は壁側胸膜由来であるが，縦隔側以外では，壁側胸膜は胸壁の筋肉と接しており軽微な変化が顕在化しづらいのに対し，縦隔側では壁側胸膜は直接縦隔脂肪に接しているため，その不整像をとらえやすい．

また，担癌患者に胸水を認め，画像的に胸膜不整を認めないが，胸水穿刺や胸腔鏡で悪性細胞や播種病変を認めるという経験は，それほど稀なことではない．胸膜中皮腫においても同様で，初期像はごく軽度の胸膜不整肥厚であり，時には胸水のみで胸膜肥厚がない症例も存在する．画像上全く不整がない症例は，画像診断の限界だが，中皮腫の予後向上には，このような超早期の段階で診断し治療することが必要と考える．原因不明の胸水が続く症例で，胸水細胞診で中皮腫と診断されれば問題ない．しかし，細胞診で診断できない場合，画像上不整所見がないことを根拠に延々と経過観察をするのではなく，積極的に胸腔鏡にて胸膜を観察・生検し，中皮腫を除外することも検討する必要がある．かといって，胸水症例全例に胸腔鏡を行うとすれば，中皮腫以外の症例に対し，必要のない侵襲を加える頻度が多くなるというジレンマに陥る．

その中で，中皮腫の可能性が高い症例を選ぶためには，胸膜の状態をより正確に評価するために造影CT・MRIを施行し，縦隔側胸膜をはじめ軽度の胸膜不整に注意して読影し，アスベスト曝露の指標となる胸膜プラークの有無にも注意する必要がある．さらに，胸水の性状，ADA値，ヒアルロン酸値なども含め，総合的に胸腔鏡下生検の必要性を判断し，施行することで，できるだけ無駄な侵襲を減らして，早期診断精度の向上を目指すべきである．

おわりに

アスベスト関連肺胸膜病変の画像診断について概説した．アスベスト曝露に特異的なものとして胸膜プラーク，胸膜中皮腫がある．特に胸膜プラークは曝露の医学的根拠として用いられ，日常診療で遭遇する機会も多いため，その正確な読影が必要とされる．胸膜中皮腫については，早期病変については画像診断のみでは，限界があることを認知しておかねばならない．具体的には，胸膜プラークを同時に認めるような原因不明の胸水症例では，積極的な胸腔鏡下生検を検討する必要がある．

石綿肺は進行すると特発性肺線維症と同様の所見を呈することもあり，その鑑別は難しいが，石綿肺に特徴的なHRCT所見を複数認めること，胸膜プラークの存在，高濃度職業性曝露歴が，石綿肺と診断するに当たってその根拠となりうる．円形無気肺は，以前は肺癌との鑑別に生検がよく施行されていたが，"comet tail sign"をはじめとした特徴的な所見に留意すれば，多くの症例は，画像のみで診断可能である．

びまん性胸膜肥厚，良性石綿胸水は画像診断的には非特異的な所見を呈するが，やはり胸膜プラークがアスベスト関連疾患の根拠となり重要な所見である．石綿肺癌については，通常の肺癌の読影と差異はないが，胸膜プラークと間質性変化の存在を画像的に指摘できれば，職業性曝露の有無に関係なく石綿救済法の対象となるので，肺癌診断時にはこれに留意しておく必要がある．

最後に，これらのアスベスト関連肺胸膜病変のうち，日常臨床において圧倒的に目にする頻度が高いのは胸膜プラークである．胸膜プラーク自体にほとんど病的意義はなく，軽微なものは見逃されやすいが，石綿曝露の医学的根拠として重要な所見となる．中皮腫，肺癌など悪性病変の指摘は当然非常に大切であるが，胸膜プラークのアスベスト関連疾患における重要性を再認識し，軽微なものもアスベスト曝露の所見としてその存在を指摘しておく必要がある．

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Summary

Asbestos-Related Pleuropulmonary Lesions

Pleural plaque is important, common finding in patients exposed to asbestos. Because it is used as a medical index of asbestos exposure, it is necessary to be familiar with pleural plaque.

Lung cancer, mesothelioma, and asbestosis accompanied by diffuse pleural thickening with marked remarkable dyspnea are targeted diseases for public compensation after asbestos exposure.

It is necessary to be familiar with the imaging findings of these diseases as well as the requirements for public compensation to be authorized.

When there is no history of asbestos exposure in the employment records of lung cancer and mesothelioma patients, it is necessary to be cautious in recommending public compensation.

Katsuya Kato*, Takumi Kishimoto**, Susumu Kanazawa*

*Department of Radiology, Okayama University Hospital, **Block Center for Asbestos-related Diseases, Okayama Rosai Hospital

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Original article

Hyaluronic acid in the pleural fluid of patients with malignant pleural mesothelioma

*Nobukazu Fujimoto**, Kenichi Gemba*[1], *Michiko Asano, Yasuko Fuchimoto, Sae Wada, Katsuichiro Ono, Shinji Ozaki, Takumi Kishimoto*

Department of Respiratory Medicine, Okayama Rosai Hospital, 1-10-25 Chikkomidorimachi, Minamiku, Okayama 7028055, Japan

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ABSTRACT

Background: We retrospectively analyzed hyaluronic acid (HA) concentrations in pleural fluid and evaluated its utility for the differential diagnosis of malignant pleural mesothelioma (MPM).

Methods: Pleural fluid HA concentrations were measured in 334 patients, including 50, 48, 85, 18, 86, 6, and 41 patients with MPM, benign asbestos pleurisy (BAP), lung cancer (LC), other malignant conditions (OMCs), infectious pleuritis (IP), collagen disease (CD), and other conditions, respectively.

Results: The median (range) HA concentrations in pleural fluid were 78,700 (7920–2,630,000) ng/ml in the MPM group, 35,950 (900–152,000) ng/ml in the BAP group, 19,500 (2270–120,000) ng/ml in the LC group, 14,200 (900–101,000) ng/ml in the OMC group, 23,000 (900–230,000) ng/ml in the IP group, 24,600 (9550–80,800) ng/ml in the CD group, and 8140 (900–67,800) ng/ml in the other diseases group. HA levels were significantly higher in the MPM group than in the other groups. Receiver operating characteristic (ROC) analysis revealed an area under the ROC curve value of 0.832 (95% confidence interval, 0.765–0.898) for the differential diagnosis of MPM. With a cutoff value of 100,000 ng/ml, the sensitivity and specificity were 44.0 and 96.5%, respectively. In the MPM group, HA values were significantly higher for the epithelioid subtype than for the sarcomatous subtype ($p=0.007$), and higher in earlier stages (I and II) than in advanced stages (III and IV) ($p=0.007$).

Conclusions: A diagnosis of MPM should be strongly considered in patients with pleural fluid HA concentrations exceeding 100,000 ng/ml.

1. Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive tumor that arises from mesothelial-lined surfaces and has a poor survival rate [1]. MPM is a rare tumor; however, it has become a very important public health issue in Japan. The industrial use of asbestos has been banned in Japan since 2006, but the incidence of MPM is expected to increase for the next few decades due to past asbestos use [2].

Patients with MPM present therapeutic and diagnostic challenges. The majority of patients with MPM exhibit pleural effusion at the time of diagnosis [3], but cytological diagnosis

*Corresponding author. Tel.: +81 86 2620131; fax: +81 862623391.
E-mail addresses: nfuji@okayamaH.rofuku.go.jp, nobufujimot@gmail.com (N. Fujimoto).
[1]Present address: Department of Respiratory Medicine, National Hospital Organization Fukuyama Medical Center, 4-14-17 Okinogamicho, Fukuyama 7208520, Japan.

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from pleural fluid is usually difficult and is of limited utility. For a definite diagnosis, thoracoscopic or percutaneous biopsy should be performed to obtain adequate specimens for pathological and immunohistochemical analyses. However, even with these invasive procedures, it is occasionally difficult to differentiate MPM from other pleural diseases, such as benign asbestos pleurisy (BAP), tuberculosis pleurisy (TB), or pleural metastasis of lung cancer (LC). Therefore, a reliable clinical marker is a requisite to support the rapid and accurate diagnosis of pleural fluid.

Several investigators sought to improve the differential diagnosis of pleural fluid by measuring molecular markers, including carcinoembryonic antigen (CEA) [4], cytokeratin 19 fragment 21-1, carbohydrate antigen (CA) 125, CA15-3, and CA19-9 [5]. Aoe et al. reported a higher concentration of receptor-binding cancer antigen expressed on SiSo cells (RCAS1) in malignant pleural fluid than that in nonmalignant effusion [6], and soluble mesothelin-related protein (SMRP) appear to be promising markers for differentiating MPM from LC [7,8]. Recently, Shiomi et al. reported that N-ERC/mesothelin could be a useful marker for the diagnosis of MPM [9]. Our group has demonstrated that the methylation profile of some tumor suppressor genes in the pleural fluid could be useful for differentiation [10]. However, the utility of these markers has not yet been fully established in clinical practice.

Glycosaminoglycan hyaluronic acid (HA) has been proposed to be functionally important in processes such as embryogenesis, angiogenesis, cell growth and migration, wound healing, and the formation of high-molecular-mass aggregates with various proteoglycans [11]. HA also plays at least 3 roles in malignancy; it functions as a template for the assembly of other pericellular macromolecules, it interacts directly with cell surface receptors that transduce intracellular signals, and it promotes anchorage-independent growth and invasiveness [12]. The association between HA and MPM was reported as early as 1939 [13]. Since then, a large number of studies have investigated the functional and diagnostic importance of HA in MPM [14–20]; however, most of these studies included only a small number of patients and detected HA using older methods, such as high-performance liquid chromatography, radiometric assay, or electrophoretic methods. Thus far, no large study has been conducted using the immunoassay analysis currently applied in clinical use.

In the present study, we retrospectively analyzed HA levels in the pleural fluid of patients who visited our institution. The aim was to investigate the clinical utility of measuring HA levels in pleural fluid for the differential diagnosis of MPM. HA levels in the pleural fluid of patients with MPM were compared with those of patients with other pulmonary diseases, such as BAP, TP, or LC. We also examined the correlations between HA levels and the clinical features of MPM, such as pathological subtype and clinical stage.

2. Material and methods

This retrospective analysis included the extraction of HA levels from the medical records of subjects that exhibited pleural effusion in Okayama Rosai Hospital between December 2005 and August 2010. This study was approved by the institutional review board of Okayama Rosai Hospital (approval No.13, Dec 21, 2009). Pleural fluid was collected, and the HA concentration was determined in clinical practice by using the latex agglutination turbidimetric immunoassay. The confirmed diagnoses of the subjects included MPM, BAP, LC, malignancies other than MPM or LC, infectious pleuritis (IP), collagen disease (CD), and other diseases. In the case of MPM, the pathological diagnosis was confirmed on the basis of normal hematoxylin-eosin staining; positive immunohistochemical reactivity to mesothelial markers such as calretinin, Wilms' tumor 1, or thrombomodulin; and negative reactivity to CEA. The clinical stage of MPM was determined according to International Mesothelioma Interest Group (IMIG) criteria [21]. LC was defined as the presence of lung cancer cells in the pleural fluid. The histological subtypes of LC were determined on the basis of the World Health Organization classification [22]. A diagnosis of BAP was made in patients with previous asbestos exposure, based on the exclusion of other specific causes and the possibility of malignant diseases via thoracoscopy.

