いけません。

江口 自然科学の実証という面では，物事がひっくり返ることは幾らでもあります。先程矢野先生が触れられたように，X線被曝などのリスクの評価はやはり十分ではないですね。

長尾 CT検診を受けようというモチベーションのためには，ポジティブな結果がほしいですが…。

市民の検診に対する意識

江口 低線量CT検診の質の問題もあると思います。

ここで，がん検診に対する社会的な意識ということに少し触れたいと思います。

現在の肺癌検診等における受診者側の意識として，検診に関心があるのでしょうか，ないのでしょうか。日本に限定した話でお願いします(笑)。

矢野 人や年齢によって様々で，私の職場は若い女性が結構多いのですが，労働者の義務だといっても色々な理由をつけて逃げ回っています。結婚している人などは「妊娠の可能性がある」ことを錦の御旗にして逃げています。そういう声は外に出にくいです。

しかし，健診は事業主の義務ですから，無料で受けさせてくれます。病院に行けば何千円かとられるわけですから，喜んで受ける人も沢山いて，様々だと思うのです。

実際，利益も害もそれぞれ集団によって違うわけです。年齢，男性，女性，喫煙で違ってくるのですが，現在そういうところを仕分けして検診がなされていないということも，非常に大きな問題点だと思います。

現状のすべての健診項目を「年に1回，全員一律」というところをもう少し改めて，弾力的に運用できるようにしなければいけないのではないかと思います。

もう1つ別な側面として，職域健診などでは異常がなく，そのあとに肺がんがみつかったということで，訴えられた例などもありましたね。そのようなことも，今後医療訴訟などがますます増えていくなかで問題になります。その判例の場合，職域健診はそこまでを目指したものではないからということで事業主が敗訴することはなかったのですが，逆にいうとその程度の検査なのかということになります。

そもそも肺がん発見についてまで事業主が責任をとらなければいけないのでしょうか。もちろん発癌性のあるような物質を取り扱っている事業所では検診は必要なことで，その場合は事業主の責任だと思いますが，個人が喫煙者なのに肺がんにまで事業主に罰則付きで義務を負わせるということは，法理上無理があるのではないでしょうか。

江口 そうですね。職域健診については，社会的な責任の所在などの問題点があるわけですね。

先程言及されたリスクグループごとに検診方法，内容を変えることは，今後検討すべき課題と思います。治療法についても，分子マーカーでのテーラーメイド治療ということが盛んにいわれるようになってきています。肺がんを考える場合，やはりこの集団ではどのくらいの死亡リスクがあるのかということをもっと細かく検討して，その集団ごとの検診を考えるというのは今後の大きな課題です。

長尾 職業よりも，危険因子として大きいのは喫煙ですから，いま先生がおっしゃったように，会社が禁煙指導に介入せずハイリスクだから肺癌検診を受けさせるというのは，少々矛盾するところではあると思います。

もう1つ，基本的に労働安全衛生法の健康診断は無料ですから，先程肺がんになっても仕方がないかというお話もありましたが，これがもし有料の肺癌検診だったら，受診者側の求めるものは非常に厳しくなりますね。かなり小さな影でも見落とすと，裁判になる可能性は十分にあると思います。

江口 職域健診では，比較的年齢の若い女性なども含まれるので，癌をみつけるというよりも，タバコを吸うなというほうの注意を喚起するべきですね。

肺がんに対する一般の人たちの危機感が足りないと思われます。もう少し肺がん予防などの啓発が必要なのではないでしょうか。佐川先生，いかがでしょう。

佐川 そうですね。啓発はもちろん重要ですし，タバコの問題として，10年くらい前までは反タバコ派と検診派みたいな感じで分かれていて，反タバコ派は「検診などしていないで，反タバコの運動をしなさい」というような状況があったのですが，それではだめで，いまは比較的，検診する人間も反タバコであるべきだろうという流れになりつつあるかなと。

実は今度のがん検診の検討会の報告書にも，検診機関による禁煙活動を入れたのです。要するに「検診する人間は，タバコについて反タバコ的なものもきちんと宣伝するようにしなさい」と入れてあるので，そういうことはやはり非常に重要だろうと思います。

いまの流れですと，高喫煙者の多くは肺がんになるから，例えばCT検診は毎年受けたほうがよいのではないかという話も出るわけですが，好きでタバコを吸っている人に余分に税金をかけるのかという話もあって，私などはタバコの値段を10倍に上げれば健康にはなるしお金はかからなくなるし，一番よいのではないかと思っているのですが。少し雑談になってしまいましたが。

江口 タバコの税収入に関しては非常に議論のあるところです。財務省の影響力が強いと聞いています。

長尾　最近の傾向として，CT 肺癌検診でみつかった気腫化病変を用いて強い禁煙指導を行うといった流れがありますね。

矢野　CT で気腫をみつけなければタバコの話ができないというのはどうなのでしょうか。お金もかかりますし。

長尾　肺癌検診の副次的な所見を利用しようというものです。

矢野　肺癌検診の benefit として肺気腫もみつかる，何々もみつかるというのですが，そんなことをしなくても分っていることを，わざわざ X 線なり放射線の被曝の結果で，というのはいかがなものでしょう。

長尾　ビジュアル的なものがあると，より効果的に指導できるというところもあります。

矢野　指導方法については，私たちも勉強していかなければと思います。

似た話で，特定健康審査の導入で話題になったことがあります。長野県の秦阜村というところで，成果が上がらなかったので健診をやめて，はじめから全戸在宅保健指導をしてみたら，全国でも有数の低医療費の村になったのです。しかし，今回の制度改正で，ともかく健診をしなければいけなくなった。同じ予算内でしますので，いままで保健師が各戸を回って様々な生活指導をされていたのができなくなって，一度は「意味がない」といっていた健診に逆戻りということになったので，本当に本末転倒です。

江口　“メタボ健診”なども例外ではないのですが，かくのごとく検診の体制はいったん決めてしまうとそれで進めざるを得ない，いままで機能していたことが逆に不都合になってくるということも，社会的に起こり得ます。

本日は肺癌検診についてその意味や，今後の展開などについてお話しいただきました。最後に検診に関する課題のポイントを，一言ずつお伺いしたい。

佐川　では 3 点だけ。1 つは現行検診の精度管理の底上げということです。仕様書を作っていますので，それが広まってくれることを期待しています。

それから CT に関しては RCT が絶対に必要だろうと思います。

3 つ目として，検診自体は日本では予防給付になっていないのです。要するに，お金を払ってやるものなのですが，予防給付にしないと受診率は上がらないのです。受診率の話は今回はあまり出ませんでしたが，受診率は先進国中で恐らく最低です。

ですから，やはり予防給付にして，あるいは incentive をつけて，受けることがメリットになるようなものにする。そうすれば，受けなくてよい検診は受けさせない，予防給付をしなければよいのですから，「全額どうぞ自費で」ということにすればよいので，とても分りやすいと思います。2 年に 1 回ですむ検診は，2 年に 1 回だけ無料にしてやればよいわけです。もう 1 年受けたい人は「ご自分で」とすればよいので，予防給付にすることが実は一番重要かなと思います。

長尾　今後どのようなモダリティーで検診が進むのか分りませんが，やはり検診機関に精度管理を義務化するような形にもっていかないとだめだと思います。

もう 1 つ，受診者側にとっての問題は「異常あり」や「経過観察」の際の不安ですね。我々はいままでこれを全然考えていなかったので，このあたりの不安をどのように取り除いていくべきか，これから考えなければいけない大きな問題だと思います。

矢野　佐川先生がおっしゃったように，わが国は一般健診の受診率は世界トップなのに，意味のあるがん検診受診率は先進国で最低であるという，非常に矛盾したところがあります。それは現在の健診や検診の社会的体制や仕組みの置く場所が間違っていて，私の職場などの場合でも，ストレスによる自殺など様々な問題が起こっています。それがあまり役に立たない健診に追いまくられて，産業保健スタッフや産業医などがそちらの仕事に時間を奪われています。臨床の先生方には検診の方法や治療に対しおおいに改善していただきたいのですが，同時にそれが置かれた仕組みなどを一緒に考えていただければと思います。

