Table 3. Nonhematological toxicity (CTCAE V3.0)[a]

	$n = 51$					
	Grade 1	Grade 2	Grade 3	Grade 4	All grades	Grade ≧3
Anorexia	16	2	2	0	39%	4%
Nausea	13	2	2	0	33%	4%
Vomiting	5	2	2	0	18%	4%
Mucositis	10	1	2	0	25%	4%
Febrile neutropenia	–	–	0	0	0%	–
Hand-foot syndrome	2	0	0	0	4%	–
Pigmentation	4	0	–	–	7%	–
Allergy	0	2	–	–	4%	–
Lethargy	13	4	0	0	33%	–
AST/ALT elevation	22	2	0	0	47%	–
Diarrhea	8	2	2	0	23%	4%
Sensory neuropathy	31	9	0	0	78%	–

[a]Other than sensory neuropathy

Table 4. Incidence of neurotoxicity in FOLFOX regimens

	1–4 Cycles ($n = 9$)			5–8 Cycles ($n = 28$)			9–12 Cycles ($n = 14$)			All cycles ($n = 51$)		
Grade	1	2	3	1	2	3	1	2	3	1	2	3
Sensory neuropathy	5	0	0	19	2	0	7	7	0	31	9	0
	56%	0%	0%	68%	7%	0%	50%	50%	0%	60%	18%	0%

Grade	The oxaliplatin-specific scale (DEB-NTC)			
	0	1	2	3
Dysesthesia and/or paresthesia	No abnormality	Transient dysesthesia and/or paresthesia lasting less than 7 days	Transient dysesthesia and/or paresthesia lasting 7 days or more	Dysesthesia and/or paresthesia with pain or function impairment that interferes with activities of daily living

was reported in 31 patients (61%). Paresthesia lasting 7 days or longer (grade 2) occurred in 9 patients (18%). Peripheral neuropathy appeared in two forms. In the first form, an acute, transient, cold-exacerbated dysesthesia or paresthesia occurred shortly after the administration of oxaliplatin; it affected the hands, feet, perioral area, and throat; and typically lasted for several days after drug administration. In the second form, a delayed-onset, cumulative, dose-related peripheral neuropathy was characterized by paresthesias affecting the hands and feet that did not remit between cycles of treatment. Investigators also reported pharyngolaryngeal dysesthesia in only one patient; however, no patients had a laryngospasm-like syndrome.

Overall, 7 of the 51 patients (14%) required dose modification during treatment; dose reduction was required for oxaliplatin alone in 4 patients, for 5FU alone in 2 patients and for both agents in 1 patient. The majority of dose reductions were by one level (reduction to 65 mg/m^2 oxaliplatin and/or 75% of the starting dose of 5FU). No patients required a second-level dose reduction. The adverse events most commonly leading to dose reduction were neurotoxicity (1 patient in FOLFOX4 and 3 patients in mFOLFOX6) and diarrhea (2 patients in mFOLFOX6). In addition, 2 patients in the mFOLFOX6 setting underwent a dose reduction of oxaliplatin due to allergic reaction. The most common reason for treatment discontinuation was PD.

Antitumor activity

All 51 patients were able to be evaluated for response. Objective responses are listed in Table 5 and Table 6. There was no complete response. The overall objective response rates (in those who underwent first-line or second-line therapy) were 50.0% and 8.7%, respectively (Table 6). Stable disease was achieved in 49% of patients. The tumor control rate (PR + SD) was 80.4%.

Discussion

The recent advent of several new agents for the treatment of metastatic CRC has markedly enhanced the therapeutic armamentarium for this disease. Oxaliplatin in combination with infusional 5FU in the FOLFOX regimens has been shown to be effective in achieving improved response rate, time to progression, and survival time compared with 5FU/LV. In addition, recent large clinical phase III studies (N9741, EFC4584, GERCOR) showed that combination chemotherapy regimens, including irinotecan and oxaliplatin, markedly improved response rates and prolonged median survival over those seen with 5FU/LV),[11–13] and these combination chemotherapy regimens have supplanted 5FU/LV as a standard systemic approach for metastatic CRC. The median survival time (MST) has been gradually pro-

Table 5. Objective responses – (1)

FOLFOX4 (n = 20)				
CR	PR	SD	PD	NE
0	5 (25%) First-line, 3; second-line, 2	12 (60%)	3 (15%)	0
mFOLFOX6 (n = 31)				
0	11 (35.5%) First-line, 11; second-line, 0	13 (41.9%)	7 (22.6%)	0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable

Table 6. Objective responses – (2)

First-line (n = 28)				
CR	PR	SD	PD	NE
0	14 (50%) FOLFOX4, 3; mFOLFOX6, 11	11 (39.3%)	3 (10.7%)	0
Second-line (n = 23)				
0	2 (8.7%) FOLFOX4, 2	14 (60.9%)	7 (30.4%)	0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable

longed through the use of 5FU/LV with irinotecan and oxaliplatin. Currently, with the addition of molecular targeted agents, an MST of over 20 months has been reported.

Since April 2005, and the approval of oxaliplatin in Japan, clinical practice in this country has been conducted in a major way by extrapolation from the results of clinical trials conducted mainly in large Western phase III studies. The results of the present retrospective study demonstrate the efficacy and feasibility of FOLFOX regimens (FOLFOX4 and mFOLFOX6) as treatment for patients with advanced CRC in the Japanese population, as has been shown in Western populations. In this retrospective analysis study in a Japanese population, neutropenia grade 3/4 occurred in 20% of the patients who were assigned to receive oxaliplatin, but it was nonfebrile, whereas grade 3/4 vomiting and mucositis affected only 4% of the patients, while diarrhea affected 4%.

Lethargy has been described as the most frequent adverse event of the mFOLFOX6 regimen in a recent report by Braun et al.[14] In our study, 17 (33%) patients experienced lethargy similar to general fatigue symptoms.

The cumulative dose-limiting toxicity of oxaliplatin is peripheral sensory neuropathy, which reportedly occurs in about 70%–80% of patients; it typically resolves a few months after discontinuation of treatment, and may be exacerbated by cold stimulation. In our series, paresthesia lasting 7 days or longer was observed in 18% of patients and led to an oxaliplatin dose reduction for four patients after they had received a minimum of seven cycles (or at least 4 months) of chemotherapy.

