-MES-1 from 1- to 10^3-fold. The mutant allele at the 154 bp band was detected up to the level of 1×10^2-fold dilution. SK-MES-1, lung cancer cell line without EGFR mutation; H1650, lung cancer cell line with EGFR mutation (del E746-A750); H1975, lung cancer cell line with EGFR mutation (L858R). *B*, PCR-RFLP was performed using either cDNA or genomic DNA (gDNA). The mutant allele in exon 19 was only detected by using cDNA in the case of malignant pleural effusion. In exon 21, the mutant allele (a 154 bp digested fragment) can be distinguished readily by using cDNA in the case of malignant pleural effusion of NSCLC. H1650; lung cancer cell line with EGFR mutation (del E746-A750), H1975; lung cancer cell line with EGFR mutation (L858R), SK-MES-1; lung cancer cell line without EGFR mutation, P; the case of malignant pleural effusion of NSCLC, M; marker.

genomic DNA derived from tumor cells occurs. To minimize the dilution caused by contaminated hematopoietic or other nontumor cells, we have chosen cDNA instead of genomic DNA for PCR/RFLP, taking into consideration that hematopoietic cells usually do not express the EGFR gene. Indeed, we could detect the mutation bands only when cDNA was used but not when genomic DNA was used from NSCLC patients with malignant pleural effusion (Figure 3*B*).

Results of PCR-RFLP Analysis of EGFR Mutations in Exons 18, 19, 21, and 20 in Clinical Samples

Only 1 patient (patient 56) showed 2 fragments (237 bp and 195 bp, corresponding to mutant and wild-type alleles, respectively) by RFLP using *Apa*I for exon 18 (Figure 4*A*). We found a novel point mutation in codon 719 (2146G>C [G719D]) confirmed by direct sequencing.

In exon 19, we found 22 deletion mutations. Patients who had a deletion mutation showed two fragments corresponding to wild-type and deletion mutant alleles (Figure 4*B*).

In exon 21 using *Msc*I and *Pvu*II, we found 14 point mutations in codon 858 by RFLP. Patients who had a 2573T>G point mutation showed 3 fragments (154 bp and 98 bp, corresponding to L858R mutant and wild-type alleles, respectively) (Figure 4*C*). No L861Q mutation was observed in our specimens.

In exon 20 using *Nla*III, we found 2 point mutations in codon 790 (2369C>T [T790M]) by RFLP. Patients who had a 2369C>T point mutation showed 2 fragments (154 bp, 114 bp, and 40 bp, corresponding to T790M mutant and wild-type alleles, respectively) (Figure 4*D*).

Finally, we found 37 EGFR mutations (34%) in exons 18, 19, and 21 in the present study.

EGFR Mutations and Clinicopathologic Features

(Table 2) The mutation status was significantly correlated with pathologic subtype (adenocarcinoma including bronchiolo-alveolar carcinomas versus nonadenocarcinoma, odds ratio = 5.56, $p = 0.035$), smoking status (never-smokers versus ever-smokers; odds ratio = 4.34, $p = 0.007$) and age (65-year-old or younger versus older than 65 years; odds ratio = 2.64, $p = 0.037$) but not with gender (female versus male; odds ratio = 1.14, $p = 0.813$) by logistic multivariate analysis.

Indeed the female never-smoker patients with adenocarcinoma had a high mutation rate (18/27, 66.6%), whereas the young never-smokers with adenocarcinoma had a higher mutation rate (13/15, 86.7%). Moreover, the young female never-smoker patients with adenocarcinoma had the highest mutation rate (10/11, 90.9%), whereas the elderly male ever-smokers with nonadenocarcinoma had the lowest mutation rate (1/11, 9.1%).

EGFR mutations were not influenced by disease extent (TNM stage) (data not shown).

EGFR Mutations and Clinical Outcome in Patients Treated with Gefitinib

(Table 3) Among 109 patients, 36 were treated with gefitinib (250 mg/d) and evaluated for their response. Eight of 36 patients were treated with gefitinib as an initial treatment. The results of the evaluation showed that 10 patients responded to the treatment. Nine of 10 responsive patients had mutations in exon 19 or exon 21, whereas one had no mutations. The response rate to gefitinib in patients with EGFR deletion mutations in exon 19 and L858R point mutation in exon 21 was 58.3% (7 of 12) and 33.3% (2 of 6, 1 of 7 patients was not evaluable due to discontinuation of treatment because of side effect), respectively. The patient