江口　やはり関係省庁の縦割り行政などとも関係してくるかもしませんね。

これからのがん検診学ということで，科学的に裏付けのある，そして合理的なかん検診を進めるべきであり，そのための課題をお話しいただきました。

お忙しいところ本当にありがとうございました。

E-cadherin expression and epidermal growth factor receptor mutation status predict outcome in non-small cell lung cancer patients treated with gefitinib

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Abstract. It is known that an epidermal growth factor receptor (EGFR) gene mutation(s) is present in a percentage of non-small cell lung cancers (NSCLCs). Gefitinib, an inhibitor of the tyrosine kinase activity of EGFR, is effective on most of them. The EGFR mutation status alone cannot fully predict the response to gefitinib and the prognosis for the patients. We hypothesized that information on the expression levels of phosphorylated-EGFR and -Akt, and E-cadherin, alone or in combination with information on the EGFR mutation, may refine our ability of prediction. We investigated 24 NSCLCs that had recurred after surgery and were treated with gefitinib. Specimens resected by surgery were subjected to the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp reaction to determine the EGFR mutation status, and to immunohistochemical staining of phosphorylated-EGFR and -Akt, and E-cadherin to determine their expression levels. The EGFR mutation status was predictive of responsive disease (complete response: CR + partial response: PR) and controlled disease (CR + PR + stable disease: SD). Positive E-cadherin staining was predictive of longer time to progression (12.4 vs. 5.9 months, $p<0.05$) and overall survival (OS) (18.4 vs. 13.0 months, $p<0.05$). Together the patients with an EGFR mutation and the patients with positive E-cadherin staining defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months. Neither p-Akt nor p-EGFR staining was associated with the response and survival. In patients with surgically resected NSCLC tumors, the EGFR mutation status and E-cadherin staining can select patients who will benefit from gefitinib therapy.

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Key words: non-small cell lung cancer, gefitinib, E-cadherin, epidermal growth factor receptor mutation

Introduction

Gefitinib (ZD1839, Iressa, AstraZeneca, Wilmington, DE) is a therapeutic reagent for non-small cell lung cancers (NSCLCs). It shows dramatic anti-tumor effects in some patients, but has no effect in others (1). The presence of an epidermal growth factor receptor (EGFR) gene mutation(s) (hereafter EGFR mutation) associates significantly with the gefitinib responsiveness (2,3) and serves as a marker in the choice of therapeutic regimens (4). Some tumors with an EGFR mutation do not respond to gefitinib therapy while those with wild-type gene do (5-7), so additional markers are required to more precisely select tumors that respond to gefitinib.

EGFR transmits signals that direct cell proliferation and survival. The wild-type EGFR preferentially transmits cell proliferation signals through Erk, while the mutant EGFR preferentially transmits cell survival signals through Akt or STAT (8). Gefitinib effectively inhibits the latter (2). This is why gefitinib selectively elicits an apoptotic response in cells with an EGFR mutation, thereby producing its clinical response (8). We hypothesized that molecules that interact with EGFR or are located downstream in the pathway modify the tumor cell response to gefitinib and therefore serve as markers that may help to more precisely predict their responsiveness to gefitinib.

In this study, three molecules were tested for their predictive ability, p-EGFR (phosphorylated at Tyr1173: pTyr1173), p-Akt (phosphorylated at Ser473: pSer473) and E-cadherin, in addition to the EGFR mutation status. p-EGFR(pTyr1173) transmits a signal that directs cell proliferation (9), p-Akt(pSer473) mediates signals that direct cell survival (10) and E-cadherin has been shown to interact with EGFR by modifying its activity (11). We investigated the expressions of these three molecules by immunohistochemistry

in 24 NSCLCs that had been resected by surgery, recurred afterward and were treated with gefitinib. The results enabled us to test their staining intensity, alone, or in combination with the EGFR mutation status. It also improved our ability to predict the responsiveness to gefitinib and patient outcome.

Materials and methods

Patients. This study was approved by the Tsuboi Cancer Center Hospital ethics board. After the written informed consent was obtained, we enrolled 24 Japanese patients who had suffered from lung cancers which were resected between 1996 and 2004 (Tsuboi Cancer Center Hospital, Fukushima, Japan) and then had recurred. The patient characteristics are summarized in Table I. Gefitinib, 250 mg per day, was initiated between July 2002 and October 2006 to treat the recurrent disease. The median time between the surgery and the start of the gefitinib treatment was 740 days (range: 113-2,012). Treatment was continued until the disease progressed, intolerable toxicity developed or a patient refused treatment for other reasons.

Evaluation of the response to gefitinib and patient outcome. Every 4 weeks chest X-rays or computed tomography (CT) scans were done to evaluate tumor response and lung toxicity, and blood tests were done to monitor systemic toxicity. Tumor response that remained stable for at least 30 days was graded according to the Response Evaluation Criteria in Solid Tumors (12). Time to progression (TTP) in these patients was defined as the interval from the start of gefitinib administration to disease progression or death. The outcomes were evaluated up to May 31, 2007, with an average follow-up time of 20.6 months (range: 1.1-50.0). Both mutation and immunohistochemical analyses were performed after completion of the response evaluation.

Table I. Patient characteristics.

Characteristic	No. of patients (n=24)	%
Gender		
Male	13	54.2
Female	11	45.8
Median age, years (range)	63.2	(44-84)
ECOG performance status		
0	6	25.0
1	18	75.0
Histology		
Adenocarcinoma	21	87.5
Squamous cell carcinoma	1	4.2
Adenosquamous cell carcinoma	2	8.4
Prior chemotherapy		
0-1 regimens	18	75.0
>2 regimens	6	25.0
Smoking history		
Never smoked	19	79.2
Smoker (current/former)	5	20.8
Stage		
I-II	19	79.2
III-IV	5	20.8

ECOG, Eastern Cooperative Oncology Group.

DNA extraction and mutation analysis. DNA was extracted from the paraffin-embedded tumor tissue (13-15). EGFR mutations were detected using the peptide nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp. This method, which has been described in detail elsewhere, is a rapid and sensitive detection system for EGFR gene mutations and can detect point mutations G719C, G719S, L858R and L861Q and deletions in exon 19 in the presence of a 100- to 1,000-fold background of wild-type EGFR (4,6,16).

Immunohistochemistry and scoring. Formalin-fixed, paraffin-embedded tumor tissue was tested for immunoreactivity to p-EGFR, p-Akt and E-cadherin. The primary antibodies used were: anti-p-EGFR that detects EGFR protein phosphorylated at Tyr1173 (Cell Signaling Technology Beverly, MA), anti-p-Akt that detects Akt protein phosphorylated at Ser473 (Cell Signaling Technology) and anti-E-cadherin (BD Biosciences, Beverly, MA). Tissue sections cut at a thickness of 5 μm were placed on glass slides, deparaffinized and then rehydrated. Antigen was quantified using the following procedure. The slides were incubated in citrate buffer in a steamer for 15 min. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 5 min and non-specific background staining was blocked by incubation in a protein block for 5 min. Sections were then reacted with primary antibody dilutions (p-EGFR a 1/400 dilution at 37˚C for 15 min, p-Akt a 1/50 dilution at 4˚C for 16 h and E-cadherin a 1/100 dilution at 37˚C for 32 min). The bound antibody was detected by biotinylated secondary antibody and visualized using diaminobenzidine (DAB) chromogen. Sections were then counterstained with Mayer's hematoxylin and mounted using the resinous mounting medium.

The p-EGFR and p-Akt stainings were scored by their cytoplasmic and nuclear staining, while E-cadherin staining was scored by its membrane staining (17-19), all without the knowledge of clinical or laboratory information. The cytoplasmic and nuclear staining of the entire tumor was scored as follows: First, 500 randomly selected tumor cells (50 cells per randomly chosen microscopic field at x40 magnification) were scored as 0 (no staining), 1 (mild), 2 (moderate) or 3 (strong staining). Second, for p-EGFR, the most intense staining observed in >1% of the cells was the staining score for the tumor. For p-Akt, the mode of the cytoplasmic or nuclear staining score, whichever was greater, was the staining score for the tumor. Tumors with staining scores of 0 or 1 were ranked negative and scores of 2 or 3 were ranked positive. The membrane staining of the entire tumor was scored as 0 when no tumor cells were stained, 1 when <10%

Table II. Response to gefitinib therapy.