Comparisons between groups were performed using the Kruskal–Wallis test and nonparametric Mann–Whitney *U* test. Areas under receiver operating characteristic (ROC) curves (AUCs) were calculated using standard techniques. Statistical calculations were performed using the SPSS statistical package version 11.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. *Patients*

A total of 334 patients visited our institution for the diagnosis and treatment of pleural effusion, and they were given a final diagnosis during the study period. The diagnoses included MPM in 50 patients, BAP in 48, LC in 85, other malignant diseases (OMDs) in 18, IP in 86, CD in 6, and other diseases in 41. Malignant diseases other than MPM and LC included 3 cases each of ovarian and gastric cancers; 2 cases each of esophagus, liver, and colon cancers; and 1 case each of breast, pancreas, gall bladder, bladder, pharynges, and submandibular gland cancers. CDs included 3 cases of systemic lupus erythematosus, 2 cases of rheumatoid arthritis, and 1 case of Wegener's granuloma. Congestive heart failure, renal failure, and liver cirrhosis were classified under other diseases.

3.2. *HA levels in pleural effusion*

The median (range) HA concentrations in pleural fluid were 78,700 (7920–2,630,000) ng/ml in the MPM group, 35,950 (900–152,000) ng/ml in the BAP group, 19,500 (2270–120,000) ng/ml in the LC group, 14,200 (900–101,000) ng/ml in the OMD group, 23,000 (900–230,000) ng/ml in the IP group, 24,600 (9550–80,800) ng/ml in the CD group, and 8140 (900–67,800) ng/ml in the other diseases group. HA levels in the MPM group were significantly higher than those in the other groups (Fig. 1). We analyzed the utility of HA for the differential diagnosis of MPM using ROC analysis (Fig. 2). The AUC value for the differential diagnosis of MPM and other groups was 0.832

(95% confidence interval [CI], 0.765–0.898). Based on a cutoff value of 100,000 ng/ml, the sensitivity was 44.0%, and the specificity was 96.5%. With a cutoff value of 150,000 ng/ml, the sensitivity and specificity were 32.0% and 99.3%, respectively.

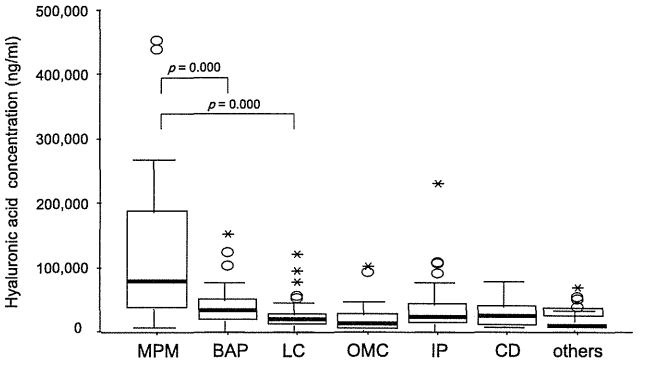

Fig. 1 – Hyaluronic acid concentrations in pleural fluid. MPM: malignant pleural mesothelioma, BAP: benign asbestos pleurisy, LC: lung cancer, OMC: other malignant condition, IP: infectious pleuritis and CD: collagen disease.

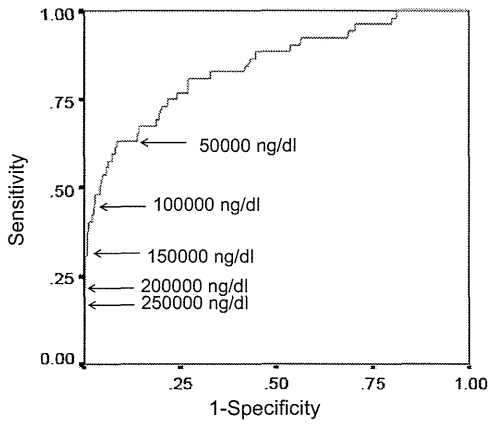

Fig. 2 – Receiver operating characteristic analysis of the use of hyaluronic acid concentrations for the differential diagnosis of malignant pleural mesothelioma. The coordinate points of a few cutoff values are indicated.

3.3. *Patients with elevated HA levels*

There were 32 (9.6%) patients with HA concentrations exceeding 100,000 ng/ml. Among them, 22 patients were diagnosed with MPM; the remaining 10 patients included 4 with BAP, 4 with IP, 1 with LC, and 1 with pharyngeal cancer (Table 1). The 4 patients with IP clinically exhibited fever elevation, leukocytosis, and elevated C-reactive protein levels; 2 of these patients were diagnosed with empyema based on the distinctive behavior of the fluid, and 1 patient was diagnosed with tuberculous pleuritis due to elevated adenosine deaminase levels (123.4 IU/l) and positive results in the mycobacterial smear and genetic tests. The patient with LC presented with an obvious tumor in the lungs, and cytological analysis of the fluid revealed adenocarcinoma cells. The patient with pharyngeal cancer had an occupational history of asbestos exposure. He underwent curative surgery and exhibited no clinical signs of recurrence, and thus, pleural mesothelioma was suspected. A thoracoscopic pleural biopsy indicated pharyngeal cancer with pleural involvement. Thoracoscopic exploration demonstrated no malignant diseases or specific findings among the 4 patients with BAP.

3.4. *HA levels in the MPM group*

Next, we analyzed the HA levels in the pleural fluid of patients with MPM. The 50 cases of MPM included the following subtypes: 34 epithelioid, 8 biphasic, 6 sarcomatous, and 2 desmoplastic. HA levels were significantly higher for the epithelioid subtype than for the sarcomatous subtype ($p=0.007$) (Fig. 3A). According to clinical stages based on the IMIG classification, HA levels were higher in earlier stages (I and II) than in advanced stages (III and IV) ($p=0.007$) (Fig. 3B). Similar results were obtained in the analyses limited to the epithelioid subtype; the HA levels were lower in more advanced stages, although this difference was not statistically significant (Fig. 3C). Finally, we compared the survival of patients with MPM between those with HA levels above or below 100,000 ng/ml, finding no differences (data not shown).

4. Discussion

In this study, we retrospectively analyzed HA concentrations in pleural fluid. Several previous studies examined HA levels

Table 1 – Patients with hyaluronic acid concentrations >100,000 ng/ml who were not diagnosed with malignant pleural mesothelioma.

Case	Final diagnosis	HA concentration (ng/ml)
1	Pharyngeal cancer	101,000
2	Benign asbestos pleurisy	103,000
3	Empyema	106,000
4	Tuberculous pleuritis	108,000
5	Benign asbestos pleurisy	118,000
6	Lung cancer	120,000
7	Benign asbestos pleurisy	123,000
8	Benign asbestos pleurisy	123,000
9	Benign asbestos pleurisy	152,000
10	Empyema	230,000

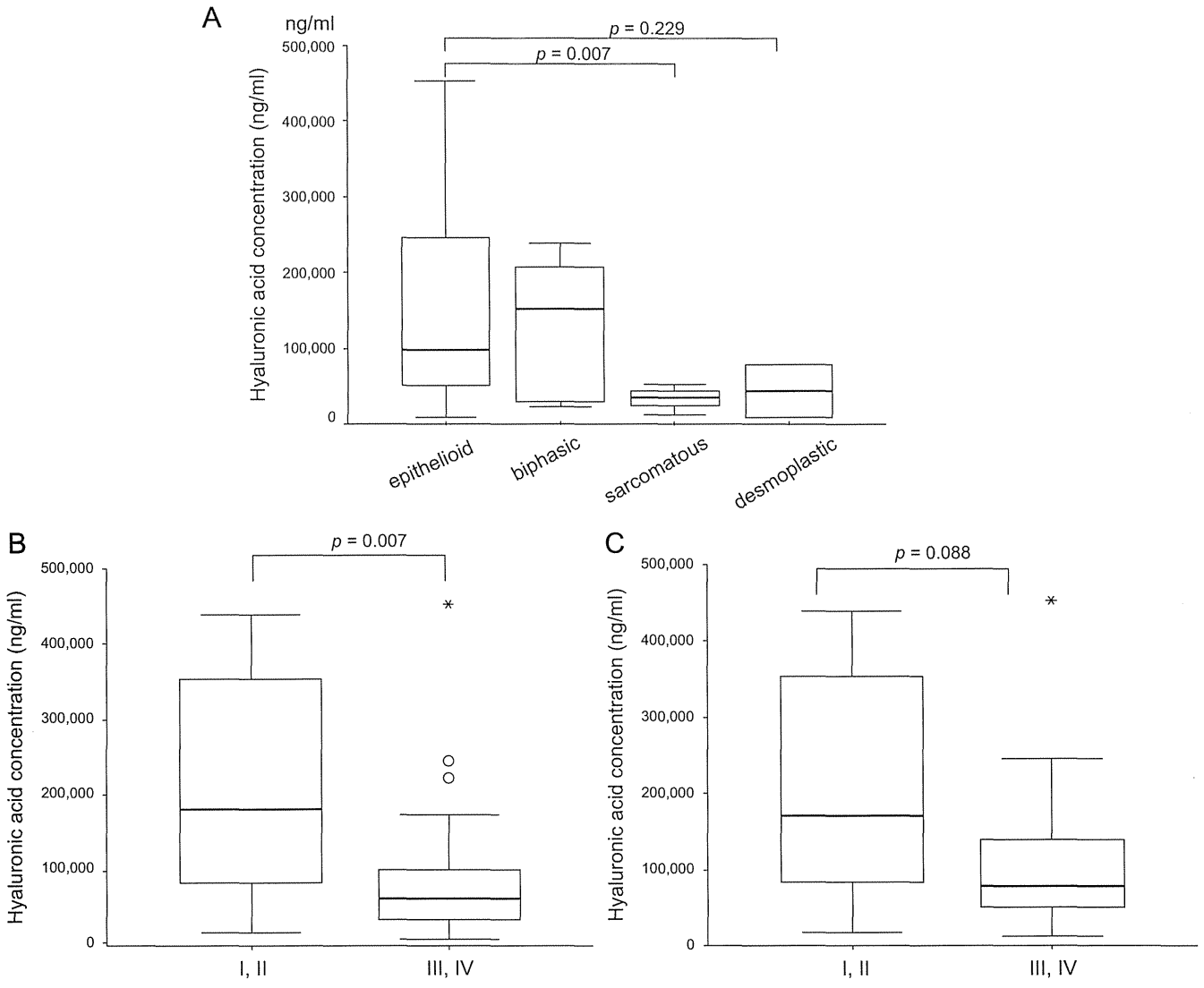

Fig. 3 – Hyaluronic acid concentrations in the pleural fluid of patients with malignant pleural mesothelioma. Comparison according to pathological subtypes (A) and clinical stages based on the International Mesothelioma Interest Group classification (B) and comparison according to clinical stages limited to the epithelioid subtype (C).

in the pleural fluid of patients with MPM. Pettersson et al. reported an HA concentration exceeding 100,000 ng/ml in the pleural effusion of 73% of patients with MPM and 23% of patients with inflammatory pleuritis; they found no HA concentrations exceeding 100,000 ng/ml in the pleural fluid of patients with other malignant or nonmalignant diseases [19]. Other studies reported elevated HA levels in the pleural fluid of patients with viral pleuritis, rheumatoid arthritis, benign asbestos pleurisy, or congestive heart failure [23,24]; however, these studies comprised a small sample size and measured HA levels using an outdated detection method. Our present study included over 300 patients (including 50 patients with MPM), and we used an HA detection method that is practical for clinical use. We believe that this is one of the largest investigations of HA levels. Another point of distinction in our study is that we determined HA levels in patients with BAP. The differentiation between MPM and BAP is critical, but BAP was not mentioned in previous studies. BAP was first described by Eisenstadt in 1964 as fibrotic pleuritis, and it may occur early or late after asbestos exposure [25,26].