The mechanism of this neurotoxicity has been elucidated to be as follows: the increased neuronal excitability is due to the action of oxaliplatin on voltage-gated sodium channels through the chelation of calcium by the oxaliplatin metabolite. The prevention of this neurotoxicity is a major goal, taking in to account the wide indications of this drug. Various different approaches have been either previously studied or are now being evaluated, based on pathogenic or practical concepts: (1) modification of the administration schedule; (2) substances acting upon sodium channels, such as calcium-magnesium, carbamazepine, gabapentine, venlafaxine; (3) detoxifying agents and antioxidants, such as glutathione, amifostine, alphalipoic acid, tocopherol; (4) substances used in other kinds of neuropathy, such as glutamine and alphalipoic acid; (5) neurotrophic factors, such as nerve growth factor (NGF), LIF; and (6) oxaliplatin analogs, with a DACH platin, without oxalate. Calcium-magnesium infusion appears to be an efficient and safe approach.

In this study, after September 2005, 32 patients (63%) were administered calcium-magnesium infusion for the prevention of the oxaliplatin-related neurotoxicity. Further studies are necessary for a better understanding and prevention of this potentially severe neurotoxicity.

In terms of antitumor activity, although the response rate (RR) in our population was slightly lower in comparison to that in previous Western clinical studies,[11–13] both of the oxaliplatin-based regimens demonstrated a promising objective RR in the first-line setting (50.0%) and in the tumor control rate (80.4%).

In a GERCOR study, the median survival was 21.5 months in 109 patients allocated to FOLFIRI then FOLFOX6 versus 20.6 months in 111 patients allocated to FOLFOX6 then FOLFIRI (P = 0.99). In first-line therapy, FOLFIRI achieved a 56% RR and 8.5-month median PFS, versus FOLFOX6, which achieved a 54% RR and 8.0-month median PFS (P = 0.26). Second-line FOLFIRI achieved a 4% RR and 2.5-month median PFS, versus

FOLFOX6, which achieved a 15% RR and 4.2-month PFS.[13] Although our study could not evaluate enough data for PFS and MST due to the short observation period after the approval of oxaliplatin in Japan, both the FOLFOX regimens we used seem to be beneficial as first-line and second-line therapy for refractory or advanced CRC in a Japanese population, with an overall response rate which is comparable to Western figures regarding first-line and second-line therapy. FOLFOX6 is the most useful of the FOLFOX regimens because it is simple and can be administered on an outpatient basis. When we use oxaliplatin in FOLFOX regimens, because 85 mg/m^2 is the approved dose for usage in Japan, the treatment is adapted for this dose, even in the mFOLFOX6 regimen.

In conclusion, the FOLFOX regimens we used were found to demonstrate good efficacy as chemotherapy regimens in our population, with an acceptable overall toxicity profile. However, attention must be paid to the occurrence of peripheral sensory neuropathy, which may influence a patient's quality of life, while also limiting the continuation of such treatment.

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perspectives Journal of proteome research

Personalized Medicine and Proteomics: Lessons from Non-Small Cell Lung Cancer

György Marko-Varga,[†,‡] Atsushi Ogiwara,[‡,§,⊥] Toshihide Nishimura,[§,⊥] Takeshi Kawamura,[§,⊥] Kiyonaga Fujii,[§,⊥] Takao Kawakami,[§,⊥] Yutaka Kyono,[⊥] Hsiao-kun Tu,[⊥] Hisae Anyoji,[⊥] Mitsuhiro Kanazawa,[⊥] Shingo Akimoto,[⊥] Takashi Hirano,[∥] Masahiro Tsuboi,[∥] Kazuto Nishio,[§] Shuji Hada,[#] Haiyi Jiang, Masahiro Fukuoka,[∇] Kouichiro Nakata,[◆] Yutaka Nishiwaki,[∥] Hideo Kunito,[§] Ian S. Peers,[○] Chris G. Harbron,[○] Marie C. South,[○] Tim Higenbottam,[▽,£] Fredrik Nyberg,[*,▽,♯] Shoji Kudoh,[∫] and Harubumi Kato[∫]

Respiratory Biological Sciences, AstraZeneca R&D Lund, SE-221 87 Lund, Sweden, Clinical Proteome Center, Tokyo Medical University, Shinjuku Sumitomo Building 17F, 2-6-1 Nishishinjuku, Shinjuku, Tokyo 163-0217, Japan, Medical ProteoScope Company, Limited, Shinjuku Sumitomo Building 17F, 2-6-1 Nishishinjuku, Shinjuku, Tokyo 163-0217, Japan, Department of Surgery, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku, Tokyo 160-0023, Japan, Clinical Division, Research & Development, AstraZeneca K.K., Umeda Sky Building Tower East, 1-88, 1-chome, Ohyodo-naka, Kita-ku, Osaka 531-0076, Japan, Clinical Science Department, Research & Development, AstraZeneca K.K., Umeda Sky Building Tower East, 1-88, 1-chome, Ohyodo-naka, Kita-ku, Osaka 531-0076, Japan, Department of Medical Oncology, Kinki University School of Medicine, 377-2,Ohno-higashi, Osakasayama-city 589-8511, Osaka, Japan, Department of Respiratory Diseases, Toho University School of Medicine, 6-11-1, Omori-nishi, Ota-ku, Tokyo 143-8541, Japan, Dept. of Thoracic Oncology, National Cancer Centre Hospital East, 6-5-1, Kashiwanoha, Kashiwa-city, Chiba 277-8577, Japan, Statistical Sciences, AstraZeneca R&D Alderley Park, Cheshire, UK, Cancer & Infection Statistics, AstraZeneca R&D Alderley Park, Cheshire, UK, Medicine & Science, AstraZeneca R&D Charnwood, Loughborough LE11 5RH, Leicestershire, UK, Sheffield University, Sheffield, UK, Epidemiology, AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden, Institute of Environmental Medicine, Karolinska Institute, Box 210, SE-171 77 Stockholm, Sweden, and 4th Department of Internal Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

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Personalized medicine allows the selection of treatments best suited to an individual patient and disease phenotype. To implement personalized medicine, effective tests predictive of response to treatment or susceptibility to adverse events are needed, and to develop a personalized medicine test, both high quality samples and reliable data are required. We review key features of state-of-the-art proteomic profiling and introduce further analytic developments to build a proteomic toolkit for use in personalized medicine approaches. The combination of novel analytical approaches in proteomic data generation, alignment and comparison permit translation of identified biomarkers into practical assays. We further propose an expanded statistical analysis to understand the sources of variability between individuals in terms of both protein expression and clinical variables and utilize this understanding in a predictive test.