	CR	PR	SD	PD	NE
No. of patients	0	8	6	5	5
Median TTP (months)		16.1	9.3	1.0	
Median OS (months)		25.9	20.8	6.5	

TTP, time to progression; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated.

showed weak membrane staining, 2 (weakly positive) when >10% showed complete membrane staining although with weak to moderate intensity and 3 (strongly positive) when >10% had complete and strong membrane staining. Entire tumors with staining scores of 0 or 1 were considered negative while those scored as 2 or 3 were considered positive.

Statistical analyses. All statistical analyses were performed by StatView version 5 software (SAS institute Inc, Cary, NC). Comparisons of the proportions between two populations utilized the χ^2 test. Comparisons of patient outcome (TTP and overall survival, OS) between patient groups utilized the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided and $P<0.05$ was considered significant.

Results

Response to the gefitinib and patient outcome. The responses to gefitinib are summarized in Table II. The responders [complete response: CR + partial response: PR, (8/24) 33%] had significantly longer TTP and OS than non-responders ($p<0.005$ and $p<0.05$, respectively). In addition, the patients with controlled disease [CR + PR + stable disease: SD (13/24) 54%] had significantly longer TTP and OS ($p<0.001$ and $p<0.001$, respectively). We found no significant differences in the OS between patients with PR and SD nor could we prove that CR + PR better defined patients who benefited from the therapy than CR + PR + SD. We therefore performed the analyses based on the two groupings.

Analyses of the EGFR mutation status and staining of p-EGFR, p-Akt and E-cadherin. We investigated the EGFR mutation status and the staining of p-EGFR, p-Akt and E-cadherin. We chose these proteins because they are intimately connected with the activity of EGFR and thus may predict responsiveness to gefitinib and/or patient outcome. In the mutation analysis, 10 patients were found to have an EGFR mutation: one had a point mutation L858R(T2573G), two had a deletion E746-A750del(2235-2249del), six had a deletion E746-A750del (2236-2250del), and one had a deletion L747-S752del, P753S(2240-2257del). All these mutations have been observed in gefitinib responders in the literature (2,3). Representative immunohistochemical staining is shown in Fig. 1 with the results summarized in Table III. The results of the EGFR mutation status are also shown. Positive p-Akt staining was associated with EGFR mutation, which is plausible because mutant EGFR stimulates the cell survival signal that is mediated by p-Akt. The staining intensity of p-EGFR and E-cadherin failed to show an association with the EGFR mutation and thus may be an independent parameter.

Predictors of the responsiveness to gefitinib. We then investigated the association between the expression of these proteins and the responsiveness to gefitinib (Table IV). The presence of an EGFR mutation significantly associates with responsive diseases (CR + PR) or controlled diseases (CR + PR + SD). This is consistent with the results presented in previous reports (20-22). We found no significant associations in the staining result for p-EGFR, p-Akt and E-cadherin.

Predictors of patient outcome. We compared the Kaplan-Meier curves to identify predictors of longer TTP and/or OS. As shown in Fig. 2A the positive staining of E-cadherin predicts a longer TTP (12.4 vs. 5.9 months, $p<0.05$) and longer OS (18.4 vs. 13.0 months, $p<0.05$). The presence of EGFR mutation(s) ($p=0.13$ and $p=0.11$, respectively, Fig. 2B), as well as p-EGFR and p-Akt staining intensity failed to predict outcome. We then looked at the EGFR mutation status in conjunction with the E-cadherin staining intensity as predictors of these same parameters. As shown in Fig. 2C in the right panel, the patients with EGFR mutation-positive tumors and those with E-cadherin-positive tumors defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months, although we failed to show a significant difference in TTP (Fig. 2C, left panel). Therefore, we consider that the patients with EGFR mutation-positive or E-cadherin-positive tumors are the most likely to benefit from gefitinib therapy.

Discussion

It was shown that NSCLC tumors with an EGFR mutation(s) respond to gefitinib at a rate of 65 to 100% (5-7,20-24). Several prospective phase II studies have shown that gefitinib therapy significantly lengthened TTP in NSCLC patients with EGFR mutation-positive tumors (5-7). Thus far, no prospective studies have reported on OS. Several retrospective studies have suggested that gefitinib therapy may result in a longer OS in patients with EGFR mutation-positive tumors (20,21,23), however, we did not observe any significant differences in either TTP or OS. This is likely due to the size of the current study, as is discussed later.

We showed that positive E-cadherin staining is significantly associated with TTP and OS. Possible mechanisms that may explain this observation include that i) tumors with a lower E-cadherin expression progress faster than those with a higher expression and ii) E-cadherin modifies EGFR function and thus contributes to the effect of gefitinib treatment. The former mechanism is supported in reports that show that tumors with a positive E-cadherin staining are more frequent in early stage than in locally advanced or metastasizing NSCLCs (25-28). Similar results have been obtained in other malignancies such as the esophagus (29,30), stomach (31,32), colon (33),

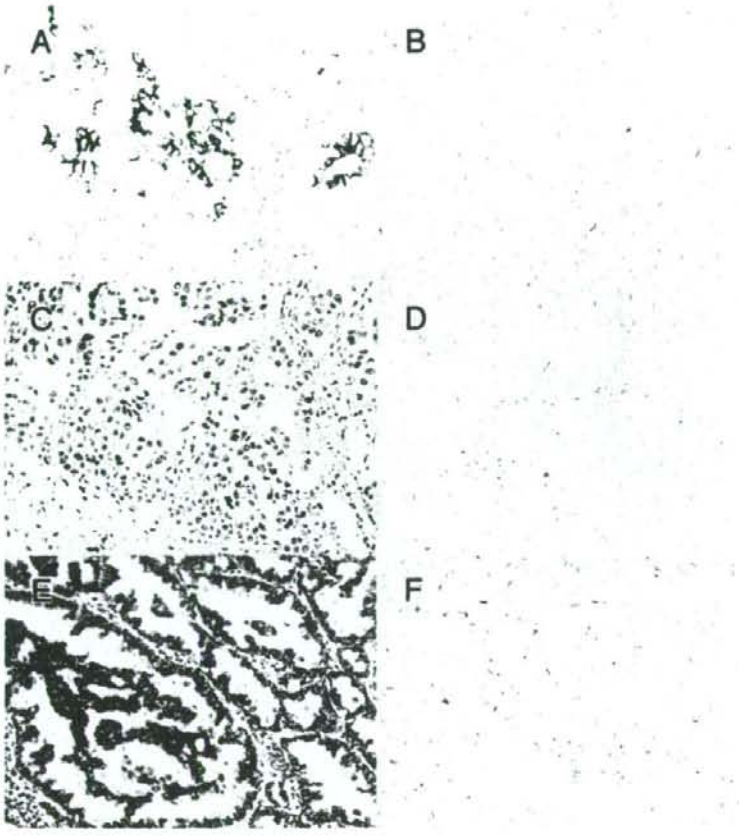

Figure 1. Immunohistochemistry. Positive (A) and negative (B) staining for p-EGFR; positive (C) and negative (D) staining for p-Akt; positive (E) and negative (F) staining for E-cadherin; magnification, x200.

Table III. EGFR mutation and staining of p-EGFR, p-Akt and E-cadherin.

	p-EGFR		p-Akt		E-cadherin	
	Positive	Negative	Positive	Negative	Positive	Negative
All patients	3	21	3	21	19	5
EGFR mutation						
Positive	2	8	3	7	9	1
Negative	1	13	0	14	10	4
P	0.35		<0.05		0.27	

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

liver (34), pancreas (35) and urinary bladder (36,37). Moreover, in NSCLCs, a positive E-cadherin expression associates with a more differentiated histology (26,28) and a better prognosis (25,27,28). The latter mechanism is supported by reports showing that E-cadherin interacts with EGFR, thereby decreasing ligand-affinity (38,39) and inhibiting activation (40) in several human tumor types including the esophageal, breast and lung (41-43). Mechanisms i) and ii) stated above are not mutually exclusive and both may contribute to a better prognosis.