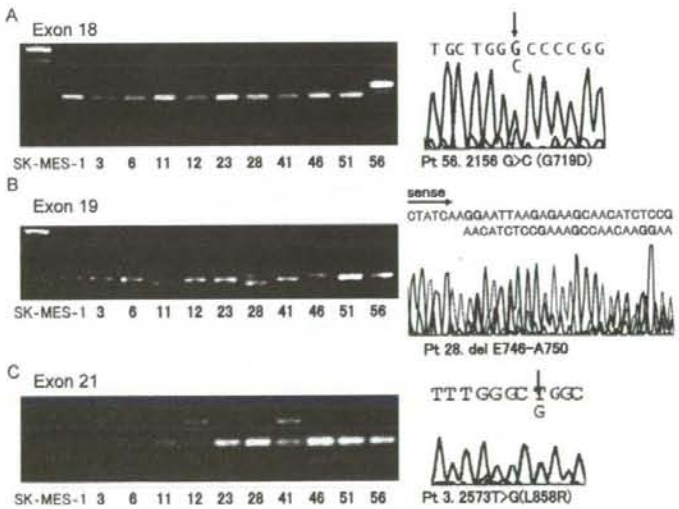

FIGURE 4. Result of PCR-RFLP analysis of EGFR mutations in exon 18, 19, and 21 in clinical samples. *A*, In exon 18, only patient 56 showed 2 fragments (237 bp and 195 bp, corresponding to mutant and wild type alleles, respectively). Direct sequencing analysis of PCR products is shown. Point mutation 2156G>C (G719D) (arrow) is detected in patient 56. *B*, In exon 19, patients 11, 28, and 46 showed 2 fragments corresponding to wild type and deletion mutant alleles. In-frame deletion was detected in patients 28. *C*, In exon 21, patients 3,12, and 41 showed 2 fragments (154 bp and 98 bp, corresponding to L858R mutant and wild type alleles, respectively). No L861Q mutation was observed in our specimens. Point mutation 2573T>G (L858R) was detected in patients 3, 12, and 41.

TABLE 2. Correlation of EGFR Mutations with Clinicopathologic Features

	EGFR Mutation		Odds Ratio	p
	+	−		
(A) Gender and EGFR mutation status				0.813
Female	22	28	1.14	
Male	15	44		
(B) Histology and EGFR mutation status				0.035
Adenocarcinoma (with BAC)	35	50	5.56	
Nonadenocarcinoma	2	22		
(C) Smoking habit and EGFR mutation status				0.007
Never smoker	21	16	4.34	
Ever-smoker	16	56		
(D) Age and EGFR mutation status				0.037
≤65	25	30	2.64	
>65	12	42		

EGFR, epidermal growth factor receptor; BAC, bronchiolo-alveolar carcinoma.

TABLE 3. Response to Gefitinib and EGFR Mutation Status

	EGFR Mutation		
Response	+	−	
CR	1	0	
PR	8	1	
SD	5	1	
PD	4	16	
CR/PR	9	1	
SD/PD	9	17	$p = 0.003$
CR/PR/SD	14	2	
PD	4	16	$p < 0.00001$

CR, complete response; EGFR, epidermal growth factor receptor; PD, progressive disease; PR, partial response; SD, stable disease.

with G719D mutation in exon 18 was not treated with gefitinib. On the other hand, 2 patients had T790M mutation and both also had activating mutations (data not shown). One patient who had L858R mutation was not treated with gefitinib. Another patient who had deletion mutation in exon 19 was resistant to gefitinib.

EGFR mutations were more frequently observed in samples from the patients who showed complete or partial responses (9 of 10 cases, 90.0%) than in samples from patients with stable disease or progressive disease (9 of 26, 34.6%; $p = 0.003$). Alternatively, the response rate to gefitinib in patients with EGFR mutations was 50% (9 of 18).

Comparison of Detection of Mutations by RFLP and Direct Sequencing

EGFR mutations were detected in 37 patients. They were detected by both RFLP and direct sequencing methods in 36 cases, whereas it was detected only by RFLP but not by direct sequencing in one case.

DISCUSSION

In the present study, we have described a reliable PCR-RFLP assay for the detection of mutations occurring in the EGFR TK domain. We have also analyzed a large series of NSCLCs for mutations in the TK domain of the EGFR gene by RFLP to assess the actual incidence of this genetic abnormality and its distribution according to histologic type, sex, smoking history, age, and TNM system parameters. In addition, we have analyzed the relationship between EGFR mutations and clinical outcome in patients treated with gefitinib.

In our analysis of 109 cases, most of the EGFR mutations were present in adenocarcinomas. Approximately 41% of adenocarcinomas showed the EGFR mutation, whereas the corresponding figure was 8% for nonadenocarcinomas. In univariate analysis, EGFR mutations were also significantly more frequent in women, younger patients, and never-smokers (data not shown). However, when the histotype, sex, smoking history, and age were tested by multivariate analysis against the presence of mutations in EGFR as a dependent variable, histotype, history of never smoking, and younger age (≤65) remained significant, while female sex did not.

EGFR mutations were more frequently observed in the samples from younger patients (45.5%) than older patients (22.2%). Tomizawa et al. also reported that EGFR mutation was significantly more frequent in younger patients (38%) than in older patients (12%, $p < 0.0001$).[13] For lung cancer, young adults are generally defined as being 40 or 45 years and under, whereas elderly patients are defined as 65 or 70 years and older. When we used 45 years as a cutoff value for age, there was no significant difference between the younger and the older patients regarding EGFR mutations, either by univariate or multivariate analysis. On the other hand, when 65-year-old was used as the cutoff value, the younger, nonelderly patients had a significantly higher prevalence of the EGFR mutation. Indeed, we found that the nonelderly (≤65 years) female never-smoking patients with adenocarcinoma had the highest mutation rate (90.9%), however, this mutation rate was almost identical to the value found in the nonelderly never-smokers with adenocarcinoma independent of sex (86.7%). Moreover, we found only one mutation in the elderly ever-smoker patients with nonadenocarcinoma (1/16, 6.3%) or in males with the above 3 indexes (9.1%). Together with the findings of multivariate analysis, the results suggest that being female may not be a significant independent factor for predicting EGFR mutations, as has been reported elsewhere. This may be partially explained by the fact that female was the predominant sex in young and adenocarcinoma patients.[14]

EGFR mutations were more frequently observed in samples from patients who showed a complete or partial response than in samples from patients with a stable or progressive disease, supporting the findings of many previous reports (Table 3). The exon 19 deletion mutations are re-