Keywords: personalized medicine • gefitinib • therapy • interstitial lung disease • non-small cell lung cancer • biomarkers • predictive test • mass spectrometry • statistical analysis • proteomics

* To whom correspondence should be addressed. Epidemiology, AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden; Tel, +46 31 706 5203; Fax, +46 31 776 3823; E-mail, Fredrik.Nyberg@astrazeneca.com.

† György Marko-Varga and Atsushi Ogiwara made equal contributions to this manuscript.

‡ Respiratory Biological Sciences, AstraZeneca R&D Lund.

§ Clinical Proteome Center, Tokyo Medical University.

⊥ Medical ProteoScope Co., Ltd.

∥ Department of Surgery, Tokyo Medical University.

Clinical Division, Research & Development, AstraZeneca K.K.

∇ Clinical Science Department, Research & Development, AstraZeneca K.K.

○ Department of Medical Oncology, Kinki University School of Medicine.

◆ Department of Respiratory Diseases, Toho University School of Medicine.

£ Dept. of Thoracic Oncology, National Cancer Centre Hospital East.

♯ Statistical Sciences, AstraZeneca R&D Alderley Park.

Cancer & Infection Statistics, AstraZeneca R&D Alderley Park.

▽ Medicine & Science, AstraZeneca R&D Charnwood.

∫ Medical School, Sheffield University.

⊥ Epidemiology, AstraZeneca R&D Mölndal.

∥ Institute of Environmental Medicine, Karolinska Institute.

Fourth Department of Internal Medicine, Nippon Medical School.

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Introduction

A personalized medicine approach uses appropriate biomarkers to select treatments best suited for an individual patient and disease phenotype. A multiple biomarker approach (e.g., proteomics) has the advantage over conventional single biomarkers of combining many different pieces of information. Here, we review the key features of state-of-the-art proteomic profiling and introduce recent analytic developments to build a proteomic toolkit for use in personalized medicine, and we describe how these may be applied in a viable method for exploiting predictive proteomic fingerprints in the clinic. The potential of our proteomics toolkit hopefully brings us one step closer to a practical personalized medicine.

Cancer therapy is moving toward individually selected treatments, chosen not only according to tumor cell type but also based on the patient's predicted responsiveness to different classes of therapy or susceptibility to therapeutic adverse events. This emerging personalized medicine approach draws on both genotype and phenotype information, including protein expression. To implement personalized medicine, we need to develop effective biomarker tests predictive of response to treatment or susceptibility to adverse events. The benefits of personalized medicine are exemplified by considering interstitial lung disease (ILD) among non-small cell lung cancer (NSCLC) patients, which is associated with various kinds of chemotherapy treatment. A personalized medicine approach, using a simple blood test to predict those NSCLC patients at risk of developing ILD, would clearly be of great value.

We review current thinking and present some novel developments in a number of areas that have to be integrated to develop and then practically apply such tests in a clinical setting:

- The large scale collection of reliable and high quality phenotypic and clinical data and blood samples.
- Protein analysis in blood.
- Data acquisition, handling, combining and analysis.
- Interpretation and utilization of results in a clinical setting.

Clinical Background

A Motivating Example: Gefitinib (IRESSA) Treatment of NSCLC. The concepts of proteomics-based personalized medicine discussed in this article are very generally applicable. A motivating example that we will refer to in order to illustrate the potential benefits of personalized medicine is ongoing work in attempting to develop a simple blood test to address the potential occurrence of ILD in seriously ill NSCLC patients, the target group for the NSCLC treatment gefitinib.

Gefitinib is a "small molecule" inhibitor of the enzyme tyrosine kinase of the epidermal growth factor receptor (EGFR) family, such as erbB1. It is an approved therapy for advanced NSCLC in many countries and offers important clinical benefits (tumor shrinkage and improvement in disease-related symptoms) for "end-stage" patients. The large phase III ISEL (IRESSA Survival Evaluation in Lung Cancer) trial demonstrated some improvement in survival with gefitinib which failed to reach statistical significance compared with placebo in the overall population and in patients with adenocarcinoma.[1] However, in preplanned subgroup analyses, a significant increase in survival was shown with gefitinib in patients of Asian ethnicity and in patients who had never smoked.[1]

Analysis of the biomarker data from a subset of patients in the ISEL study suggested that patients with pretreated advanced NSCLC who have tumors with a high EGFR gene copy number (detected by fluorescent in situ hybridization [FISH]) have a higher likelihood of increased survival when treated with gefitinib compared with placebo.[2] Increased HER2 gene copy number has also been seen in tumors from patients who are responsive to gefitinib.[3] Somatic-activating mutations of EGFR in tumor tissue have also been associated with increased gefitinib responsiveness in patients with NSCLC.[4-7] Such mutations are more commonly found in tumor samples from patients of Asian origin and non-smokers.[8]

Following the ISEL subgroup analyses, and the biomarker evidence, it has become important to clarify which patients are more suitable for treatment with gefitinib. Analyses for both somatic-activating mutations and gene copy number require tumor tissue, which is not always available from the time of diagnosis; therefore, a blood test may represent a more versatile option and be of great value to clinicians.

With respect to tolerability, the search for a blood test that might include both genetic and proteomic biomarkers to define patients at risk of adverse effects from a drug, for example interstitial lung disease with gefitinib, is a focus of research.