Our results confirmed that HA values in pleural fluid were significantly higher in patients with MPM than in those with other diseases, verifying the utility of HA as an assistant diagnostic marker of MPM. The sensitivity for the differential diagnosis (44.0% with a cutoff value of 100,000 ng/ml) was lower than that previously reported [18], but the specificity was high (96.5%). These results indicate that patients with pleural fluid HA levels greater than 100,000 ng/ml should be highly suspected as having MPM. In our previous report, the SMRP concentration was also significantly higher in MPM than in other diseases. In the analysis, the AUC value was 0.75 (95% CI, 0.615–0.884) for the differential diagnosis of MPM from other diseases [8]. In the current study, the AUC value of HA was 0.832 (95% CI, 0.765–0.898) for the differential diagnosis of MPM. From this point of view, HA might be a better diagnostic marker than SMRP.

The present study included 32 patients with pleural fluid HA levels exceeding 100,000 ng/ml, including 22 patients with

MPM. Among the 10 cases that were patients with other diseases, a differential diagnosis was conducted for 4 using practical approaches based on clinical features, blood tests, and pleural fluid examination; they included 2 patients with empyema, 1 with tuberculous pleuritis and 1 with lung cancer. Thoracoscopic exploration was required for the remaining 6 patients, including 1 patient with pharyngeal cancer and 5 with BAP. Our findings indicate that most patients with HA concentrations exceeding 100,000 ng/ml have MPM and that it was possible to make a clinical diagnosis using a practical approach for most patients that did not have MPM, even among those with HA concentrations exceeding 100,000 ng/ml. Furthermore, we found that differentiating between MPM and BAP requires thoracoscopic exploration. In fact, approximately 10% of the patients with BAP presented with an HA concentration exceeding 100,000 ng/ml, and thus, physicians should proceed carefully when making a differential diagnosis between MPM and BAP.

In Japan, patients with MPM should be compensated by worker's compensation or "the relief benefits." The approval of compensation requires pathological diagnostic confirmation with a biopsy specimen. However, MPM usually develops after a long latent period of approximately 30–40 years, and thus, most cases are discovered in elderly patients with concomitant medical problems that disallow invasive diagnostic procedures. In such cases, the approval of compensation should be based on clinical features, including imaging findings and/or pleural fluid analysis. Our results indicate that HA in pleural fluid could be one of the features used to approve MPM compensation.

In the current study, we also demonstrated 2 important features of HA concentrations in patients with MPM. HA levels were higher for the epithelioid subtype than for the sarcomatous or biphasic subtype, indicating that a more accurate diagnostic marker is needed for the detection of sarcomatous MPM. We also found that HA levels decreased in the advanced stage of the disease, indicating that HA determination might only be useful in the early stage of MPM. The source of HA in MPM has been a subject of controversy. Asplund et al. proposed that normal mesothelial cells rather than mesothelioma cells were the source of HA and that the secretion of HA into pleural fluid was induced by growth factors [27]. We consider this one of the reasons why HA levels do not correlate with the clinical stage of MPM. A further understanding of the mechanisms of HA production in MPM will be essential for better understanding the carcinogenesis of MPM.

5. Conclusion

We have demonstrated that the HA concentration in pleural fluid is higher in patients with MPM than in those with other pulmonary diseases. Physicians should consider the possibility of MPM in patients with elevated HA levels.

Conflict of interest

The authors have no potential conflicts of interest.

Acknowledgments

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ORIGINAL ARTICLE

Treatment and survival analyses of malignant mesothelioma in Japan

KENICHI GEMBA[1,*], NOBUKAZU FUJIMOTO[1], KEISUKE AOE[2], KATSUYA KATO[3], YUKIO TAKESHIMA[4], KOUKI INAI[4] & TAKUMI KISHIMOTO[1]

[1]*Department of Respiratory Medicine, Okayama Rosai Hospital, Okayama, Japan,* [2]*Department of Medical Oncology, National Hospital Organization Yamaguchi-Ube Medical Center, Ube, Japan,* [3]*Department of Radiology, Okayama University Hospital, Okayama, Japan and* [4]*Department of Pathology, Hiroshima University Graduated School of Medicine, Hiroshima, Japan*

Abstract

Background. There are few reports concerning treatment strategies and their contributions to survival of patients with malignant mesothelioma (MM) in Japan. *Material and methods.* We extracted all death cases due to MM between 2003 and 2008. The diagnosis of MM was confirmed in 929 cases. Histological subtypes was determined in 709 cases, including 396 (55.9%) epithelioid, 154 (21.7%) sarcomatoid, 126 (17.8%) biphasic, and 33 (4.7%) other types. *Results and conclusion.* Median overall survival (OS) of all MM cases was 7.7 months (95% confidence interval, 7.1–8.3). Median OS of patients with epithelioid MM was significantly longer than that of patients with biphasic ($p = 0.030$) or sarcomatoid ($p < 0.001$) MM. Surgical resection was performed in 172 patients (18.5%) and 449 (48.3%) received systemic chemotherapy. Survival of patients treated with both surgery and systemic chemotherapy was favorable. Median OS of patients in the late phase of the study period (2006–2008) was significantly longer than that in the early phase (2003–2005) (8.1 vs. 7.5 months, $p = 0.008$). Independent favorable prognostic factors included age younger than 70 years, female gender, epithelioid subtype, and clinical stage I–III. Multivariate analysis demonstrated that patients who had radical surgery and systemic chemotherapy showed a longer survival, though this could be due to selection bias of patients.

Malignant mesothelioma (MM) is an aggressive tumor that develops from mesothelial cells of the pleura, peritoneum, pericardium, or testicular tunica vaginalis. It is generally associated with a history of asbestos exposure [1] and has a very poor prognosis [2]. Management of MM is controversial, and there is no definitive standard of care. Until recently, individual modalities such as surgery, radiotherapy, and chemotherapy have failed to prolong survival. Intensive multi-modality treatment including extrapleural pneumonectomy (EPP), systemic chemotherapy, and radiotherapy has been evaluated at several institutions with encouraging results [3]. However, this approach is feasible only for a small proportion of patients. Reported adverse predictors of survival in patients with MM are sarcomatoid histology, older age, advanced International Mesothelioma Interest Group (IMIG) stage, and absence of surgery or chemotherapy [4]. However, most of this evidence is reported from Europe, and there are few reports concerning the clinical features of MM in Japan. A newspaper article published in June 2005 reported that five residents who lived near the now-closed asbestos cement pipe plant in Amagasaki, Japan, developed pleural mesothelioma. Since this report, asbestos-related problems have raised significant social concern. We performed a nationwide retrospective survey to investigate all MM cases in Japan. As a result, we analyzed more than 6000 MM cases registered in the Vital Statistics yearly survey carried out by the Japanese Ministry of Health, Labour, and Welfare, between 2003 and 2008. To our knowledge, this is the largest study concerning MM in Japan. We have already reported the clinical features of MM [5]. We confirmed that more than 70% of MM cases in Japan were associated with asbestos exposure.

*Current address: Department of Respiratory Medicine, National Hospital Organization Fukuyama Medical Center, 4–14-17 Okinogamicho, Fukuyama 7208520, Japan.

Correspondence: N. Fujimoto, Department of Respiratory Medicine, Okayama Rosai Hospital, 1–10-25 Chikkomidorimachi, Minamiku, Okayama 7028055, Japan. Tel: +81 86 2620131. Fax: +81 86 2623391. E-mail: nfuji@okayamaH.rofuku.go.jp

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In this paper, we report the treatment and survival data of patients with MM in Japan. The aim of this study was to characterize the treatment strategy and its contributions to survival. Changes in treatment modality and survival data within the study period were examined. Prognostic factors including age, gender, histological subtype, and treatment modality are also analyzed.

Material and methods

Patients

Methods of this retrospective survey were previously described [5]. In brief, we requested and received authorization to view the death records of Vital Statistics in Japan, and extracted all cases of death due to MM between 2003 and 2008. There were 6030 deaths due to MM. We contacted the closest living relatives of each case to obtain consent for our study by postal mail. As a result, the informed consent was obtained by postal mail from 2069 cases (34.3%). Based on authorization from relatives, we contacted each patients' medical institution to obtain information including medical records, x-ray films, and/or computed tomographic (CT) images by postal mail. Data from 1111 cases were obtained. We reviewed the medical records and radiological images to confirm the clinical and pathological diagnosis of MM. As a result, the clinical diagnosis of MM was confirmed in 929 cases, including 753 males (81.1%) and 176 females (18.9%). Median age at diagnosis of MM was 67.0 years (range, 16–94). The origin of MM was the pleura in 794 cases (85.5%), peritoneum in 123 cases (13.2%), pericardium in seven cases (0.8%), and testicular tunica vaginalis in five cases (0.5%). Histological subtypes of MM based on World Health Organization criteria [6] was determined in 709 cases, included 396 (55.9%) epithelioid, 154 (21.7%) sarcomatoid, 126 (17.8%) biphasic, and 33 (4.7%) other types. The clinical stage of pleural MM was determined according to IMIG criteria [7] in 603 cases, including 172 cases (28.5%) of stage I and II, 279 cases (46.3%) of stage III, and 152 cases (25.2%) of stage IV.

Statistical analysis

Mean values were compared using the t-test. Comparisons between independent groups were performed using χ^2-test. Survival time was defined as the period from diagnosis to death. Survival curves were calculated using the Kaplan and Meier method. The log-rank test was used to evaluate differences in survival. The Cox proportional hazard model was employed for multivariate analysis. Statistical calculations were performed using SPSS statistical package version 11.0 (SPSS Inc., Chicago, USA).

Results

Treatment of MM

Treatment modalities in 929 MM cases were reviewed. Among them, radical surgical resection was performed in 172 cases (18.5%) and systemic chemotherapy was delivered in 449 cases (48.3%). In 374 cases (40.3%), only palliative treatment was given. Radical surgical resection was performed in 154 (19.4%) of the 794 cases of pleural MM. Among them, EPP was performed in 103 cases (66.9%). Systemic chemotherapy was delivered in 386 cases (48.6%). Among patients that underwent EPP, systemic chemotherapy was delivered in 80 cases (51.9%) as either neoadjuvant therapy, adjuvant therapy, or at disease relapse.