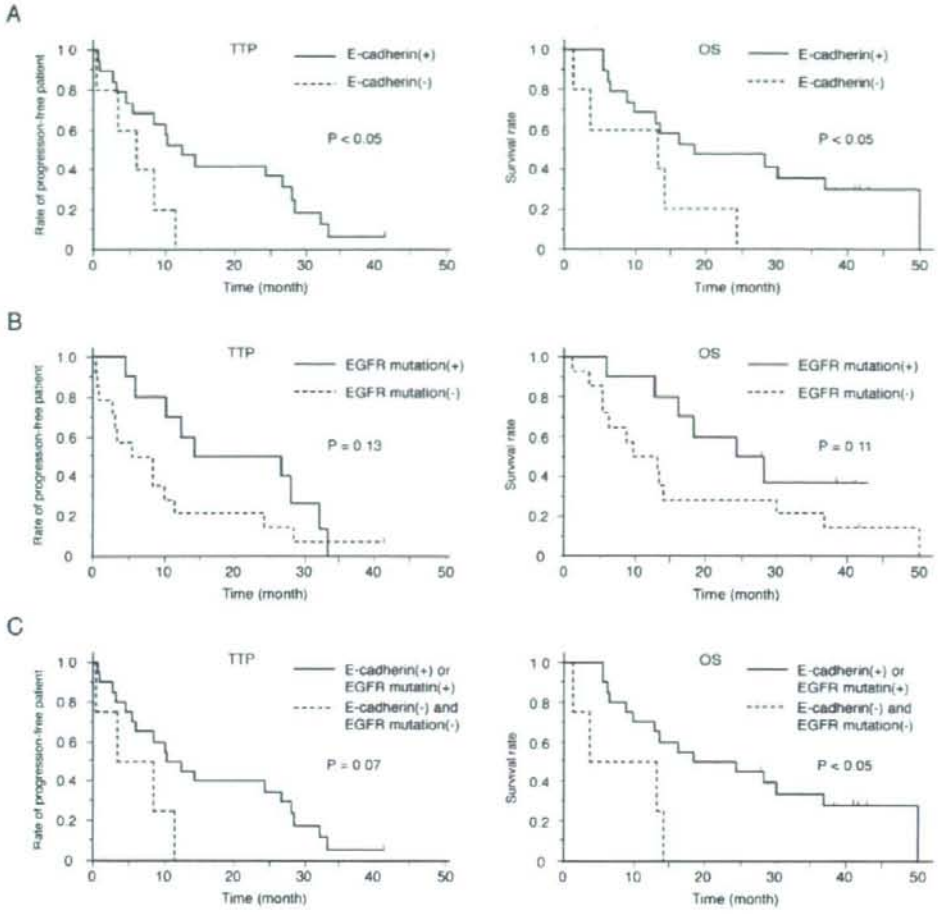

Figure 2. (A) Kaplan-Meier plots of TTP and OS where patients are grouped by the E-cadherin staining of their tumors. (B) Kaplan-Meier plots of TTP and OS where patients are grouped by the EGFR mutation status of their tumors. (C) Kaplan-Meier plots of TTP and OS where the two groups of patients have i) tumors which stain positively for E-cadherin or have an EGFR mutation(s) and ii) tumors which are negative for E-cadherin staining and EGFR mutation.

Table IV. Gefitinib response summarized by the EGFR mutation status and by the staining of p-EGFR, p-Akt or E-cadherin.

	EGFR mutation		p-EGFR		p-Akt		E-cadherin	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
All patients	10	14	3	21	3	21	19	5
Responsive disease (CR + PR)	6	2	1	7	1	7	6	2
P	<0.005		0.23		0.23		0.72	
Controlled disease (CR + PR + SD)	7	7	1	13	1	13	11	3
P	<0.05		0.54		0.54		0.95	

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

The current study warrants a larger one and presents an important question. We have six panels in Fig. 2, three of which showed significant differences and three of which did not. It is calculated that, if twice as many patients had been enrolled and had shown similar responsiveness and prognoses, all six sets of the two groups compared in Fig. 2 would have shown significant differences. To investigate this, a study should be scheduled where more than twice the number of patients is enrolled. We showed that tumors with a positive E-cadherin staining have a better prognosis after gefitinib therapy. It is, however, not clear whether the E-cadherin expression and EGFR mutation(s) contribute to it independently or synergistically. Basic and clinical researches addressing this issue may provide important information on the role of E-cadherin and EGFR in carcinogenesis.

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Antitumor activity of histone deacetylase inhibitors in non-small cell lung cancer cells: development of a molecular predictive model

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Abstract

To ascertain the potential for histone deacetylase (HDAC) inhibitor-based treatment in non-small cell lung cancer (NSCLC), we analyzed the antitumor effects of trichostatin A (TSA) and suberoylanilide hydroxamic acid (vorinostat) in a panel of 16 NSCLC cell lines via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. TSA and vorinostat both displayed strong antitumor activities in 50% of NSCLC cell lines, suggesting the need for the use of predictive markers to select patients receiving this treatment. There was a strong correlation between the responsiveness to TSA and vorinostat ($P < 0.0001$). To identify a molecular model of sensitivity to HDAC inhibitor treatment in NSCLC, we conducted a gene expression profiling study using cDNA arrays on the same set of cell lines and related the cytotoxic activity of TSA to corresponding gene expression pattern using a modified National Cancer Institute program. In addition, pathway analysis was done with Pathway Architect software. We used nine genes, which were identified by gene-drug sensitivity correlation and pathway analysis, to build a support vector machine algorithm model by which sensitive cell lines were distinguished from resistant cell lines. The prediction performance of the support vector machine model was validated by an additional nine cell lines, resulting in a prediction value of 100% with respect to determining response to TSA and vorinostat. Our results suggested that (*a*) HDAC inhibitors may be promising anticancer drugs to NSCLC and (*b*) the nine-gene classifier is useful in predicting drug sensitivity to HDAC inhibitors and may contribute to achieving individualized therapy for NSCLC patients. [Mol Cancer Ther 2008;7(7):1923–30]

Introduction

Several chemotherapy regimens have proven to be effective (1) and are widely applied to treatment for unresected non-small cell lung cancer (NSCLC) (2). However, at present, the effect of these therapies on improving patient survival remains far from satisfactory (1–3). Recently, new therapeutic strategies targeting specific tumor-related genes in NSCLC have been developed, such as the use of small molecules that inhibit epidermal growth factor receptor tyrosine kinase, which show a dramatic antitumor effect in a proportion of patients (1). It is consequently desirable to find more novel therapeutic agents to target NSCLC.

Histone deacetylase (HDAC) and histone acetylase catalyze deacetylation and acetylation, respectively, of histone in eukaryotes, whose dynamic balance is important for the accurate regulation of gene expression in eukaryotes (4). Imbalance in these key enzymes can bring disorder to proliferation and differentiation in normal cells and then lead to tumor initiation. Various HDAC inhibitors, including suberoylanilide hydroxamic acid (vorinostat), MS-275 (Schering), and trichostatin A (TSA), have been reported to exhibit antitumor activities against hematologic, breast, and bladder malignancies (5–9). Although the antitumor activity of HDAC inhibitors against NSCLC has been indicated previously (10–13), these prior studies have been somewhat limited in relation to the number of cell types examined. Here, we examined the sensitivity of a series of NSCLC cell lines to HDAC inhibitors *in vitro* via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our study showed that TSA and vorinostat displayed strong antitumor activities in a proportion of NSCLC cell lines.

This result indicates the need for the development of biomarkers to predict response of HDAC inhibitor treatment in NSCLC. HDAC inhibitors have been reported to be highly effective in up-regulating expression of tumor suppressor genes, reducing tumor growth, and inducing programmed cell death. However, it seems to be difficult to list predictive biomarkers of HDAC inhibitors only by the status of tumor suppressors. In this study, we built a support vector machine (SVM) algorithm model, by which sensitive cells were distinguished from resistant cells, using

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biomarkers identified by gene expression-TSA drug sensitivity correlation and pathway analysis. A separate set of cancer cell lines validated the prediction performance of this novel SVM model.