Interstitial Lung Disease as a Complication in NSCLC Patients. ILD is a disease that afflicts the parenchyma or alveolar region of the lungs.[9] The alveolar septa (the walls of the alveoli) become thickened with fibrotic tissue. Associated with drug use, it can present precipitously with acute diffuse alveolar damage (DAD). The lungs show so-called "ground glass" shadowing on chest radiology, and patients complain of severe breathlessness. There are no effective treatments but patients can be supported by oxygen supplementation, corticosteroid therapy, or assisted ventilation. The process of alveolar damage is however fatal in some patients. ILD is a comorbidity in patients with NSCLC.[10-16] Both diseases are associated with cigarette smoking,[17-20] and ILD is also considered to be associated with various kinds of lung cancer chemotherapy.[21-26]

In the ISEL study of gefitinib in NSCLC mentioned above, ILD-type events occurred in 1% of both placebo and gefitinib-treated patients.[1] Most ILD-type events occurred in patients of Asian origin, where placebo and treated patients had similar prevalences of respectively 4% and 3%. The rate observed in the gefitinib-treated arm was in line with earlier safety data from Japan and a large gefitinib post-marketing surveillance study in Japan (3322 patients), where the reported rate of ILD-type events was 5.8%.[27]

A simple blood test to predict the potential occurrence of ILD in seriously ill NSCLC patients before initiating treatments would clearly be of great value. This article describes the personalized medicine approach, which could be used to provide such a test. Consequently, the proteomics objectives of the preliminary phase of the study we describe were to verify the protein expression alterations in blood plasma from case patients (who developed ILD) and control patients (without ILD) treated by gefitinib, using a liquid chromatography−mass spectrometry/mass spectrometry (LC−MS/MS) proteomics platform.

Data and Sample Collection

To develop a personalized medicine test, it is essential to have access to an adequately sized collection of high quality tissue samples on which to perform proteomics analysis, with corresponding reliable diagnostic and clinical data.

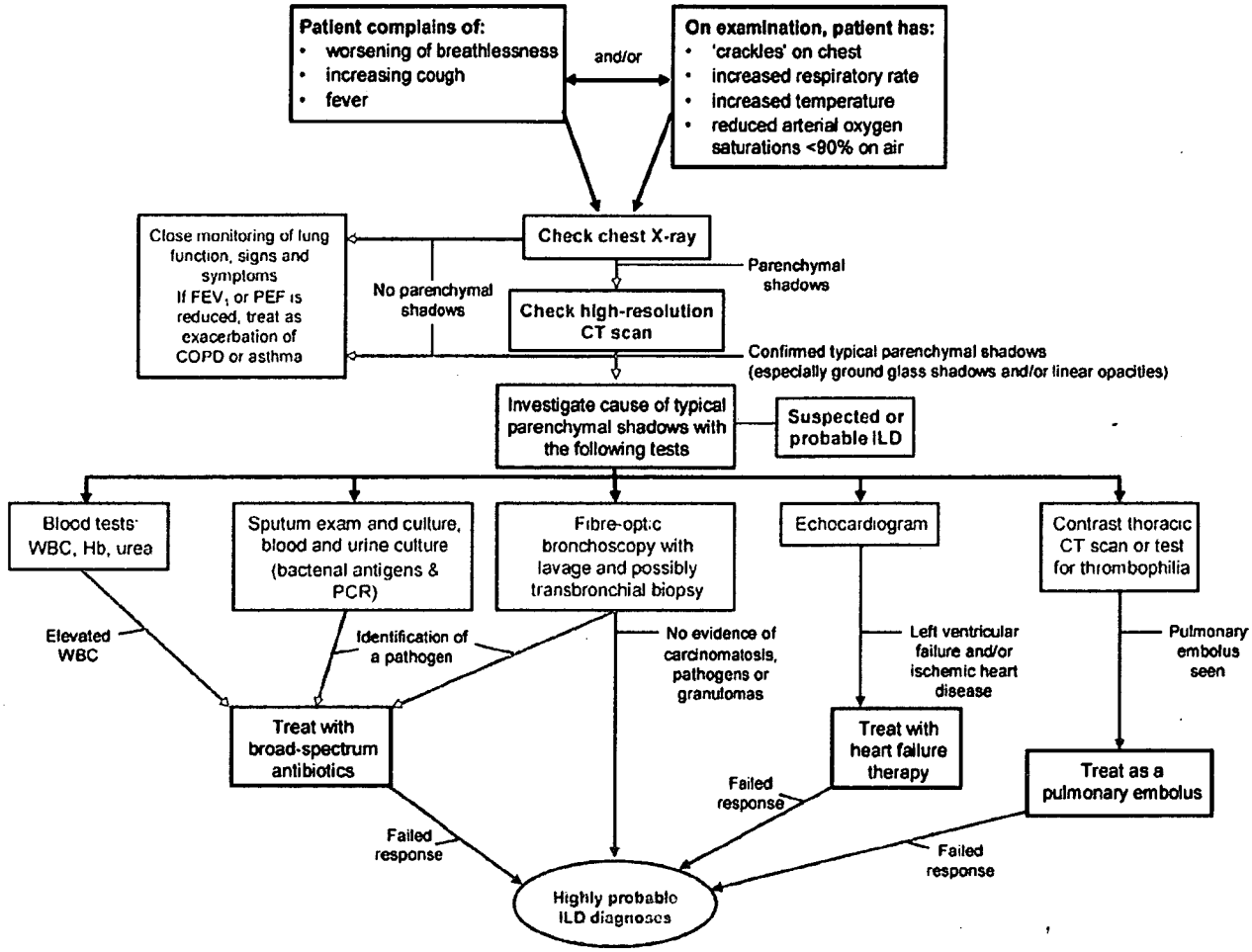

Figure 1. Algorithm for diagnosis of interstitial lung disease (ILD) in non-small cell lung cancer (NSCLC) patients.

As an example, in our work with gefitinib, samples were taken after obtaining informed consent from a nested case-control study, i.e., a case-control study performed within a prospective pharmacoepidemiological cohort of several thousand patients with advanced or recurring NSCLC who had received at least one prior chemotherapy regimen, and who were to be treated with gefitinib or chemotherapy. The main objective of this study was to measure the relative risk of ILD in Japanese patients with NSCLC using gefitinib compared with conventional therapy, with the associated aims of determining the incidence rate of ILD in late stage NSCLC patients and the principal risk factors for this complication.