The chemotherapy regimen of the initial treatment was identified in 432 cases. Among them, platinum-based chemotherapy was delivered in 337 cases (78.0%), non-platinum combination chemotherapy in 48 cases (11.1%), and non-platinum monotherapy in 47 cases (10.9%). Platinum-based regimens included cisplatin/gemcitabine in 134 cases (39.8%), cisplatin/pemetrexed in 74 cases (22.0%), carboplatin/gemcitabine in 54 cases (16.0%), carboplatin/paclitaxel in 15 cases (4.5%), and others. The non-platinum combination regimen delivered most frequently was gemcitabine/vinorelbine (37/48, 77.1%). Gemcitabine was the most frequently used non-platinum monotherapy (27/47, 57.4%).

Next, we examined the changes in treatment modalities over time. The proportion of patients treated with surgical resection was 18.2% (110/605 cases) in the early phase (2003–2005) of the study and 19.1% (62/324 cases) in the late phase (2006–2008). The proportion of patients treated with systemic chemotherapy significantly increased from 42.6% (258/605 cases) in the early phase to 59.0% (191/324 cases) in the late phase ($p < 0.001$). The proportion of patients receiving palliative treatment alone decreased from 43.5% (263/605 cases) in the early phase to 34.3% (111/324 cases) in the late phase ($p = 0.005$).

Survival of MM

Among the 929 patients with MM, date of diagnosis was unknown in eight cases; survival analysis was performed on the remaining 921 cases. Median overall survival (OS) was 7.7 months [95% confidence interval (CI), 7.1–8.3]. Median (95% CI) OS of patients with pleural MM (n = 789) was 7.9 (7.3–8.5)

months and longer than that of patients with peritoneal MM [4.7 (3.8–5.7); n = 123], but the difference was not statistically significant (p = 0.069). Median (95% CI) OS of epithelioid, biphasic, and sarcomatoid types of MM was 9.4 (7.1–10.7), 7.9 (6.6–9.2), and 4.1 (3.2–5.0) months, respectively. Median OS of epithelioid MM was significantly longer than both biphasic (p = 0.030) and sarcomatoid (p < 0.001) MM, and median OS of biphasic MM was significantly longer than that of sarcomatoid MM (p < 0.001).

Next, survival was analyzed according to treatment modality. For this purpose, patients were divided into those treated with both radical surgery and systemic chemotherapy (group A), those with radical surgery but no systemic chemotherapy (group B), those with systemic chemotherapy but no radical surgery (group C), and those without radical surgery or systemic chemotherapy (group D). Median (95% CI) OS was 15.1 (12.0–19.0), 8.6 (6.6–10.5), 9.3 (8.4–10.2), and 4.1 (3.4–4.8) months in group A, B, C, and D, respectively. Survival of group A was significantly longer than that of the other groups (A vs. B; p = 0.018, A vs. C and A vs. D; p < 0.001) and the survival of group D was significantly shorter than that of group B (p = 0.008) and group C (p < 0.001).

Survival of pleural MM

We next analyzed survival of patients with pleural MM only. Histological subtype was determined in 607 cases. Median (95% CI) OS was 10.2 (8.9–11.6), 8.0 (6.2–9.8), and 4.2 (3.0–5.5) months for the epithelioid (n = 325), biphasic (n = 111), and sarcomatoid (n = 141) subtype, respectively. OS was significantly shorter for the sarcomatoid subtype compared to both epithelioid (p < 0.001) and biphasic subtypes (p < 0.001) (Figure 1). According to IMIG stage, median (95% CI) OS was 11.2 (9.4–13.0), 7.9 (7.1–8.7), and 3.9 (3.0–4.6) months for stages I and II, stage III, and stage IV, respectively. OS of stage III patients was significantly shorter than that of stage I and II (p = 0.001); OS of stage IV patients was significantly shorter than that of stage III (p < 0.001). Patients were then divided according to treatment as those receiving radical surgery and systemic chemotherapy (group E), radical surgery but no systemic chemotherapy (group F), systemic chemotherapy but no radical surgery (group G), and without radical surgery or systemic chemotherapy (group H). Median (95% CI) OS was 15.1 (11.6–18.6), 8.8 (6.7–10.8), 9.4 (8.4–10.4), and 4.3 (3.3–5.3) in group E, F, G, and H, respectively (Figure 2). Survival of group E was significantly longer than that of the other groups (E vs. F; p = 0.035, E vs. G and E vs. H; p < 0.001) and survival of group H was significantly shorter than that of group F (p = 0.024) and group G (p < 0.001).

Then we analyzed the survival according to the chemotherapy regimen. Median OS of cisplatin/gemcitabine, carboplatin/gemcitabine, and cisplatin/pemetrexed was 10.2, 10.8, and 7.8 months, respectively. The survival of cisplatin/gemcitabine was statistically superior to that of cisplatin/pemetrexed (Hazard ratio 1.62, 95% CI 1.06–2.45). The survival of group F was similar to that of cisplatin/gemcitabine (Hazard ratio 0.988, 95% CI 0.97–1.00).

Changes in survival of MM

OS according to year of death was analyzed. Median (95% CI) OS was 6.9 (5.8–8.0), 7.3 (6.1–8.5), 8.3 (6.3–10.3), 8.4 (6.7–10.0), 8.8 (6.8–10.8), and

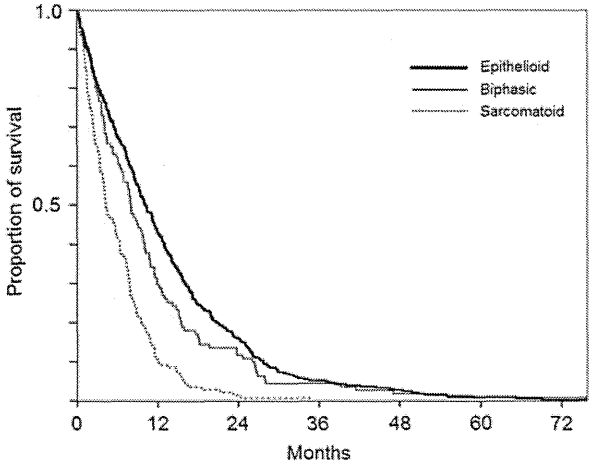

Figure 1. Overall survival of patients with malignant pleural mesothelioma according to histological subtype.

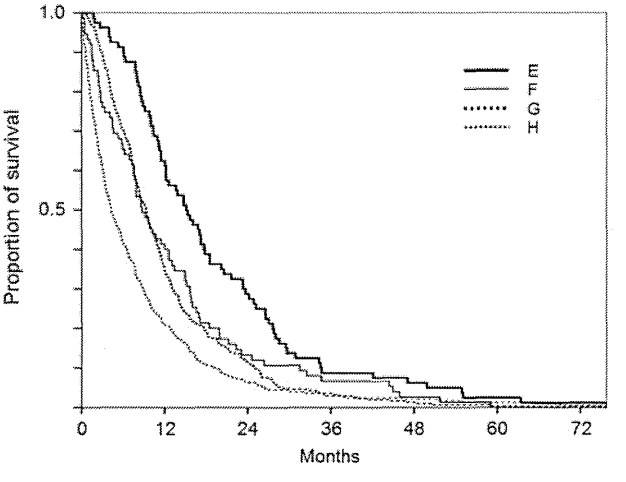

Figure 2. Overall survival of patients with malignant pleural mesothelioma according to treatment modality. E. both radical surgery and systemic chemotherapy, F. radical surgery but no systemic chemotherapy, G. systemic chemotherapy but no radical surgery, and H. without radical surgery or systemic chemotherapy.

7.2 (5.9–8.6) months for patients who died in 2003, 2004, 2005, 2006, 2007, and 2008, respectively. OS of patients who died in 2005, 2006, and 2007 was significantly longer than those died in 2003. Median (95% CI) OS of patients who died in the late phase (n = 321) was significantly longer than that of patients who died in the early phase (n = 600) [8.1 months (7.1–9.0) vs. 7.5 months (6.7–8.3), p = 0.008] (Figure 3).

Prognostic factor analyses

In univariate log-rank analysis age <70 years, epithelioid subtype, and clinical stage I–III were favorable prognostic factors (Table I). These factors and female gender were independent favorable factors on multivariate Cox regression analysis. Further multivariate analysis was conducted to clarify the contribution of radical surgery and systemic chemotherapy to the survival of pleural MM, these factors were included in the analysis as explanatory variables. Both modalities contributed to longer survival (Table II).

Discussion

We analyzed the treatment and survival data of patients with MM in Japan. The aim of this study was to characterize the treatment modalities, survival, and risk factors affecting OS. There were about 6000 deaths due to MM in our study period. We contacted the closest living relatives of each case to obtain consent for our study by postal mail. And we contacted patients' medical institution to obtain medical information including medical records, x-ray films, and/or CT images by postal mail. Data from 1111 cases were obtained. We have to accept the low collection rate of postal mail method; however, there is no selection bias through the process of our data collection. We determined the treatment and survival data of 929 MM patients. To our knowledge, this is the largest study conducted in Japan and one of the largest studies of MM worldwide.

We demonstrated that MM carries a poor prognosis with a median OS of only 7.7 months in Japan. Median OS of epithelioid MM was significantly longer than either of biphasic or sarcomatoid MM, and median OS of biphasic MM was significantly longer than that of sarcomatoid MM. We also found that female gender, age under 70 years, epithelioid subtype, and clinical stage I–III were favorable prognostic factors. In previous reports, the Cancer and Leukemia Group B (CALGB) [8] identified pleural involvement, elevated lactate dehydrogenase, poor performance status, chest pain, thrombocytosis, non-epithelial histology, and age older than 75 years as poor prognostic factors. The European Organization for Research and Treatment of Cancer (EORTC) [9,10] reported poor performance status, probable diagnosis of MM, leukocytosis, male gender, and sarcomatoid subtype as indicators of poor prognosis. In the Surveillance, Epidemiology, and End Results (SEER) Program [4], the outcomes for 1475 patients with histologically confirmed MM were analyzed;

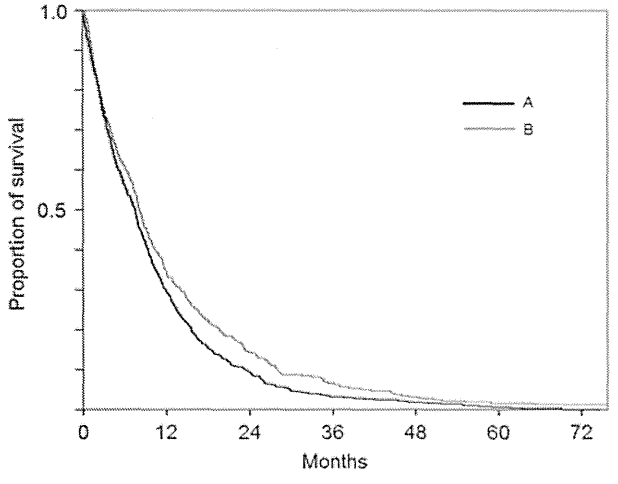

Figure 3. Overall survival of patients with malignant mesothelioma according to year of death. A. patients died between 2006 and 2008, B. patients died between 2003 and 2005.

Table I. Univariate analysis of clinical variables and survival of patients with malignant pleural mesothelioma (Log-rank test).