Materials and Methods

Cell Lines

We analyzed the expression profiles and sensitivity to HDAC inhibitor treatment of separate two sample sets of human NSCLC cell lines. The training sample set consisted of the following 16 cell lines: PC9, PC7, PC14, A549, LK-2, RERF-LC-KJ, RERF-LC-MS, RERF-LC-AI, PC1, PC3, PC10, ABC-1, EBC-1, LC2/ad, SQ5, and QG56 (set 1). The test set consisted of the following 9 cell lines: Lu65, VMRC-LCD, LCOK, NCI-H1650, NCI-H1975, LCI-sq, LC-1F, NCI-H441, and Calu-6 (Set 2). PC7, PC9, PC14, A549, RERF-LC-KJ, RERF-LC-MS, PC3, ABC-1, LC2/ad, VMRC-LCD, LCOK, NCI-H1650, NCI-H1975, and NCI-H441 are adenocarcinoma cell lines. LK-2, RERF-LC-AI, PC1, PC10, EBC-1, LCI-sq, LC-1F, SQ5, and QG56 are squamous cell carcinoma cell lines. Lu65 is a large-cell carcinoma cell line. Calu-6 is an anaplastic carcinoma cell line. The PC1, PC3, PC6, PC7, PC9, PC10, PC14, and QG56 cell lines were obtained from IBL. The A549, NCI-H1650, NCI-H1975, NCI-H441, and Calu-6 cell lines were obtained from the American Type Culture Collection (14). The Lu65, LCOK, and VMRC-LCD cell lines were provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute; ref. 14). The LK-2 cell line was obtained from the Health Science Research Resources Bank. PC1, PC3, and PC10 cell lines were provided by S. Hirohashi (National Cancer Center Research Institute). RERF-LC-KJ, LC2/ad, SQ5, LCI-sq, LC-1F, and RERF-LC-AI cell lines were obtained from the RIKEN Cell Bank. RERF-LC-MS, EBC-1, and ABC-1 cell lines were purchased from the Health Science Research Resources Bank.

MTT Assay for Drug Activity

Estimation of cytotoxicity in the above-mentioned cell types was mediated by a rapid colorimetric assay for mitochondrial dehydrogenase activity as described previously (15–17). Briefly, cell suspensions (200 μL; 10^5 cells/mL) were seeded into 96-well microtiter plates (Falcon), and 10 μL drug solution was added at various concentrations (0.1-20 μmol/L). Following 72-h (37°C) exposure to either TSA (Sigma-Aldrich Japan) or vorinostat (Alexis Biochemicals), RPMI 1640 containing 10% FCS, 20 μL MTT solution (5 mg/mL in PBS) was added to each well and incubation was then continued for another 4 h at 37°C. Samples were then subjected to spectrophotometric analysis at 560 nm (Ultraspec 4050; LKB).

RNA Isolation, cDNA Array Hybridization, and Analysis of Hybridization Signals

Total RNA was isolated from untreated cell line using standard protocols described previously (18–20). We did high-density oligonucleotide array analysis using Affymetrix HG-U133A (22,282 probe sets) expression array (Affymetrix; refs. 16, 20). Total RNA was used to synthesize double-strand cDNA together with SuperScript II and a T7-oligo(dT) primer. Then, biotinylated cRNA was synthesized from the double-stranded cDNA using the RNA Transcript Labeling kit and was purified and fragmented. The fragmented cRNA was hybridized to the oligonucleotide microarray, which was washed and stained with streptavidin-phycoerythrin. Scanning was done with GeneChip Scanner 3000 (Affymetrix). GeneChip analysis was done based on the Affymetrix GeneChip Manual with GeneChip Operating Software version 1.0 (Affymetrix), and Microarray Database software. For GeneChip analysis, the signal intensity was normalized by using the average of all probe sets. Only present call was used. The transcriptomic data we generated for set 1 was deposited previously in Gene Expression Omnibus (GEO accession no. GSE4127). That for set 2 was also deposited in Gene Expression Omnibus (GEO accession no. GSE10089).

Data Analysis for Transcriptomic Data

Data analysis for the correlation coefficients that related the drug activity patterns to the expression patterns of the genes was principally done by a modified National Cancer Institute program (CIM-Maker; ref. 21). $[A]$ (IC_{50}) refers to the drug activity matrix in which the rows represent the anticancer drugs and the columns represent the NSCLC cell lines. $[T]$ (gene expression) refers to the gene expression matrix in which the rows represent individual genes and the columns represent the cell lines. To analyze the relationship between gene expression and drug activity, we generated a gene-drug correlation matrix [AT] (correlation coefficient) in which the rows represent the genes and the columns represent the drugs. First, we subtracted its mean value from the matrix $[A]$ in the direction of row and columns for a pretreatment. Secondly, we normalized each element in the matrix $[A]$ by subtracting its row-wise mean and dividing by its row-wise SD; normalized $[T]$ was generated in a similar way. Finally, we took the inner product of the matrix $[A]$ and the transpose of the matrix $[T]$. The resulting matrix $[AT]$ implied the Pearson correlation coefficients that reflected the relationship between drug activity and gene expression.

Pathway Analysis

We used pathway analysis to provide a viewpoint of the biological function of genes within the proposed classifier. Pathway analysis was done using the Pathway Architect software (Stratagene). All of the known TSA target genes (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7A, HDAC9, and HDAC11) were added to the list of genes identified by gene-drug sensitivity correlation. The pathways showing the relationships among the genes on the list was drawn by selecting all molecules on the pathway edit window. All relationships among the molecules were retrieved from the database, with this information being derived from PubMed abstracts by natural language processing technology. The function was done by selecting the data of maximum reliability (MAX) by choosing all modes of interactions including "Promoter Binding", "Regulation", "Protein Modification", and "Expression" and by taking the relationships supported by

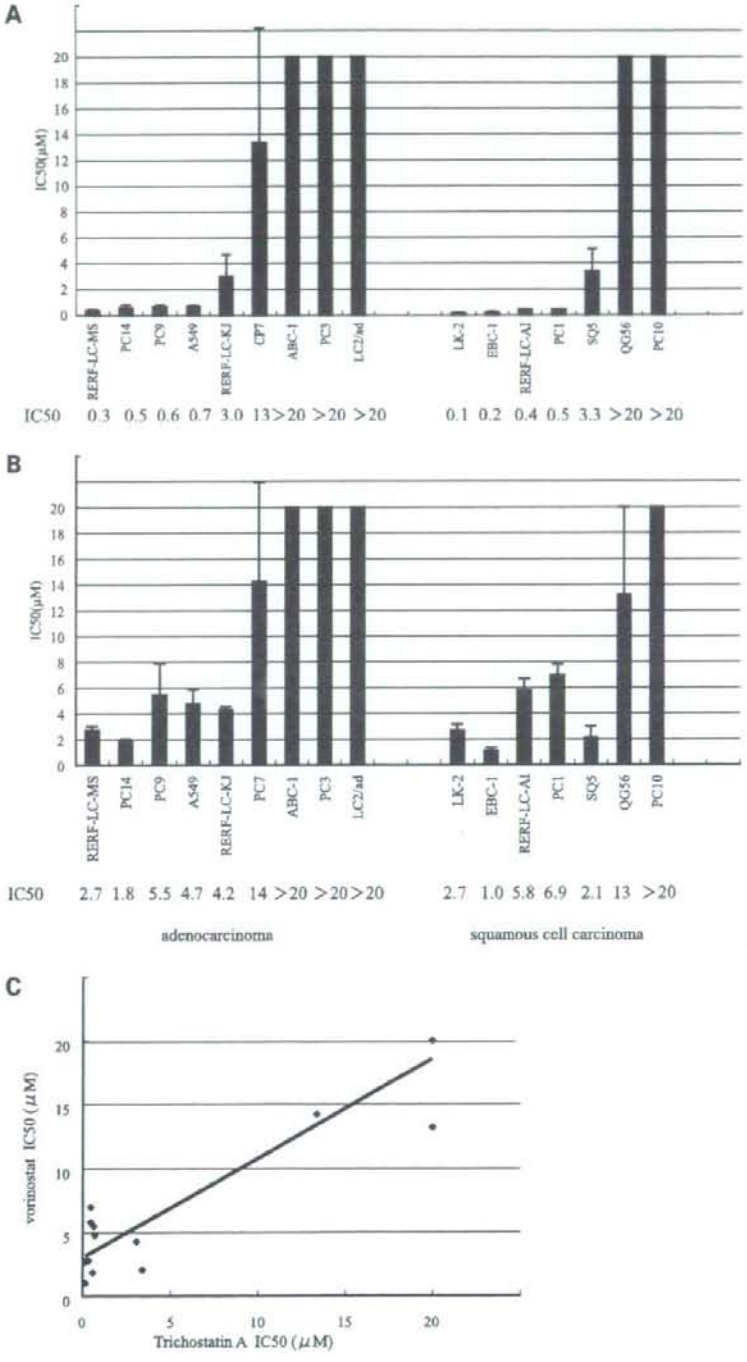

Figure 1. **A,** IC_{50} values for a panel of 16 NSCLC cell lines responding to TSA treatment as determined via MTT assay. Cell lines were classified as highly sensitive ($IC_{50} \leq 1$ μmol/L) and resistant ($IC_{50} < 15$ μmol/L) to TSA. **B,** IC_{50} values for a panel of 16 NSCLC cell lines responding to vorinostat treatment as determined via MTT assay. Cell lines were classified as highly sensitive ($IC_{50} \leq 3$ μmol/L) and resistant ($IC_{50} < 15$ μmol/L) to vorinostat. **C,** correlation between the responsiveness to TSA and vorinostat in a panel of 16 NSCLC cell lines (Spearman rank correlation $r = 0.949$, $P < 0.0001$).

three or more consistent data sources. Next, we picked up the incorporated genes out of the imported gene list used at the onset of the pathway analysis, except the subunits of the target gene. Thus, the list of the genes associated with drug response was established in view of not only gene expression profile data but also the biological functions of altered/associated genes. The data from the listed genes were used to build a SVM model with ArrayAssist software (Stratagene) to predict the drug response (IC_{50}).