Central to both the case-control study and the proteomics analysis was the use of internationally agreed criteria for the diagnosis of ILD and an algorithm of diagnostic tests to exclude alternative diseases.[26] Principal investigators in the study were asked to assess all patients for possible ILD using the diagnostic algorithm (Figure 1). Two case review boards of experts from oncology, radiology, and pulmonary medicine were set up to independently establish a consistent final diagnosis of ILD. In addition, extensive standard clinical and demographic risk factor data were collected on all registered cases and controls.

This degree of rigor in establishing accurate phenotypic diagnosis is critical to develop a robust and reliable personalized medicine test, as inaccuracies at this stage will affect all subsequent data analyses. The availability of clinical and risk factor data, and a rigorous epidemiological study design setting for the collection of proteomics samples is also of great value to fine-tune the statistical analysis.

Is Proteomics Ready for Personalized Medicine Applications?

The Human Proteome Map in Plasma. The impetus to develop personalized medicine based on blood samples has encouraged proteomic profiling that identifies individual proteins and multiple "fingerprint" protein patterns. A remaining limitation has been the lack of integration of the technology of protein separation with bioinformatics and statistical methods. Extensive national and international[29,30] collaborations are being implemented to address some of these needs. An important component in this development is the Human Proteome Organization (HUPO; www.HUPO.org), a scientific consortium that supports various programmes to map the proteins expressed in various human tissues, disease states, etc.[31-33] One of these is the Plasma Proteome initiative started in 2002, aiming to annotate and catalog the many thousands of proteins and peptides[31-37] of the human plasma proteome. Recently results from the pilot phase with 35 collaborating laboratories from 13 countries[38-42] and multiple analytical

groups were made publicly available on the Internet (www.bioinformatics.med.umich.edu/hupo/ppp; www.ebi.ac.uk/pride). The combined efforts have generated 15 710 different MS/MS datasets that were linked to the International Protein Index (IPI) protein IDs, and an integration algorithm applied to multiple matches of peptide sequences yielded 9504 IPI proteins identified with one or more peptides[40] and characterized by Gene Ontology, InterPro, Novartis Atlas, and OMIM. Such advances provide an important platform for transforming proteomics from a technology to a useful biomarker tool applicable to personalized medicine.

Protein Analysis in Blood The Methods. With respect to automated studies, multidimensional chromatography is the main technology used for protein analysis in blood. It is coupled to mass spectrometry either by electrospray ionization (ESI) for analysis in solution or matrix assisted laser desorption/ionization (MALDI) in solid phase applications.[39,41,42,47] Alternatively, ion-trap mass spectrometers are gaining recognition for high-throughput sequencing.[46,48-53] Linking a Fourier transform ion cyclotrone resonance (FTICR) unit to the linear trap can increase the resolution profoundly,[36,54-56] one of several novel principles for strengthening the assignment of protein annotations with the most commonly used protein search engines.[36,47,54-61] For protein annotation, the recent development of a human protein reference database complements these technologies.[61] Studies of protein expression in a variety of biological compartments ranging from sub-cellular to whole organisms have been undertaken with these analytic approaches.[62-70] Some key findings from the HUPO initiatives that impact on methodology include:

- For studies using blood samples, plasma rather than serum is preferred, with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.[49]
- The abundant proteins in plasma should be depleted prior to analysis.[49]
- Acceptance of protein annotation, i.e., accepted protein identities[39,40] should use standard criteria. These include having two identified peptide sequences from each protein, both with a statistical significance score high enough to ensure a correct sequence confirmation when compared with the corresponding gene sequence entity.[39]

Despite the advances in methodology, important hurdles to using proteomics in a personalized medicine context remain.

Protein Expression Analysis in Blood: Some Important Hurdles. Although protein profiling technology is highly automated and interfaced with database search engines to relate peptide sequences to protein identities and function,[39,40] there are many practical reasons why determining the relative abundance of proteins relevant for prediction purposes is difficult:

- About 90% of proteins are believed to be present only in low copy numbers, i.e., at medium and low abundance levels.[49]
- There can be variation both in the quantity and form of protein expression within normal physiological function.
- Between 300 000 and 3 million human protein species exist as direct gene products or post-translational modifications.[34]
- The relative abundance of the post-translational modifications occurring within the cell is called a Cell-Protein-Index Number (CPIN).[29,30] As an example, if one considers that there are 30 types of phosphorylation variants of a single phosphoprotein, and a hundred possible fold forms of glycosylation of a single glycoprotein, the theoretical CPIN varies considerably depending on the sample complexity.
- The dynamic range of protein expression within cells, between levels of most and least abundant proteins, is in the order of 10^{6} - 10^{10}.[34-36]
- In a typical clinical proteomics study the total cellular protein material in a sample seldom exceeds 10 - 20 milligrams. Therefore, the least abundant proteins would be present at starting levels not exceeding picograms.
- Recent studies use technology that can identify several thousand proteins in plasma samples,[29] but this still probably only represents a small fraction of the intermediate and processed protein forms. This is due to the current limitation of mass spectrometry not being able to ionize all amino acid sequences and protein modifications with equal efficiency. In most situations, a limited region of the full length protein is sequence annotated.
- The detection of differences in protein expression between groups of interest (e.g., cases and controls) takes place against a background of high variation between individuals within a group, within individuals over time and possible analytic run-to-run variation. Any method used to address this hurdle (which will involve "alignment" for spectral methods) directly impacts the ability to find good protein biomarkers.

Beyond the hurdles above, the fundamental challenge of protein biomarkers is to link the relative abundance of single markers or a fingerprint to clinically important biological processes based on some direct or indirect cause-effect link[29] related to normal or aberrant biological pathways.[47-49] In the following sections, we present the approach used for the identification of protein biomarkers potentially associated with development of ILD in NSCLC patients within the case-control study used as our motivating example. We build on the foundations described above and introduce further analytic developments and ideas relating to proteomic data generation, assaying and alignment to build a proteomics toolkit that can be applied today for personalized medicine approaches.

A State of the Art Clinical Biomarker Analysis System

In the previous section, we described several challenges in proteomic analysis. Here we describe a system and analysis approaches that we have successfully implemented to address some of these issues.