Factor	N	MST§ (95% CI)	p
Gender			0.6435
Male	654	7.9 (7.2–8.5)	
Female	135	7.7 (6.3–9.0)	
Age			<0.001
<70	438	10.0 (8.6–11.4)	
≥70	351	6.1 (5.0–7.1)	
Subtype			<0.001
Epithelioid	326	10.0 (8.4–11.5)	
Non-epithelioid	251	6.3 (5.1–7.5)	
Stage			<0.001
I–III	451	9.4 (8.5–10.3)	
IV	152	3.8 (3.0–4.6)	

CI, confidence interval. §Median survival time (months).

Table II. Multivariate analysis of clinical variables and survival of patients with malignant pleural mesothelioma.

Factor	Exp (β)	95% CI	p
Gender	1.55	1.20–2.01	0.001
Age	1.28	1.05–1.57	0.016
Subtype	1.74	1.45–2.09	<0.001
Stage	1.83	1.48–2.27	<0.001
Surgery	1.31	1.03–1.66	0.026
Chemotherapy	1.58	1.31–1.91	<0.001

CI, confidence interval.

the most important prognostic factors identified were age, gender, tumor stage, treatment, and geographic area of residence. Our results confirmed that the characteristics of MM in Japan are similar to those in the US and Europe.

Regarding treatment modalities, both radical surgery and systemic chemotherapy contributed to prolonged survival of MM based on multivariate prognostic analysis. These results should be interpreted with caution, as choice of therapy is potentially biased by age, clinical stage, physical condition, and other factors. The role of surgical resection in the management of MM is controversial. Among surgical procedures, EPP, in which the lung and ipsilateral parietal pleura, pericardium, and hemidiaphragm are resected, is considered the only procedure of curative content. It is usually integrated in a multimodality strategy combined with chemotherapy and radiotherapy. In the largest series of the trimodality consisting of EPP, adjuvant chemotherapy, and radiotherapy, median survival was 19 months, and five-year survival was 15%. The best outcome was a median survival of 51 months and five-year survival of 46%, obtained in the subset of the patients with epithelial histology, no extrapleural lymph node metastasis, and negative margins [3]. However, Treasure et al. [11] argued that the primary reason for these positive results was patient selection, and they recently reported a lack of survival advantage with EPP [12]. In our study, patients treated with surgery alone demonstrated almost equivalent survival as those receiving systemic chemotherapy alone. Based on currently available data, we believe that surgery should be applied within an integrated, multimodality strategy combined with chemotherapy and radiotherapy, and physicians should be extremely careful about the indication.

In our analysis, median OS of patients who died in the late phase of the study (2006–2008) was significantly longer than that of patients who died in the early phase (2003–2005). We have two explanations for this survival prolongation. One is increased prevalence of definitive diagnostic confirmation of MM. As we described in a previous report [5], there were many more cases in the early phase in which MM was diagnosed clinically based on radiologic or laboratory findings, without pathological confirmation. For most of these cases, only palliative treatment was delivered even for patients with good performance status (PS). The number of these 'suspected-MM' cases decreased in the late phase, possibly due to increased use of accurate pathological techniques such as immunohistochemical analysis [13] and the widespread dissemination of less-invasive diagnostic procedures such as thoracoscopy [14] or laparoscopy. The increase in definitive diagnosis of MM would result in an increased number of cases diagnosed at an earlier stage and in patients with good PS. As a result, the proportion of the patients treated with palliative management alone decreased from 43.5% in the early phase to 34.3% in the late phase. These changes might contribute to the prolongation of survival seen here. Another possibility is the impact of systemic chemotherapy. Platinum-based regimens consisting of cisplatin/gemcitabine or carboplatin/gemcitabine was mainly delivered with limited efficacy [15] before the approval of the antifolate agent pemetrexed in 2007 in Japan. Since then, combination therapy with platinum and pemetrexed is considered a standard regimen based on favorable clinical trial results [16,17]. At the same time, the proportion of cases treated with systemic chemotherapy significantly increased from 42.6% in the early phase to 59.0% in the late phase. The introduction of pemetrexed might explain the increased application of chemotherapy, and contribute to the longer survival in the later phase of the study. In our analysis, the OS of cisplatin/pemetrexed was inferior to that of cisplatin/gemcitabine. This is because our analysis included patients who had died from 2003 to 2008 and pemetrexed was approved in 2007 in Japan, hence the long survivors of cisplatin/pemetrexed were not included.

In conclusion, we report treatment and survival data of patients with MM in Japan. The survival rate has increased due to improved definitive diagnosis and more widespread treatment. Novel approaches for the early diagnosis and improved treatment strategies, based on the biology of the disease, are essential to further improve survival.

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Integrated analysis of genetic and epigenetic alterations reveals CpG island methylator phenotype associated with distinct clinical characters of lung adenocarcinoma

Keiko Shinjo[1,2], Yasuyuki Okamoto[1], Byonggu An[1], Toshihiko Yokoyama[3], Ichiro Takeuchi[4], Makiko Fujii[1], Hirotaka Osada[1,2], Noriyasu Usami[5], Yoshinori Hasegawa[3], Hidemi Ito[6], Toyoaki Hida[7], Nobukazu Fujimoto[8], Takumi Kishimoto[8], Yoshitaka Sekido[1,2] and Yutaka Kondo[1,9,*]

[1]Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan, [2]Department of Cancer Genetics, [3]Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan, [4]Graduate School of Engineering, Nagoya Institute of Technology, Nagoya, Japan, [5]Division of General Thoracic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan, [6]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan, [7]Department of Thoracic Oncology, Aichi Cancer Center Hospital, Nagoya, Japan, [8]Department of Respiratory Medicine, Okayama Rosai Hospital, Okayama, Japan and [9]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama, Japan

*To whom correspondence should be addressed. Tel: +81 52 764 2994; Fax: +81 52 764 2994;
Email: ykondo@aichi-cc.jp

DNA methylation affects the aggressiveness of human malignancies. Cancers with CpG island methylator phenotype (CIMP), a distinct group with extensive DNA methylation, show characteristic features in several types of tumors. In this study, we initially defined the existence of CIMP in 41 lung adenocarcinomas (AdCas) through genome-wide DNA methylation microarray analysis. DNA methylation status of six CIMP markers newly identified by microarray analysis was further estimated in a total of 128 AdCas by bisulfite pyrosequencing analysis, which revealed that 10 (7.8%), 40 (31.3%) and 78 (60.9%) cases were classified as CIMP-high (CIMP-H), CIMP-low and CIMP-negative (CIMP-N), respectively. Notably, CIMP-H AdCas were strongly associated with wild-type epidermal growth factor receptor (*EGFR*), males and heavy smokers ($P = 0.0089$, $P = 0.0047$ and $P = 0.0036$, respectively). In addition, CIMP-H was significantly associated with worse prognosis; especially among male smokers, CIMP-H was an independent prognostic factor (hazard ratio 1.7617, 95% confidence interval 1.0030–2.9550, $P = 0.0489$). Compellingly, the existence of CIMP in AdCas was supported by the available public datasets, such as data from the Cancer Genome Atlas. Intriguingly, analysis of AdCa cell lines revealed that CIMP-positive AdCa cell lines were more sensitive to a DNA methylation inhibitor than CIMP-N ones regardless of *EGFR* mutation status. Our data demonstrate that CIMP in AdCas appears to be a unique subgroup that has distinct clinical traits from other AdCas. CIMP classification using our six-marker panel has implications for personalized medical strategies for lung cancer patients; in particular, DNA methylation inhibitor might be of therapeutic benefit to patients with CIMP-positive tumors.

Introduction

Lung cancer is the leading cause of human cancer death worldwide (1). Recent targeted therapies have improved the survival of patients with certain types of lung cancer, especially adenocarcinoma (AdCa), a common type of non-small cell lung cancer. AdCas with epidermal growth factor receptor (*EGFR*) mutation benefit particularly from EGFR tyrosine kinase inhibitors (TKIs) (2), whereas those harboring *EML4–ALK* fusion are highly sensitive to anaplastic lymphoma kinase (ALK) inhibitors (3). Despite these recent advances in targeted therapy for AdCas, a considerable number of patients with lung cancer still suffer from recurrence of disease. AdCas without such mutations are generally less sensitive to these targeted therapies than tumors with mutations. Given the evidence that the frequency of *EGFR* mutations account for up to 30% of AdCas, and even *EML4–ALK* fusions are found in AdCas albeit at a lower frequency, elucidating the underlying mechanisms other than such gene alterations in lung carcinogenesis is desirable to facilitate the development of new strategies for lung cancer treatment.

Studies have shown that in addition to genetic alterations, accumulation of epigenetic alterations play an important role in tumorigenesis of lung cancer (4). DNA methylation, an important epigenetic factor, affects the chromatin structure and is closely associated with gene regulation (5). Simultaneous dysregulation of multiple genes, including those involved in cell cycle, cell growth, cell death or cell adhesion, by DNA methylation may be a strong driving force to undergo transformation, sometimes in correlation with potentiated aggressiveness of the tumors (6).

Recent studies in colon cancer have shown that a subset of tumors suffer from a remarkably high rate of aberrant promoter DNA methylation at a large number of loci, referred to as CpG island methylator phenotype (CIMP) (7). CIMP tumors in colon exhibit distinct genetic and clinical features, such as high rates of *BRAF* and *KRAS* mutations, low frequency of *TP53* mutation, specific histology, proximal location and characteristic clinical outcome, suggesting that CIMP-related cancers may proceed through a unique pathway (8). In lung cancer, some studies have shown the existence of a subgroup of tumors with the characteristic methylation status of CIMP (9–13). However, in comparison with the considerable research of CIMP markers performed in colon cancers (7,14–16), no studies have assessed which DNA methylation markers can predict the most extensively methylated subgroups (i.e. CIMP) in lung AdCas due to the lack of accompanied genome-wide DNA methylation analysis in multiple samples. Since different panels of markers may lead to different classification of lung cancer (9,11), it is important to define markers that can accurately identify CIMP.

To examine whether CIMP exists as a characteristic subgroup in AdCas, we initially performed global screening for genes with aberrant DNA hypermethylation by the methylated CpG island amplification microarray (MCAM) analysis, which provides reproducible results with a high validation rate (16–20). Using the six CIMP markers newly identified by MCAM analysis, we characterized a distinct subgroup of AdCas exhibiting CIMP. In addition, several AdCa cell lines with different CIMP status were treated with a DNA methylation inhibitor, and the relationship between DNA methylation status and drug sensitivity was assessed. Our data provide evidence for a new strategy for lung cancer treatment.

Abbreviations: AdCa, adenocarcinoma; CIMP, CpG island methylator phenotype; CIMP-H, CIMP-high; CIMP-L, CIMP-low; CIMP-N, CIMP-negative; EGFR, epidermal growth factor receptor; MCAM, methylated CpG island amplification microarray; TCGA, the cancer genome atlas; TKI, tyrosine kinase inhibitor;

Materials and methods

Cell lines

A549 was purchased from the American Type Culture Collection (Rockville, MD) and PC9 was purchased from Immuno-Biological Laboratories (Fujioka, Gunma, Japan). NCI-H23, NCI-H358, NCI-H920, NCI-H2009, NCI-H1573, NCI-H1650 and HCC4011 were kind gifts from Dr Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX) and Dr Mitsuo Sato (Nagoya University Graduate School of Medicine, Nagoya, Japan). Y-ML13 (ML13) and ACC-H1 (H1) were established in our institute. All cell lines were maintained in RPMI-1640 medium (Sigma–Aldrich, St Louis, MO) supplemented with 10%

fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotic–antimycotic reagent (Invitrogen) at 37°C in a humidified incubator with 5% CO_2.