Real-time PCR Analysis

Real-time PCR using ABI PRISM 7700 Sequence Detector system (Perkin-Elmer/Applied Biosystems) was done to quantitate the expression of genes associated with HDAC inhibitor response (NQO1, Sec23A, PSME2, MYL6, HNRPDL, TM9SF1, PDCD4, and PSMB5). All of the PCR primers and TaqMan fluorogenic probes were obtained from Applied Biosystems. Total RNA was extracted from cultured cells and reverse transcribed using the RevaTra Ace Kit, with a random hexamer being used as primer (Toyobo). A portion of the resulting cDNA was used for quantitative PCR in a 25 μL total volume incorporating using the primers, TaqMan probes, and Master Mix, which was composed of PCR buffer, $MgCl_2$, dATP, dCTP, dGTP, dUTP, AmpErase UNG, and AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems). The initial thermal cycle conditions were 50°C for 2 min and 95°C for 10 min, as recommended by the manufacturer, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression levels were expressed as ratio of mRNA in a particular sample to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA in that sample. Real-time quantitative reverse transcription-PCR was each done in triplicate for each sample (22).

Validation Assays

The predictive analysis of the SVM algorithm model was validated by using a separate set of test cell lines. The above-mentioned nine NSCLC cell lines in set 2 were used for this testing process, with the SVM model being applied to classify cell lines as sensitive or resistant based on gene expression profiling data.

Results

Effect of HDAC Inhibitors on Cell Growth *In vitro*

Drug sensitivity tests of HDAC inhibitors (TSA and vorinostat) were done on an initial panel of 16 human NSCLC cell lines via MTT analysis. Figure 1 shows the sensitivity of TSA (Fig. 1A) and vorinostat (Fig. 1B) against the training set of cell lines. Accordingly, the concentrations used in the present study are clinically achievable. In our study, TSA and vorinostat both displayed strong antitumor activities in 8 of 16 NSCLC cell lines. There was a strong correlation between the responsiveness to TSA and vorinostat (Spearman rank correlation $r = 0.949$, $P < 0.0001$) in the panel of 16 NSCLC cell lines tested (Fig. 1C). However, the responsiveness to HDAC inhibitors was different from that observed previously with other classes of anticancer agents (16, 17, 20). Clinical trials with vorinostat showed that serum levels in treated patients reached 0.43 to 2.98 μmol/L (6, 7). The pharmacokinetic

Table 1. Factors associated with TSA sensitivity based on expression profiles, sensitivity, and pathway analyses in the 16 NSCLC cell line panel and their functions

Probe set ID	Gene symbol	Gene title	Genes incorporated by pathway analysis	Correlation coefficients
201064_s_at	*PABPC4*	Poly(A) binding protein, cytoplasmic 4 (inducible form)		
201737_s_at	*MARCH6*	Membrane-associated ring finger (C3HC4) 6		
209339_at	*SIAH2*	Seven in absentia homologue 2 (*Drosophila*)		
212887_at	*SEC23A*	Sec23 homologue A (*Saccharomyces cerevisiae*)	+	−0.734
214857_at	*C10orf95*	Chromosome 10 open reading frame 95		
217100_s_at	*UBXD7*	UBX domain containing 7		
201762_s_at	*PSME2*	Proteasome (prosome, macropain) activator subunit 2 (PA28 β)	+	−0.683
201919_at	*SLC25A36*	Solute carrier family 25, member 36		
201993_x_at	*HNRPDL*	Heterogeneous nuclear ribonucleoprotein D like	+	0.678
202731_at	*PDCD4*	Programmed cell death 4 (neoplastic transformation inhibitor)	+	0.724
208799_at	*PSMB5*	Proteasome (prosome, macropain) subunit, β type, 5	+	−0.688
208912_s_at	*CNP*	2′,3′-cyclic nucleotide 3′ phosphodiesterase		
209149_s_at	*TM9SF1*	Transmembrane 9 superfamily member 1	+	−0.672
209150_s_at	*TM9SF1*	Transmembrane 9 superfamily member 1	+	−0.672
210519_s_at	*NQO1*	NAD(P)H dehydrogenase, quinone 1	+	−0.690
211730_s_at	*POLR2L*	Polymerase (RNA) II (DNA directed) polypeptide L, 7.6-kDa polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa		
212082_s_at	*MYL6*	Myosin, light polypeptide 6, alkali, smooth muscle and nonmuscle	+	−0.718
219717_at	*FLJ20280*	Hypothetical protein FLJ20280		
220200_s_at	*SETD8*	SET domain containing (lysine methyltransferase) 8		

analysis of the phase I trial in patients with solid tumor showed that vorinostat was rapidly eliminated and had linear pharmacokinetics with dose-proportional increases in C_{max} in the dose range of 75 to 900 mg/m^2. The C_{max} at 900 mg/m^2 was 5674 ± 545 ng/mL (19.4-23.5 μmol/L; ref. 5). In relation to sensitivity to vorinostat, five of these cell lines (RERF-LC-MS, PC14, LK-2, EBC-1, and SQ5) had an IC_{50} of ≤3 μmol/L (highly-sensitive), four cell lines (PC3, PC10, ABC-1, and LC2/ad) had an IC_{50} of >15 μmol/L (resistant), and the remaining seven cell lines had an IC_{50} of 3 to 15 μmol/L (intermediate sensitive). In the case of TSA, no clinical trials were reported. According to the correlation data (Fig. 1C), cell lines were classified into three groups. Eight of these cell lines (RERF-LC-MS, PC14, PC9, A549, LK-2, EBC-1, RERF-LC-AI, and PC1) had an IC_{50} of ≤1 μmol/L (highly sensitive), five cell lines (PC3, PC10, ABC-1, LC2/ad, and QG56) had an IC_{50} of >15 μmol/L (resistant), and the remaining three cell lines had an IC_{50} of 1 to 15 μmol/L (intermediate sensitive).

Gene Expression-Drug Sensitivity Correlation

We previously used Affymetrix GeneChip technology to perform gene expression profile analysis of the same set of 16 NSCLC cell lines (set 1; ref. 20). To avoid the influence of cell culture artifacts, we separately cultured each cell line in six bottles (22). Signal intensities were normalized by comparison with the average values of all probes. As most of all cell lines belonged to highly sensitive or resistant group in the antitumor sensitivity to TSA, we used the MTT results for TSA for the development of a molecular model of sensitivity to HDAC inhibitors. The top 19 genes associated with TSA sensitivity are listed in Table 1.

Pathway Analysis

In addition, pathway analysis was done with Pathway Architect software to provide a viewpoint of the biological function of genes within the proposed classifier. All subunits of the target gene of the compound used in this study (TSA), namely HDAC, were added to Table 1. To try to develop the classifier by the molecules with the biological relation to HDAC, the molecules not incorporated in the drawn pathway in these steps were removed and picked up the incorporated genes out of the imported gene list used at the onset of the pathway analysis, except the subunits of the target gene (Supplementary Fig. S1).[4] Thus, the list of the genes including nine genes associated with the drug response was established in view of not only gene expression profile data but also the biological functions of altered/associated genes (Table 1).