The Components of the Analysis System. The analysis system (Figure 2) uses liquid chromatography-based high-resolution separation of peptides with an interface to tandem MS/MS, a technology which has been attracting great attention as the "shotgun" method of proteome analysis.[44,68-70] With this technology, after depletion of albumin and immunoglobulin G (IgG), all extracted plasma proteins are digested into their specific peptide components by proteolytic enzyme treatment.

The generated peptides are subjected to capillary reverse-phase submicro- to micro-flow liquid chromatography (capillary RP μLC), separated by retention times due to their physicochemical properties, and then detected and sequenced by a linear ion-trap tandem mass spectrometer[71] (LTQ, Thermo Fisher Scientific, San Jose, CA) interfaced with a spray needle tip for ESI of peptides.[70] A two-dimensional quadrupole ion trap mass spectrometer[71] is used, operated in a data-dependent acquisition mode with operational m/z range limits set at 450 - 2000 (Figure 3, graphs A and B). Automatic switching to MS/MS acquisition mode is made in 1-second scanning cycles, controlled by the XCalibur software. The actual differences between annotated peptide fragment peaks shown in Figure 3, graph C, correspond to the amino acid residue mass, i.e.,

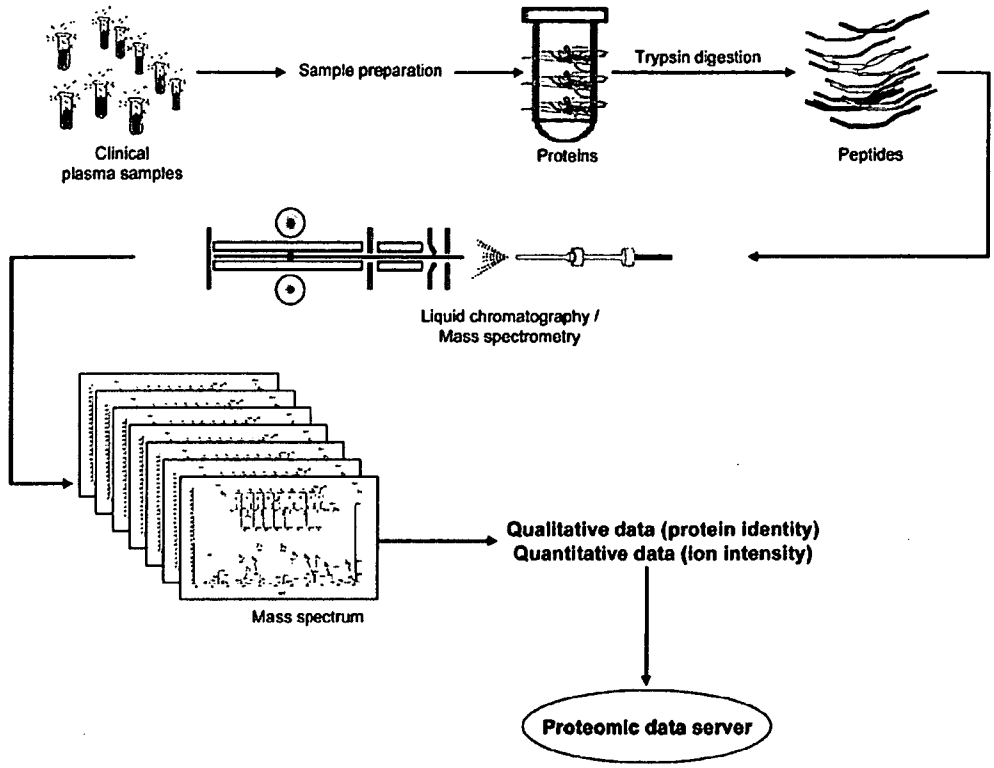

Figure 2. Schematic illustration of the clinical proteomics screening process.

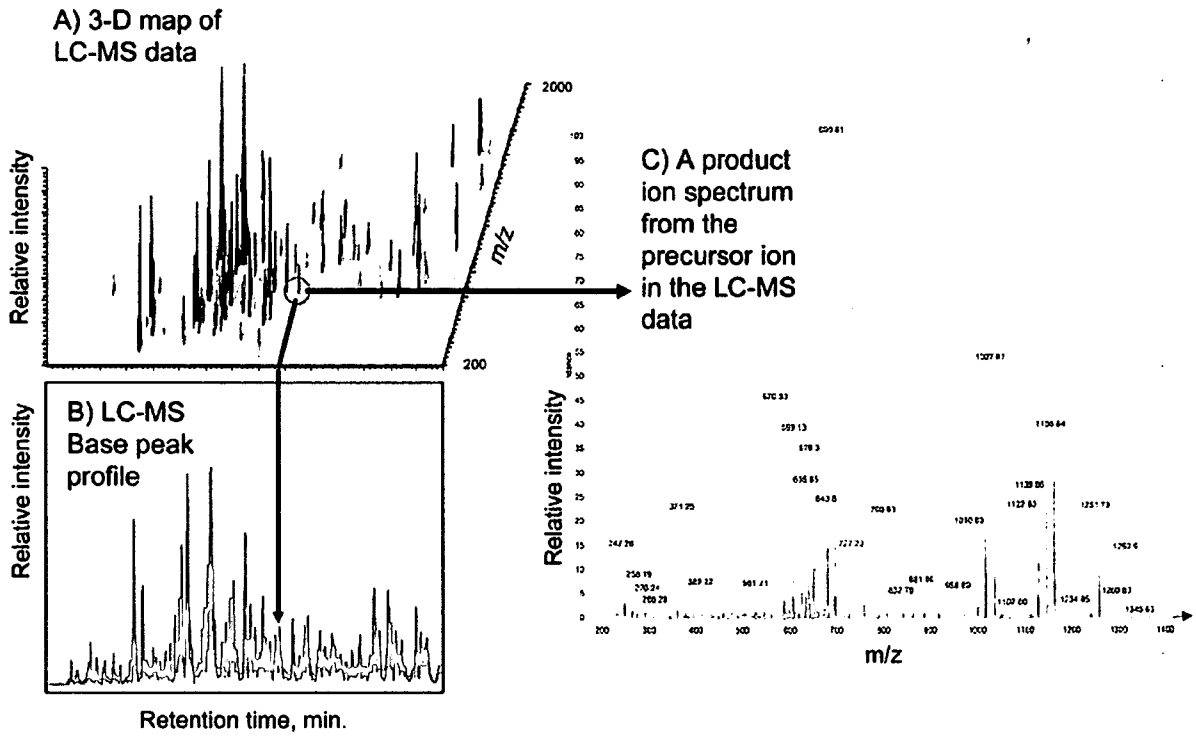

Figure 3. Profile of LC MS data: (a) the three-dimensional view of LC-MS data, (b) the base-peak mass chromatogram, and (c) a product ion spectrum measured for a precursor ion in data-dependent acquisition mode (with MS acquisition operational *m/z* range set at 450 2000).