5-Aza-dC treatment of cells

Cells were treated with 50 nM–1 μM 5-aza-2′-deoxycytidine (5-Aza-dC; Sigma–Aldrich) as described previously (17) or the TKI AG1478 (Calbiochem, San Diego, CA) for 72 h. DNA was extracted on the seventh day following treatment. Changes in proliferation were determined by using the TetraColor ONE (Seikagaku, Tokyo, Japan) system, containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt and 1-methoxy-5-methylphenazinium methylsulfate as the electron carrier. After 1 h incubation at 37°C, absorbance was read at 450 nm with a multiplate reader. Growth inhibition was expressed as a mean ratio of absorbance reading from treated versus untreated cells. Cell numbers were also counted under a light microscope at the same time point.

Tissue samples

We collected 128 AdCa samples and 26 normal lung tissues from patients who underwent surgical resection at the Aichi Cancer Center Central Hospital, Okayama Rosai Hospital, Nagoya University Hospital and its affiliated hospitals in Japan, in accordance with the institutional policy. Samples and clinical data were collected after appropriate institutional review board approval was received and written informed consent had been obtained from all the patients. Histological and cytological examination of normal lung tissues, which were obtained from the lung cancer patients, revealed no remarkable findings as malignant tissues. In the normal tissues, no aberrant methylation was detected in 10 genes by pyrosequencing analysis (Supplementary Table 1, available at *Carcinogenesis* Online). A sample size of 124 patients was calculated to be sufficient to provide a survival rate difference of 25% with a significance level of 0.05 and power of 80%, when the frequency of CIMP-AdCa was estimated to be ~20% as is observed in colorectal cancer (8); therefore, we collected 128 AdCas to analyze the significance of CIMP (Table I).

DNA preparation

Genomic DNA was extracted using a standard phenol–chloroform method. Fully methylated DNA was prepared by treating genomic DNA with *Sss*I methylase (New England Biolabs, Ipswich, MA), and unmethylated DNA was prepared by treating genomic DNA with phi29 DNA polymerase (GenomiPhi DNA Amplification Kit; Amersham Biosciences, Uppsala, Sweden) according to the manufacturers' protocol.

DNA methylation analysis

We performed bisulfite treatment as described previously (21,22).The DNA methylation levels were measured using pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden). Primer sequences and polymerase chain reaction conditions are shown in Supplementary Table 2, available at *Carcinogenesis* Online. All the primers were designed to examine the methylation status of CpGs within 0.5 kb of the transcription start site. A methylation level >15% was considered methylation positive since lower values could not be easily distinguished from background (17–20).

Methylated CpG island amplification microarray

For MCAM analysis, we randomly selected 41 cases from the 128 AdCas without any bias (average age was 61.9 years, ranging from 36 to 83 years old; Table I). A detailed protocol of MCA has been described previously (16–20). Briefly, amplicons from individual AdCas were labeled with Cy5 dye and cohybridized against amplicons from normal controls labeled with Cy3 dye on 15 K custom-promoter microarrays from Agilent Technologies (G4497A; Agilent Technologies, Santa Clara, CA) containing 6157 unique genes, which we had initially validated in a previous study (17). A Cy5/Cy3 signal in excess of 2.0 in MCAM was considered methylation positive (17–19). Comparison of the MCAM signal ratio (Cy5/Cy3 > 2.0 or Cy5/Cy5 ≤ 2.0) with the methylation status (positive or negative) from the pyrosequencing analysis showed a good concordance between the two analyses (sensitivity, 68.0% and specificity, 88.7%; Supplementary Table 3, available at *Carcinogenesis* Online).

Hierarchical clustering analysis

Cluster analysis was performed using an agglomerative hierarchical clustering algorithm (18,23). For specimen clustering, pairwise similarity measures among specimens were calculated using the Cluster 3.0 software (http://rana.lbl.gov/EisenSoftware.htm) or Minitab 15 statistical software (http://www.minitab.com), based on the DNA methylation intensity measurements

Table I. Clinical and molecular features according to the CIMP status

	All cases (%)	CIMP-N (%)	CIMP-L (%)	CIMP-H (%)	*P* value
Cases	128 (100)	78 (60.9)	40 (31.3)	10 (7.8)	
Age (mean ± SD)	64.7 ± 9.8	63.9 ± 10.0	66.4 ± 9.8	63.8 ± 7.0	0.4043
Gender					
Male	71 (55.5)	35 (44.9)	27 (67.5)	9 (90.0)	0.0047
Female	57 (44.5)	43 (55.1)	13 (32.5)	1 (10.0)	
Stage[a]					
I	75 (59.5)	51 (67.1)	21 (52.5)	3 (30.0)	0.2259
II	19 (15.1)	7 (9.2)	8 (20.0)	3 (30.0)	
III	29 (23.0)	16 (21.1)	10 (25.0)	4 (40.0)	
IV	3 (2.4)	2 (2.6)	1 (2.5)	0 (0)	
Smoking status					
Heavy smoker	41 (32.3)	21 (27.6)	12 (30.8)	8 (80.0)	0.0036
Light smoker	29 (22.8)	15 (19.7)	13 (33.3)	1 (10.0)	
Never-smoker	57 (44.9)	42 (55.3)	14 (35.9)	1 (10.0)	
Differentiation[a]					
Well	15 (17.4)	11 (21.2)	3 (10.7)	1 (16.7)	0.3552
Moderate	55 (64.0)	30 (57.6)	22 (78.6)	3 (50.0)	
Poorly	16 (18.6)	11 (21.2)	3 (10.7)	2 (33.3)	
Recurrence[a]					
(−)	41 (42.3)	27 (43.5)	14 (46.7)	0 (0)	0.1394
(+)	56 (57.7)	35 (56.5)	16 (53.3)	5 (100)	
EGFR mutation					
(−)	80 (62.5)	42 (53.8)	28 (70.0)	10 (100)	0.0089
(+)	48 (37.5)	36 (46.2)	12 (30.0)	0 (0)	
KRAS mutation					
(−)	118 (92.2)	74 (94.9)	36 (90.0)	8 (80.0)	0.2113
(+)	10 (7.8)	4 (5.1)	4 (10.0)	2 (20.0)	
p53 mutation[a]					
(−)	89 (69.5)	57 (74.0)	28 (73.7)	4 (40.0)	0.16
(+)	35 (27.3)	20 (26.0)	10 (26.3)	5 (50.0)	
BRAF mutation					
(−)	86 (100)	56 (100)	23 (100)	7 (100)	NA
(+)	0 (0)	0 (0)	0 (0)	0 (0)	

[a]Data were not available in some cases. Recurrence was observed within 5 years after surgery.

across all genes. *K*-means consensus clustering was performed with the R statistical package. A dendrogram and heat map were constructed using either TreeView (http://rana.lbl.gov/EisenSoftware.htm) or R statistical computing environment (http://cran.r-project.org).

Nearest neighbor classification

Using the DNA methylation status of six CIMP markers (positive or negative by pyrosequencing analysis), nearest neighbor classification was employed to classify the validation set consisting of 87 independent AdCas (24). In this analysis, each validation case was classified into one of the three clusters identified in the training set. The number of nearest neighbors was set as $k = 4$ because the smallest cluster (cluster 1) was consisted of four cases. The analysis was conducted using R statistical computing environment (http://cran.r-project.org).

Mutation analysis

Mutations in *KRAS* (codons 12 and 13) were analyzed by direct sequencing and the pyrosequencing method (25,26). *EGFR* mutations (exons 18–21) and *TP53* mutations (exons 5–8) were examined using direct sequencing (19,25). Mutation of *BRAF* (codon 600) was determined by the pyrosequencing method as previously reported (27). The polymerase chain reaction primer sequences used are listed in Supplementary Table 2, available at *Carcinogenesis* Online.

The Cancer Genome Atlas data

We obtained the methylation data of AdCa samples from the The Cancer Genome Atlas data (TCGA) web site (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp), and data of 85 AdCa samples (batches 34 and 37) were included in the analysis, which was conducted using the Illumina Infinium Human DNA Methylation 27 platform. The 3833 most variant probes from 27 578 CpG dinucleotides were used for further analysis, and a β value > 0.4 was considered as methylation positive.

Statistical analysis for clinical features

All statistical analyses were performed using the JMP statistical software version 5.1 (SAS Institute, Cary, NC). Fisher's exact test was used to determine non-random associations between two categorical variables. Kruskal–Wallis analysis was used to evaluate the extent of differences among more than three groups. All reported *P* values are two sided, with $P < 0.05$ taken as statistically significant. Patients were followed until incidence of death or until October 2010, whichever came first. Survival information was available for 118 of the 128 cases. Overall survival was calculated from the date of surgery until the date of death or the date the patient was last known to be alive (censored). The median follow-up time was 42.5 months. Overall survival curves were generated via the Kaplan–Meier method, and the log-rank test was used for statistical analysis. A multivariate analysis using the Cox proportional hazards model was performed to estimate the hazard ratio. All variables for the multivariate analysis were categorical variables (age, stage and CIMP status).

Results

Identification of a distinct subgroup with characteristic DNA methylation profiling in AdCas

First, we evaluated the genome-wide DNA methylation status in a training set of 41 AdCas using MCAM analysis (18–20). Among 6157 genes on the microarray, we selected 1156 genes that are commonly methylated across >10% of AdCas and performed consensus average linkage hierarchical clustering analysis (28). In terms of DNA methylation, AdCas could be divided into three clusters, with clustering stability increasing for $k = 2$ to $k = 3$ but not for more than $k = 3$ (Figure 1A, Supplementary Figure 1, available at *Carcinogenesis* Online). Intriguingly, all four cases in cluster 1 stably fell into the same cluster regardless of *k* values (2–5), whereas 12 cases (92%) and 24 cases (96%) fell into clusters 2 and 3, respectively, indicating a high similarity of their methylation profile among each of the three-cluster member. The number of DNA methylated genes showed bimodal distribution in AdCas; DNA methylation was highly accumulated in two AdCas, both of which fell into cluster 1# (Figure 1B). Consistently, a majority of the genes were commonly methylated across more than half of the AdCas in cluster 1, whereas common methylation targets were detected in ≤50% of the AdCas in clusters 2 and 3 ($P < 0.001$, Figure 1C). In addition, the average number of methylated genes was 766.8 ± 70.4, 485.7 ± 40.6 and 319.2 ± 28.1 in clusters 1, 2 and 3 (k=3), respectively ($P < 0.001$) (Figure 1D). These observations indicated that extensively methylated AdCas exist, which appear to be characterized by correlated CpG island DNA methylation of a subset of genes in a subset of tumors, whereas AdCas with less extensive DNA methylation or with rare DNA methylation were classified into discrete subgroups (7,14–16).