Building a SVM Algorithm Model

We used nine genes, which were listed by gene-drug sensitivity correlation and pathway analysis, to build a SVM algorithm model by which eight sensitive cell lines were distinguished from five resistant cell lines (Supplementary Fig. S2A-C).[4] The nine-gene signature was an independent predictor of TSA activity. In this classifier, *PDCD4* and *HNRPDL* were up-regulated and *NQO1, SEC23A, PSME2, MYL6, PSMB5*, and *TM9SF1* were down-regulated (Table 1). Of these, three genes (*NQO1, SEC23A*, and *PSME2*) were particularly associated with drug activity in Partial Least Squares analysis (Fig. 2).

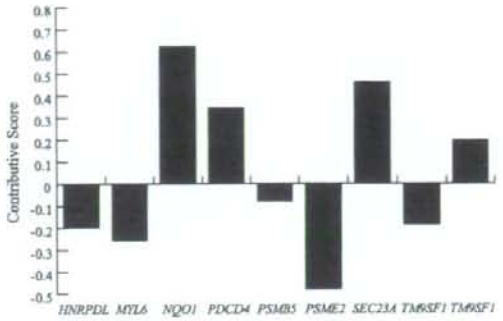

Figure 2. Contribution of the nine genes associated with HDAC inhibitor sensitivity. It was calculated based on the independent Partial Least Squares analysis.

All training set samples were correctly classified concordant with the preclinical response to TSA treatment (Supplementary Fig. S2A-C).[4] Three cell lines with intermediate sensitivity (IC_{50}; $1 < X < 20$) were categorized into the responsive group (Supplementary Fig. S2D).[4] We also validated the prediction performance of this SVM system by testing against an additional nine cell lines, resulting in a prediction value of 100% for determining the response to TSA and vorinostat (Table 2). The nine genes categorized two lines with intermediate sensitivity to TSA treatment into the responsive group. The expression level of these genes, as quantified by GeneChip-based DNA microarray analysis, was validated using real-time PCR (Spearman rank correlation $r = 0.701$, $P < 0.0001$) in the training sample cell lines (Supplementary Table S1).[4]

Discussion

In our study, HDAC inhibitors displayed strong antitumor activities in 8 of 16 NSCLC cell lines tested, suggesting the need for predictive markers to select patients. With a view toward developing predictive markers for determining response to HDAC inhibitor treatment in the context of individualized therapy for NSCLC, we did a gene expression profiling study using cDNA arrays and related the cytotoxic activity of TSA to corresponding gene expression patterns using a modified National Cancer Institute program. Pathway analysis was also done to reduce substantial false positives based only on the expression level of altered genes. From this analysis, we identified nine genes to build a SVM algorithm model. The

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 2. Validation of predictive performance of the nine genes by examining the SVM value in an independent set of nine NSCLC cell lines

	Cell lines	IC_{50} (TSA)	IC_{50} (vorinostat)	Predicted class	True class (TSA)	True class (vorinostat)
1	LCI-sq	0.19	2.14	Sensitive	Highly sensitive	Highly sensitive
2	VMRC-LCD	0.27	0.87	Sensitive	Highly sensitive	Highly sensitive
3	Lu65	0.34	3.74	Sensitive	Highly sensitive	Intermediate sensitive
4	LCOK	0.52	3.66	Sensitive	Highly sensitive	Intermediate sensitive
5	NCI-H1650	0.89	9.37	Sensitive	Highly sensitive	Intermediate sensitive
6	LC1F	1.26	4.82	Sensitive	Intermediate sensitive	Intermediate sensitive
7	NCI-H1975	1.56	3.96	Sensitive	Intermediate sensitive	Intermediate sensitive
8	NCI-H441	0.77	8.30	Sensitive	Highly sensitive	Intermediate sensitive
9	Calu-6	0.58	2.10	Sensitive	Highly sensitive	Highly sensitive

NOTE: Cell lines were classified as highly sensitive ($IC_{50} \leq 1$ μmol/L), intermediate sensitive (1 μmol/L < $IC_{50} \leq 15$ μmol/L), and resistant ($IC_{50} < 15$ μmol/L) to TSA. In relation to response to vorinostat, cell lines were classified as highly sensitive ($IC_{50} \leq 3$ μmol/L), intermediate sensitive (3 μmol/L < $IC_{50} \leq 15$ μmol/L), and resistant ($IC_{50} < 15$ μmol/L).

prediction performance of the SVM model was validated by an additional nine NSCLC cell lines, resulting in a prediction value of 100% for determining the response to TSA and vorinostat (Table 2).

In previous studies, HDAC inhibitors have been shown to inhibit the proliferation of a wide variety of transformed cells *in vitro*, including lymphoma, myeloma, leukemia, and NSCLC (6), and inhibit tumor growth in rodent models of a variety of solid tumors and hematologic malignancies by both parenteral and oral administration, including prostate cancer (23), leukemia (24), breast cancer (25, 26), glioma (27), and lung cancer (28). In lung cancer, vorinostat and TSA were reported to suppress cell growth of a small number of NSCLC cell lines (12, 29, 30). In our study, these two HDAC inhibitors had distinct and differential activities in the panel of NSCLC cell lines tested. These results suggested that clinical studies in selected NSCLC patients would be required for a more refined evaluation of these drugs.

In this study, nine genes [*NQO1, SEC23A, PSME2, MYL6, PSMB5, TM9SF1(1), PDCD4, HNRPDL*, and *TM9SF1(2)*: *TM9SF1(1)* and *TM9SF1(2)* were exons 3 and 6 of the *TM9SF1* gene, respectively] were identified that were associated with the response of HDAC inhibitors in NSCLC cell lines, and three genes (*NQO1, SEC23A*, and *PSME2*) were particularly associated with drug activity (Table 1). The *NQO1* gene is a flavoenzyme that catalyzes the two-electron reduction of quinones and nitrogen oxides (31, 32). A major function of this enzyme may be to decrease the formation of reactive oxygen species by decreasing one-electron reductions and associated redox cycling (33). It has been shown to activate some anticancer drugs (34). In addition, it was reported previously that inhibition of NQO1 reduces the malignant phenotype of pancreatic cancer cells *in vitro* (35). Additionally, another mechanism involved in p53 turnover, apart from the Mdm-2-ubiquitin-proteasome degradation pathway, was regulated by NQO1 (36). Inhibition of NQO1 activity by dicoumarol induces p53 and p73 proteasomal degradation, indicating that NQO1 plays a role in p53 stabilization (37). Moreover, stress-induced NQO1 and NQO2 transiently stabilize p53, which leads to protection against the adverse effects of stressors (38). In addition, interactions of p53 and HDAC were reported to result in p53 deacetylation, thereby reducing its transcriptional activity (39). Therefore, NQO1 expression may be involved in the activities of HDAC inhibitors.

PDCD4 is a recently discovered tumor suppressor protein that inhibits protein synthesis by suppression of translation initiation (40). PDCD4 is ubiquitously expressed in normal tissues, but its expression is lost or suppressed in several tumors, including lung, breast, colon, brain, and prostate cancers (41). Loss of PDCD4 expression in human lung cancer cells correlates with tumor progression and poor prognosis (42). In addition, ATRA-induced PDCD4 expression is mediated by inhibition of the phosphatidylinositol 3-kinase/Akt/mTOR survival pathway that constitutively represses PDCD4 expression in AML cells (43). PDCD4 was reported to block phosphorylation of c-JUN (44), and inhibition of HDAC may activate mitogen-activated protein kinase pathways such as stress-activated signal transduction pathways by c-Jun NH_2-terminal kinase leading to AP-1 activation (45). Therefore, PDCD4 overexpression may influence on the activity of HDAC inhibitors through mitogen-activated protein kinase pathway. Other genes [*SEC23A* (46), *PSME2* (47, 48), *MYL6* (46), *PSMB5* (49), *TM9SF1* (46), and *HNRPDL* (49)] have been reported to interact with HDAC signaling in several profiling studies and network analyses. It is unclear how the expression of these genes might be related to the sensitivity of HDAC inhibitors. Otherwise, proteasome subunits, derived from *PSME2* and *PSMB5* genes, are multicatalytic proteinase complexes, which are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process via a nonlysosomal pathway (50). The *SEC23A* and *TM9SF1* genes contribute transporter activity. Other genes were not reported to the associated with drug resistance,

apoptosis, or proliferation. The contributive scores of *TM9SF1* gene were small but on opposite direction. *TM9SF1(1)* and *TM9SF1(2)* are exons 3 and 6, respectively. The transcript variants of this gene were reported.[5]

When using DNA microarray-based gene expression profiling and clinical response data, it is sometimes difficult to consistently reproduce gene-drug sensitivity correlation data. There seem to be several reasons for this difficulty. First, these data are often influenced by sampling methods, sample preservation status, tumor size, tumor environment status including tumor vessels and inflammation, etc. In our study, these influences were minimized due to the use of cultured cell lines. However, cell lines differ from tumors and should therefore be considered as surrogates that may contain information on the molecular cell biology and molecular pharmacology of cancer. Second, the relative list between gene expression and drug activity might contain statistical false positives, in general, even if the precision of the data analysis method is high enough, because all analyses are based only on the expression data originally containing certain dispersion. Here, we used pathway analysis with a view to taking into account the biological function of each gene in an effort to reduce false positives. We showed that the biomarkers listed by gene expression-TSA drug sensitivity correlation and pathway analysis can be confidential if the prediction performance of a SVM model only by these biomarkers was validated.