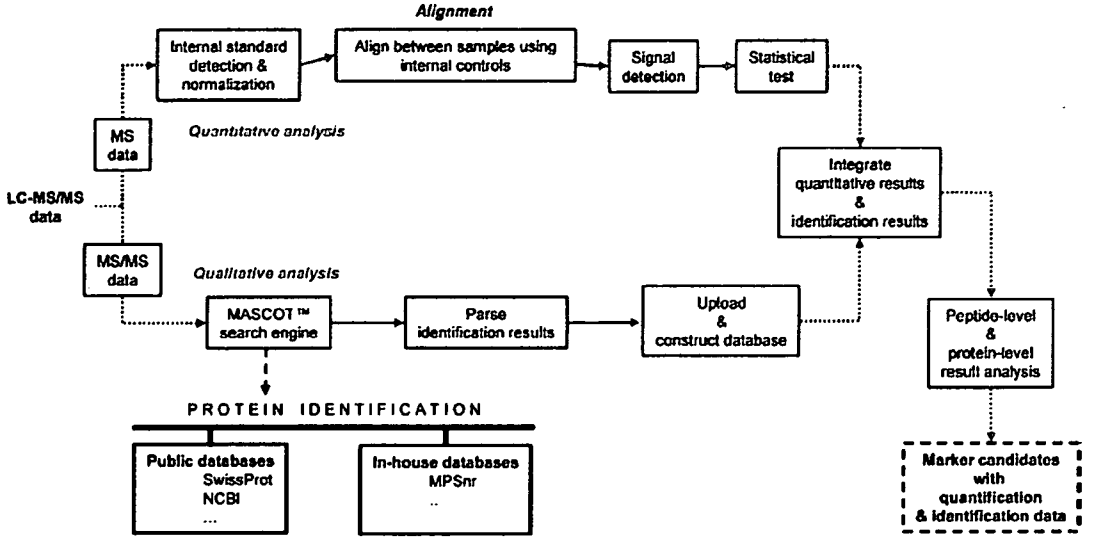

Figure 4. Overview of the data acquisition and database mining process developed within the gefitinib biomarker study.

identify the correct amino acid sequence. Internal standards are used for alignment of retention-times.

How the Methodology Overcomes Some of the Hurdles. The system described above addresses some of the hurdles noted previously. The digestion of all extracted plasma proteins into peptides will reduce the complexity by combining high-resolution nanoflow chromatographic fractionation with the separation power of modern mass spectrometry, performing automated and unattended shotgun sequencing in plasma.[35] Peptides are also more soluble and easier to handle than intact proteins. In addition, the two-dimensional quadrupole ion trap mass spectrometer[71] operates with a high-volume quadrupole electric field that makes it highly efficient to trap ions. The result is high sensitivity, high scanning speed, and better quantification over a wide dynamic range in comparison with the conventional three-dimensional ion-trap instruments.

Finding signals against a background of high variation is a further challenge, and the next section describes some approaches for addressing these.

Initial Data Handling, Processing, and Analysis

Proteomic data analysis process can be considered as consisting of two components (Figure 4). *Quantitative analysis* is used to discover significant differences in peptide signal intensities by comparing two (or more) sample groups. This process uses data collected from an entire MS run to quantify the amount of peptide ions by their respective ion signal intensity. *Qualitative analysis* is used to identify the amino acid sequence of each peptide ion, from the respective product ion spectra. To maximize their value, the results from the two component analyses should be considered in combination.

A typical quantitative analysis may consist of several steps:

1. Normalization: To account for differences in the original sample concentrations. Typically, the total signal intensity is scaled to a constant value for each analyzed sample.

2. Alignment: Correcting for nonlinear fluctuation in retention time between different samples. A variety of methodologies are available for aligning LC−MS data sets. We have found the i-OPAL algorithm (Patent # WO 2004/090526 A1), which is based on the single linkage clustering algorithm[72] and which makes use of internal standard signals, to perform well. Other alignment algorithms include xcms.[73]

3. Peak picking or signal detection: Identifying individual peptide ions within the data.

4. Identify discriminating peptides: A number of methods can be used, often in combination. A common approach is to apply a Student's *t*-test and select peptides which are significant, i.e., with a *p*-value less than the chosen cutoff, and which also show a fold-change or intensity ratio greater than another criterion. Further developments of this aspect are discussed in the Principled Statistical Analysis section.

A popular choice for qualitative analysis is the MASCOT MS/MS ion search program.[74] This may be run against a number of different peptide sequence databases, for example the NCBI Nr, Refseq, Gene Ontology, HUGO, and Swiss-Prot sequence databases. The results of the quantitative analysis can then be combined with the qualitative analysis so that, for example, a peptide must be both discriminating and have annotation i.e., have achieved a high MASCOT score showing confidence in identification to be considered a candidate biomarker.

The approaches we have discussed above are focused on finding potentially discriminating proteins of clinical utility. In the following section, we describe the next stage in our thinking, namely how we could rapidly deploy in the clinic a viable method for exploiting a predictive proteomic fingerprint.