Identification of CIMP markers in AdCas

MCAM analysis suggested the existence of a distinct subgroup with extensive DNA methylation in AdCas. Because CIMP status is closely associated with clinical outcome in forming a distinct subgroup in colon cancer, glioma and breast cancer (29–31), it is useful to accurately identify CIMP tumors without performing microarray analysis, which may reveal the etiology and clinical correlates of CIMP in AdCas. First, we examined whether the DNA methylation status of the classical CIMP markers (*p16*, *MINT1*, *MINT2*, *MINT31* and *MLH1*), which are effective for diagnosis of CIMP in colon cancer (32), reflected the methylation profile, especially CIMP, determined by MCAM analysis in AdCas (Figure 2A, left panel). CIMP-positive AdCas defined by the classical CIMP markers were not consistent with all the extensively methylated AdCas in cluster 1, suggesting that these markers are not always accurate for diagnosis of the extensively methylated AdCas.

To establish a new panel of a minimum number of CIMP markers without reducing the classification power, which was readily applied to a large number of tumor samples, we eliminated the genes from the target genes of methylation in MCAM analysis (Figure 2B). The initial definition of CIMP was based on concordant methylation of Type C loci (cancer-specific methylation) (7). Therefore, we first excluded markers that showed evidence of DNA methylation in normal tissues, which means Type A loci (age-related methylation) (7). Then, we selected 232 genes that were methylated in >75% of AdCas in cluster 1 but were methylated in <30% in the other clusters. Among these genes, 10 genes fulfilled the criteria of (i) concomitant array signals in all the probes for the same genes on the microarray, (ii) methylation-positive probes were located within 500 bp from transcription start sites and (iii) enable to design the stable and reproducible pyrosequencing assay using the candidate genes. Finally, we selected six candidate markers, *CCNA1*, *ACAN*, *GFRA1*, *EDARADD*, *MGC45800* and *p16* (*CDKN2A*), to determine CIMP in AdCas using a statistical model based on recursive descent partition analysis with the pyrosequencing data of 10 genes (33).

DNA methylation status of this panel of six markers was examined in 41 AdCas using pyrosequencing analysis. These six markers were frequently methylated in four (10%) AdCas of cluster 1, which showed DNA methylation in five or more of the six markers (Figure 2A, right panel). We designated this subgroup as CIMP-high (CIMP-H). In contrast, 26 (63%) AdCas were rarely methylated; none or one marker was methylated in this subgroup. The majority of AdCas (21 cases, 81%) in this subgroup fell into cluster 3, which showed the lowest frequency of DNA methylation. We defined AdCas with methylation in none or only one of the six markers as CIMP-negative (CIMP-N). The intermediate subgroup (11 cases, 27%) between CIMP-H and CIMP-N showed DNA methylation in two to four markers of the six selected CIMP markers. We designated this subgroup as CIMP-low (CIMP-L), in which 7 (64%) of 11 AdCas fell into cluster 2 and showed intermediate frequency of DNA methylation.

Validation analysis of newly identified CIMP markers

It is important to note that this initial selection of the six candidate markers did not introduce a bias for detecting CIMP only in the training set. Therefore, the newly identified panel of six markers was independently confirmed in a validation set of 87 AdCas. Among them, we found 6 (7%) CIMP-H, 30 (34%) CIMP-L and 51 (59%) CIMP-N tumors, which were of a similar frequency as observed in the training set of 41 AdCas (Supplementary Figure 2, available at *Carcinogenesis* Online). To estimate whether the classification by the six CIMP markers in the validation set was compatible with the CIMP classification in the training set, we performed the nearest four neighborhood prediction analysis (Figure 2C, see Materials and Methods). This

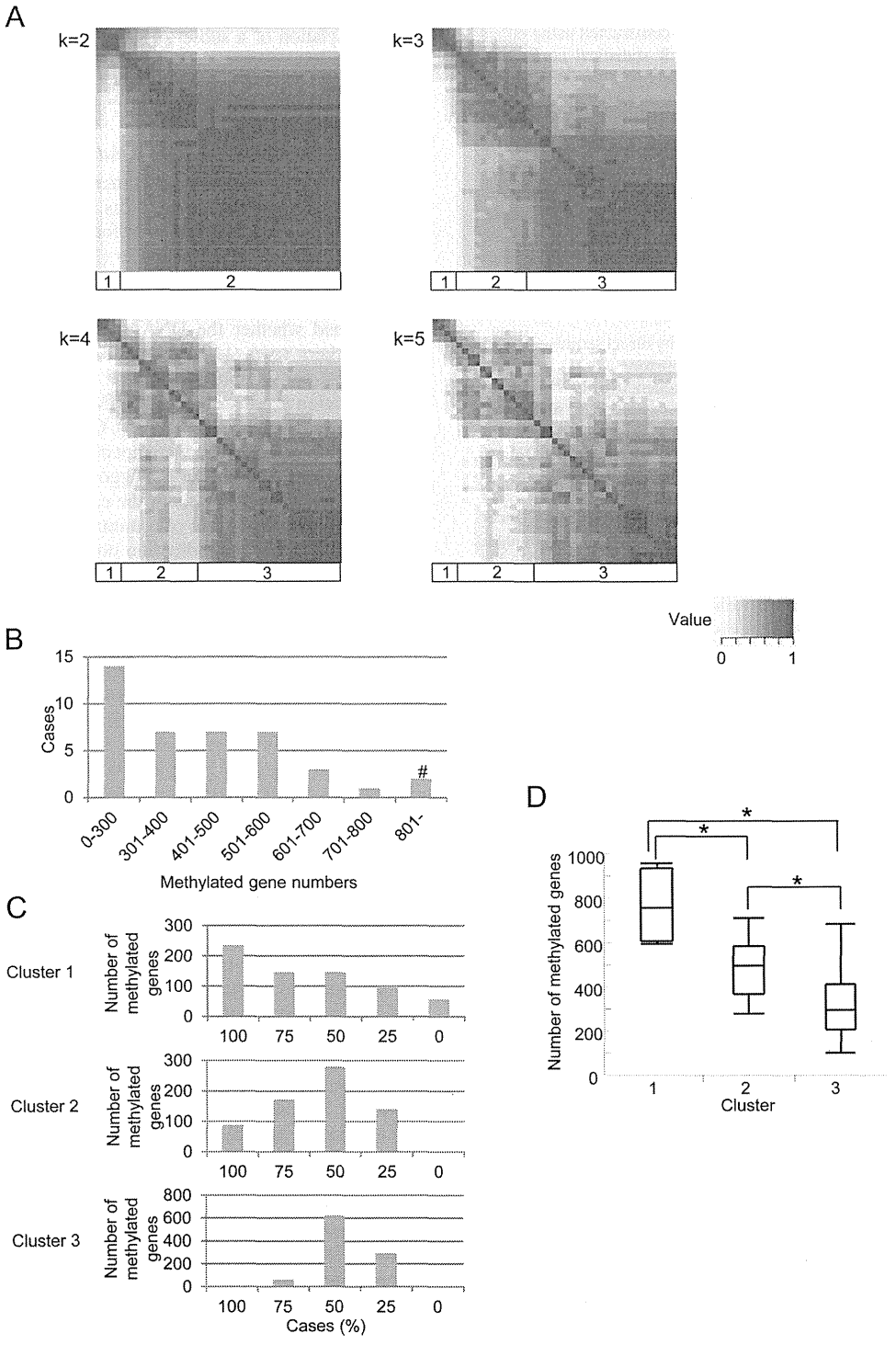

Fig. 1. DNA methylation profiling by MCAM analysis. (**A**) Consensus clustering analysis was performed with the 1156 genes from the AdCa cases for $k = 2, 3, 4$ and 5. The samples are listed in the same order on the *x*- and *y*-axes. Clusters are designated as are indicated at the bottom of each panel. Consensus index values range from 0 (highly dissimilar) to 1 (highly similar). (**B**) The distribution of number of methylated genes. *X*-axis indicates the number of methylated genes. *Y*-axis indicates cases. Two AdCas were highly methylated (#). (**C**) Distribution of number of methylated genes in each cluster. In total of 679 genes, in which methylation was observed in 20–80% of 41 AdCas, were analyzed. (**D**) Box and whisker plots of the number of methylated genes in each cluster. The mean is marked by a horizontal line inside the box whose ends denote the upper and lower quartiles. Error bars represent 5th and 95th percentile values, $^*P < 0.001$.

analysis defined 6 of 87 AdCas in the validation set as cluster 1, all of which were also categorized as CIMP-H tumors according to our criteria using the six-marker panel. Furthermore, 51 AdCas that were classified as cluster 3 with a probability >80% by the nearest neighbor classification analysis were also classified as CIMP-N tumors by our six CIMP marker panel. These results indicate that the three clusters are highly reproducible, and our panel of six markers is capable of accurately categorizing cases into these three clusters.

Identification of AdCa–CIMP in the Cancer Genome Atlas data set

Next, we examined whether the six CIMP markers could also be applicable in classifying AdCas deposited in TCGA (http://tcga-