In conclusion, our results suggested that (*a*) HDAC inhibitors may be promising anticancer drugs to NSCLC and (*b*) the nine-gene classifier is useful in predicting drug sensitivity to HDAC inhibitors in NSCLC and may contribute to achieving individualized therapy for NSCLC patients.

Disclosure of Potential Conflicts of Interest

A. Kawakami: Genetic Lab Co., Ltd., employee. H. Uesaka and H. Nakae: MediBIC employees. The other authors reported no potential conflicts of interest.

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—Review—

Unique Medical Education Programs at Nippon Medical School

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Abstract

In an attempt to improve the content of the educational programs offered by Nippon Medical School and to better prepare our students to work in the rapidly changing world of medicine, the school has recently revamped its teaching methodology. Particular emphasis has been placed on 1) simulator-based education involving the evaluation of students and residents in a new clinical simulation laboratory; 2) improving communication skills with the extensive help of simulated patients; 3) improving medical English education; 4) providing early clinical exposure with a one-week clinical nursing program for the first year students to increase student motivation at an early stage in their studies; 5) a new program called Novel Medical Science, which aims to introduce first-year students to the school's fundamental educational philosophy and thereby increase their motivation to become ideal physicians. The programs have been designed in line with 2006 guidelines issued by the Ministry of Education, Culture, Sports, Science and Technology to allow flexibility for students to take part in education outside their own departments and year groups as part of the Ministry's program to encourage distinctive education at Japanese universities.
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Key words: medical education, simulation, simulated patient, medical English, early clinical exposure

Introduction

Medical education in the 21st century is undergoing significant changes in terms of both content and methodology[1]. To keep up with these changes, Nippon Medical School (NMS) has designed unique medical education programs in line with the guidelines issued by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in 2006 to allow flexibility for students to take part in education outside their own departments and year groups[2] as part of MEXT's program to encourage distinctive education at Japanese universities. Plans are also underway to integrate the basic medical science and clinical medical science departments[3] and to make more effective use of students' evaluations of the courses they take[4]. The NMS curriculum is still being revised, and the present report summarizes our unique medical education programs.

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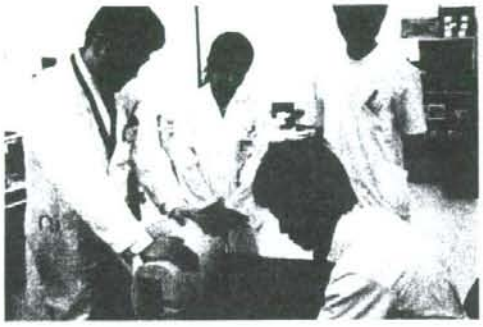

Fig. 1 Lumbar puncture training with a lumbar puncture simulator

Fig. 3 Delivery simulation

Fig. 2 Emergency resuscitation training for students and residents in the laboratory

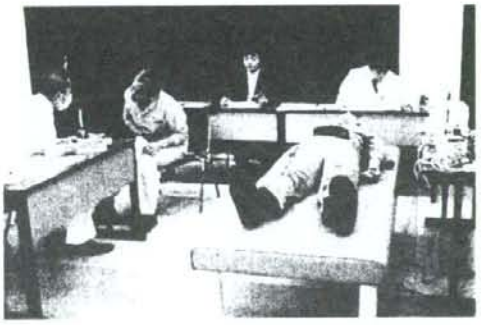

Fig. 4 How to use make-up in SP-attended education and a simulator for the advanced OSCE

New and Distinctive Medical Education at NMS

1. Simulator-based Medical Education

The Clinical Simulation Laboratory (C. S. Lab.) established in April 2005 is a place where students, residents, and other medical staff can acquire and practice basic clinical skills; it plays a very important role in our new programs and is used for various purposes. The first-year students receive a thorough orientation there and train for the Objective Structured Clinical Examination (OSCE, a test assessing students' basic clinical skills) and advanced OSCE. It is used as part of the fourth-year basic clinical practice course to provide training in gynecological, eye, ear, breast, lung and heart examinations, colonoscopy and venipuncture. It is an invaluable training venue for part of the fifth- and sixth-year students' bedside learning course. And interns use it to learn how to carry out thoracocentesis, intubation, and lumbar punctures (**Fig. 1**). In 2006, a total of 1,368 users attended 142 sessions there. Use of the laboratory drastically increased when simulator-based education was officially incorporated into the NMS curriculum. As can be seen on the website *http://www.nms.ac.jp/csl/*, the C. S. Lab. is equipped with various simulation devices together with panels explaining their use. These devices include the Sim-Man, Ichiro, Mr. Lung, dummies for internal examinations, delivery, and breast cancer examinations, rectal examination simulators, devices simulating otoscopy, fundoscopy, blood sampling and venipuncture, and devices simulating various medical techniques such as endoscopic surgery and suture. To allow users to learn clinical skills, various clinical situations are recreated, such as consciousness disorders and difficult intubation due to drug overdose (**Fig. 2**), delivery, neonatal resuscitation[5] (**Fig. 3**), anaphylactic

Table 1 The Code of Behavioral Standards for Simulated Patients at Nippon Medical School

1. Nurturing physicians and researchers with a spirit of humanity and passion for research is the underlying principle of Nippon Medical School's educational philosophy.
2. Those who volunteer to act as Simulated Patients at Nippon Medical School should sympathize with this educational philosophy and be committed to helping foster physicians who seek to provide patient-oriented healthcare.
3. By acting as patients in medical interviews, physical examination training and student evaluation, Simulated Patients play an important role in helping Nippon Medical School students and healthcare providers develop their skills in medical practice and communication in the medical setting. These activities contribute significantly to the fostering of physicians who are trusted by their real patients.
4. Simulated Patients should make every effort to acquire the knowledge and skills necessary to improve their ability to act as patients.
5. Simulated Patients are required to treat as confidential all the documents and information they acquire through participation in training programs at Nippon Medical School.
6. Simulated Patients should at all times act in accordance with these and other guidelines promulgated by the Nippon Medical School Committee for the Recruitment and Training of Simulated Patients.
7. Simulated Patients are encouraged to provide feedback and suggestions to the Nippon Medical School Committee for the Recruitment and Training of Simulated Patients whenever they feel it appropriate to do so.

Committee for the Recruitment and Training of Simulated Patients,
Nippon Medical School

Fig. 5 SPs and students role playing medical interviews

Fig. 6 English medical interview training with native English speakers

shock, and stroke. The C. S. Lab. is operated and managed by a committee consisting of members of the Academic Quality and Development Office, faculty members from each clinical department, and representatives from the student, resident, and nursing bodies. The C. S. Lab. is important not only in providing training in basic clinical skills but also in improving awareness of safety issues in medical settings. It has greatly contributed to improving medical and general healthcare education at NMS.

2. Training in Medical Communication with Simulated Patients

Training with simulated patients (SPs) helps students to acquire the communication skills they will require as physicians, and it is not an overstatement to say that SPs are at the core of the new medical communication program. In recent years, the importance of communication skills in clinical settings has been more widely recognized, and medical interview training with SPs is indispensable if students are to meet the goal of becoming effective communicators. NMS developed