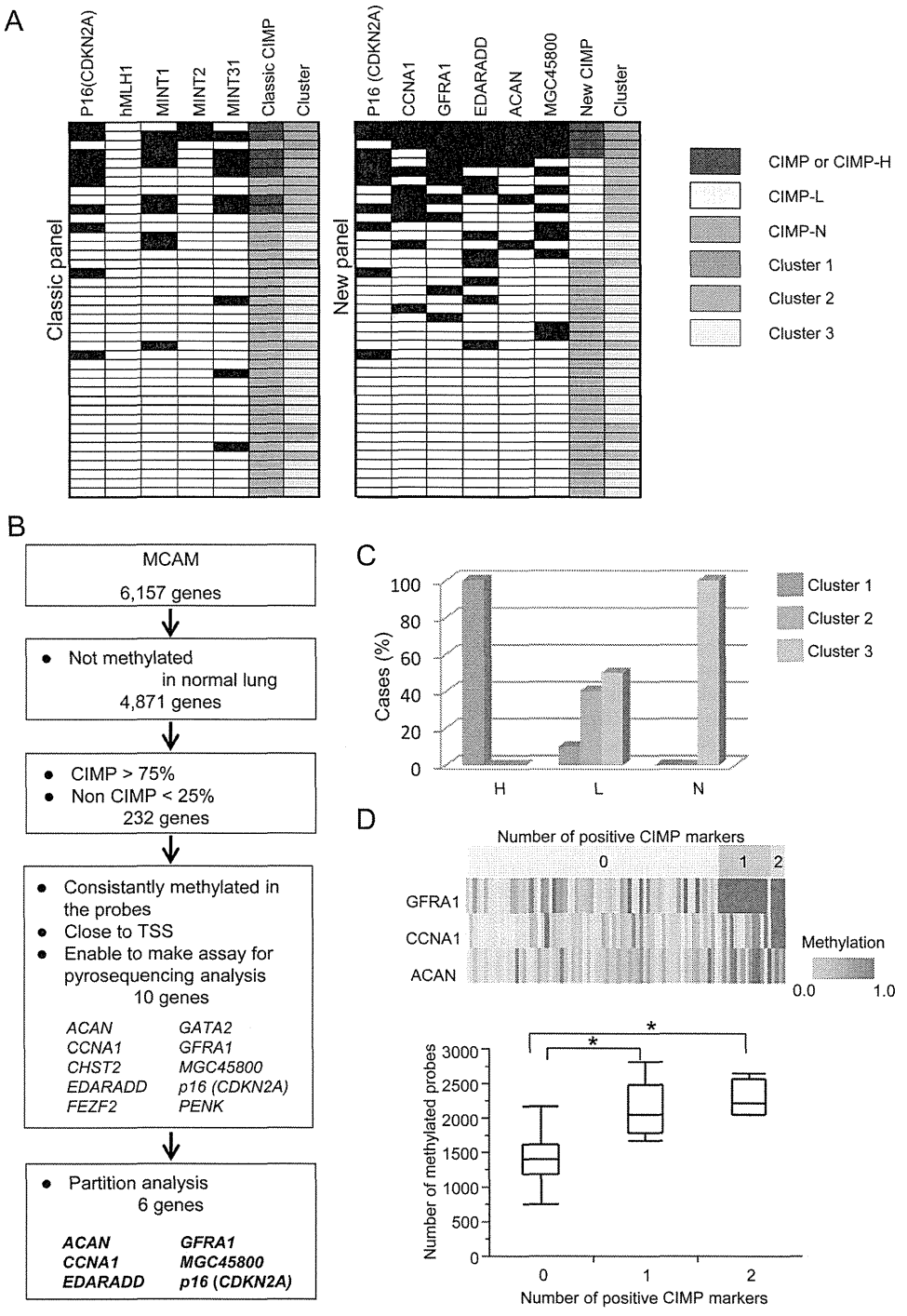

Fig. 2. DNA methylation profiling in AdCa using pyrosequencing analysis. (**A**) Comparison of classification by CIMP markers with the clusters determined by consensus clustering analysis (Figure 1A). Left: tumors with methylation in at least two of the five classical CIMP markers were determined as CIMP. Right: a panel of new CIMP markers classified CIMP-H (five or six of six markers), CIMP-L (two to four of six markers) and CIMP-N (zero or one of six markers), respectively. Black, methylation positive and white boxes, methylation negative. Clusters 1, 2 and 3 correspond to clusters 1, 2 and 3 in Figure 1A. The samples are listed in the same order on the *y*-axis in both panel. (**B**) Schema of selection for a new panel of six CIMP markers. TSS, transcription start site. (**C**) Nearest neighbor classification analysis in the validation set. Relationship between CIMP status determined by six CIMP markers, and the cluster determined by consensus cluster analysis are shown. H, CIMP-H; L, CIMP-L and N, CIMP-N. Clusters 1, 2 and 3 correspond to clusters 1, 2 and 3 in Figure 1A. (**D**) Analysis of DNA methylation status of three CIMP markers, GFRA1, CCNA1 and ACAN using the data set (3833 probes) from the Cancer Genome Atlas data set. Upper panel, heat map overview of three genes in 85 AdCas. Color corresponds to the methylation level as indicated; red is high (β value = 1.0) and yellow is low (β value = 0.0) levels of DNA methylation. Lower panel: relationship between number of methylated probes (β value > 0.4) among 3833 probes and the number of methylated genes of CIMP markers. $^{*}P < 0.001$.

data.nci.nih.gov/tcga/tcgaHome2.jsp) (30). DNA methylation data of 85 AdCas were obtained from the TCGA database, which were analyzed by the Infinium BeadChip containing 27 578 probes corresponding to 14 473 genes. Among those probes, we found that some probes corresponding to three of our new CIMP markers (*ACAN*, *CCNA1* and *GFRA1*) were located close to the regions where we conducted the pyrosequencing analysis. In contrast, no probes corresponding to the other three CIMP markers (*CDKN2A*,

MGC45800 and *EDARADD*) were located within 500 bp of transcription start sites, which was a condition of our pyrosequencing analysis. Therefore, we examined the classification power of the three CIMP markers (*ACAN*, *CCNA1* and *GFRA1*) in the 85 AdCas obtained from the TCGA. None, 4 (4.7%), 14 (16.5%) and 67 (78.8%) AdCas showed DNA methylation in three, two, one or none of the three CIMP markers, respectively (DNA methylation positive, β value > 0.4, Figure 2D, upper panel). AdCas with DNA methylation in one or two CIMP markers had more methylated probes among the 3833 most variant probes than those who had no methylation in the CIMP markers (average methylated probes: 1430.8 ± 39.5, 2116.4 ± 86.3 and 2288.8 ± 161.5, in AdCas with none, one or two methylated CIMP markers, respectively, $P < 0.001$; Figure 2D, lower panel). These data suggested that the methylation status of the three CIMP markers is also predictive for highly methylated AdCas in the TCGA data set.

Clinical significance of CIMP tumors in lung AdCa

Next, we assessed whether CIMP-positive AdCas form a distinct subgroup with characteristic clinical features. We combined two sets of AdCa cohorts (training set and validation set) for the analysis of CIMP signatures, with a total of 128 cases. We did not access any clinical information before CIMP classification of both sets of AdCas to avoid any bias in the analysis of the clinical significance of CIMP tumors. Of the 128 pooled AdCas, 10 (7.8%) were classified as CIMP-H, 40 (31.3%) as CIMP-L and 78 (60.9%) as CIMP-N (Figure 3A). CIMP-H tumors were more prevalent in males ($P = 0.0047$) and associated with frequent exposure to smoking (pack year > 40, $P = 0.0036$). Intriguingly, we found a tight association between CIMP and *EGFR* status ($P = 0.0089$; Table I). None of the 10 CIMP-H AdCas contained any *EGFR* mutations, whereas 36/78 (46.2%) CIMP-N and 12/40 (30.0%) CIMP-L AdCas had *EGFR* mutations. In contrast, no such tendency was observed between CIMP status and *KRAS*, *TP53* and *BRAF* mutations.

To investigate whether CIMP status had any impact on overall survival, we performed Kaplan–Meier survival analysis and found that CIMP-H was a significantly negative prognostic factor ($P = 0.0115$, log-rank test; Figure 3B). Since *EGFR* mutations have been indicated as a potential positive prognostic factor for survival in advanced non-small cell lung cancer patients treated with chemotherapy with or without TKI (34), we analyzed overall survival according to the *EGFR* mutation status. Among the AdCas harboring wild-type *EGFR*, CIMP-H tumors still correlated with poor survival ($P = 0.0312$, log-rank test; Figure 3C). In addition to a worse prognosis in patients with AdCas who were smokers ($P = 0.0373$, log-rank test; Figure 3D), we found that CIMP-H was an independent prognostic factor among male smokers (hazard ratio 1.7617, 95% confidence interval 1.0030–2.9550, $P = 0.0489$; Figure 3E). Taken together, these findings indicated that CIMP-H tumors have unique clinical features that distinguish them from the other AdCas.

Clinical implication of epigenetic therapy for lung AdCa

To evaluate the relationship between CIMP status and effects of the DNA methylation inhibitor, 5-Aza-dC, as an antitumor agent, we first analyzed DNA methylation status of the six CIMP markers in 14 AdCa cell lines, including one CIMP-H (H358), seven CIMP-L (H23, H1, PC9, H2009, H3255, H1975 and H1650) and six CIMP-N cell lines (H920, A549, HCC827, ML13, H1573 and HCC4011) (Supplementary Table 4, available at *Carcinogenesis* Online). CIMP-H cells (H358) harbor wild-type *EGFR*, whereas the CIMP-L and CIMP-N cells harbor either wild-type (H23, H1, H2009, H1975, H920, A549, HCC827, ML13 and H1573) or mutated (PC9, H3255, H1650 and HCC4011) *EGFR*. Regardless of the CIMP status, cells with wild-type *EGFR* showed resistance to the TKI, AG1478 (Figure 4A). Intriguingly, antitumor activity of 5-Aza-dC appeared to be associated with CIMP status. Each cell line showed different IC50, which were significantly lower in the CIMP-positive cells (average, CIMP-H and CIMP-L) than in the CIMP-N cells ($P = 0.02$, average IC50 was 68, 229 and 982 nM, respectively, Figure 4B). To determine a more accurate relationship between DNA demethylation and antitumor activity, we used level of *LINE-1* demethylation, which represents the global level of methylation, as a surrogate marker of 5-Aza-dC treatment. We examined the power of growth inhibition at a concentration of ~20% of *LINE-1* demethylation. Cell growth of CIMP-H and CIMP-L cells was significantly inhibited at each concentration of 5-Aza-dC, in contrast to CIMP-N cells, the majority of which did not respond to the treatment (Figure 4B). These data suggest that in addition to CIMP-H AdCas, tumorigenesis of CIMP-L AdCas may also depend on DNA methylation silencing pathway to some extent.

Discussion

In the current study, we performed a comprehensive genome-wide DNA methylation analysis and identified a distinct molecular subgroup (CIMP-H) in human AdCas. This subgroup showed a remarkably high rate of DNA methylation in correlated cancer-specific CpG island hypermethylation of a subset of genes, indicating the existence of CIMP in AdCas (7).

Previously, studies suggested the existence of CIMP in lung cancer. The first study defined a CIMP-positive case as having a tumor with aberrant methylation in either *CDH13* or *CRBP1* and found that CIMP-positive cases showed poorer prognosis than the CIMP-N ones (9). Although a consistent clinical feature, poor prognosis of CIMP-positive cases, was observed between our study and the previous ones, frequencies of CIMP and the other clinicopathological features associated with CIMP were varied, probably due to the different panels of CIMP marker examined (9,11–13). Thus, it is still unclear which DNA methylation markers can define the most extensively methylated subgroups (CIMP) due to the lack of accompanied genome-wide analysis in the previous AdCa studies. If CIMP affects only a subset of CpG islands in a subgroup of AdCas, collection of data for a large numbers of markers from numerous tumor samples is required to identify CIMP in AdCas. Indeed, we found in the current study that CIMP-positive tumors diagnosed by the original CIMP markers defined in the colon cancer study (7) were not consistent with the extensively methylated AdCas, suggesting that these markers are not always applicable for diagnosis of CIMP tumors other than colon cancers. Thus, the existence of CIMP in AdCas from the global point of view has remained elusive before the current study. Our genome-wide MCAM analysis successfully identified six practical and representative markers for the prediction of CIMP in AdCas.

Integrated analysis of the DNA methylation status of the six CIMP markers with several cancer-associated gene mutations, including *EGFR*, *TP53*, *KRAS* and *BRAF*, revealed that CIMP-H tumors in both the training and the validation sets did not harbor any *EGFR* mutations, suggesting that the two events are mutually exclusive, whereas mutations in the other three genes did not show such strong associations with CIMP status. Thus, our six novel markers enabled us to decipher the negative association between CIMP and *EGFR* mutations, which had been only suggested by previous studies (35). Indeed, CIMP-H tumors are significantly associated with males, frequent exposure to smoking and high relapse rate of disease, which clearly differ from the typical features of *EGFR*-mutant AdCas, such as association with females, non-habit forming smoking and better prognosis. Interestingly, colorectal cancers also show strong association between CIMP status and smoking (36,37). Thus, smoking may be one of the potential causes of CIMP. These data suggested that a particular set of genes are methylated in CIMP-H AdCas, and their target genes are involved in activation of an alternative pathway, in which tumorigenesis may be minimally dependent on *EGFR* mutation.

The association of better clinical outcome with CIMP-positive tumors has been reported in breast cancer, colon cancer and glioma. Fang *et al.* (31) showed that there was significant overlap of CIMP targets in those different types of cancers. Among the 33 overlapped CIMP targets between the cancers, we found that the DNA methylation status of 19 genes was available from our MCAM analysis. Using