A Proposal for Proteomics in the Clinical Setting: Mass Spectrometric Biomarker Assays - MSBA

Although today's technology allows for high-throughput analyses of many proteins rather than a single protein,[30] the details of how such multiplexing assays will be adapted for clinical use have not been well clarified. The Mass Spectrometric Biomarker Assay (MSBA) platform described here was conceived as one example of a rapid and seamless method to progress from identification of a diagnostic more directly to a clinically useful test. MSBA requires only a minute sample amount (5−20 μL) to obtain a read-out from a handful of quantified protein biomarkers (typically 3−35) and automatically analyzes proteins using liquid-phase separation and tandem mass spectrometry with simultaneous quantitation and identification.

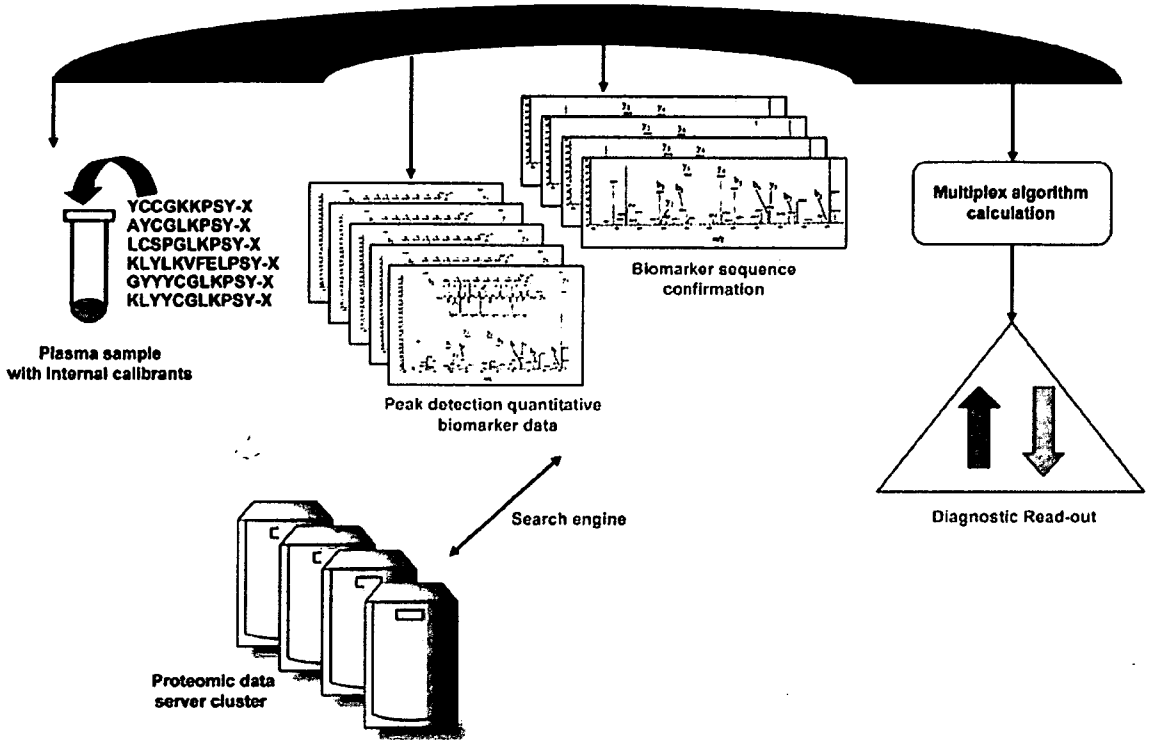

Figure 5. Entire flow of the operational components of Mass Spectrometric Biomarker Assays (MSBA).

The MSBA builds on a pre-defined Multiplex Biomarker list, which is stored within the MSBA database. Each marker entity has the values of masses and the relative retention time index with tolerance parameters. In running a patient sample, the predefined biomarker list is scanned to pick up patient sample signals that match with one of the predefined biomarker signals by satisfying the tolerance criteria (in general ±1 for m/z value and ±2% for relative retention time index). The selected candidate signals are further confirmed using the product ion spectrum. That is, the product ion spectrum is represented as a vector by binning (grouping) the m/z ratio values. Using the cosine correlation between the sample vectors and the reference vectors, we can confirm whether the selected candidate signals are truly assigned as target biomarkers. (A standard threshold value of the cosine correlation is 0.8.)

The process steps within the MSBA cycle are outlined in Figure 5. The calculation of the final multiplex biomarker assay read-out from all of the individual markers can be performed by a variety of applications, as discussed in more detail in the Principled Statistical Modeling Approach section. Figures 6A and B illustrate one approach, calculating a distance score which indicates to what extent a measured sample is distant from the case or control template in terms of predefined multiplex biomarkers.

$$S_{\text{case or control}} = \sqrt{\left[\frac{1}{n(n-2)}\right]\left[n\sum_i y_i^2 - \left(\sum_i y_i\right)^2 \frac{\left[n\sum_i x_i y_i - \left(\sum_i x_i\right)\left(\sum_i y_i\right)\right]^2}{n\sum_i x_i^2 - \left(\sum_i x_i\right)^2}\right]}$$

If the ratio of S_{case} and S_{control} exceeds an MSBA threshold parameter, then the test sample is predicted to be a patient susceptible to develop ILD (ILD case); if not, the test sample is predicted to be a non-susceptible patient (control). We are currently evaluating the MSBA approach in practice.

A Principled Statistical Modeling Approach

We have described an analytical approach based on proteomic data, with various novel developments. However, additional insight is needed to further improve model discrimination and to broaden the focus from the proteomic data to the ultimate goal of prediction using combinations of data. Statistical analysis can be used to provide further refinement by combining information from the full clinical and laboratory datasets.

An advantage of a multiple biomarker approach (e.g., proteomics) compared with standard single biomarker development is the capability to combine information from many different entities. An example is illustrated in Figure 7A. Considering each biomarker alone fails to separate the two groups of subjects, as there is considerable overlap for both biomarkers. Use of two biomarkers in combination completely separates the two groups.

We can also use clinical variables to advantage in the analysis of the peptide patterns. For example, the efficacy of gefitinib appears to be greater in non-smokers, women, patients of Asian origin, and patients with adenocarcinomas.[8] Figure 7B illustrates how, instead of two protein biomarkers, the combination of clinical data (e.g., age) and a proteomic biomarker is able to separate two groups.

On this basis, we propose using a principled statistical analysis approach to first explore and understand the data and

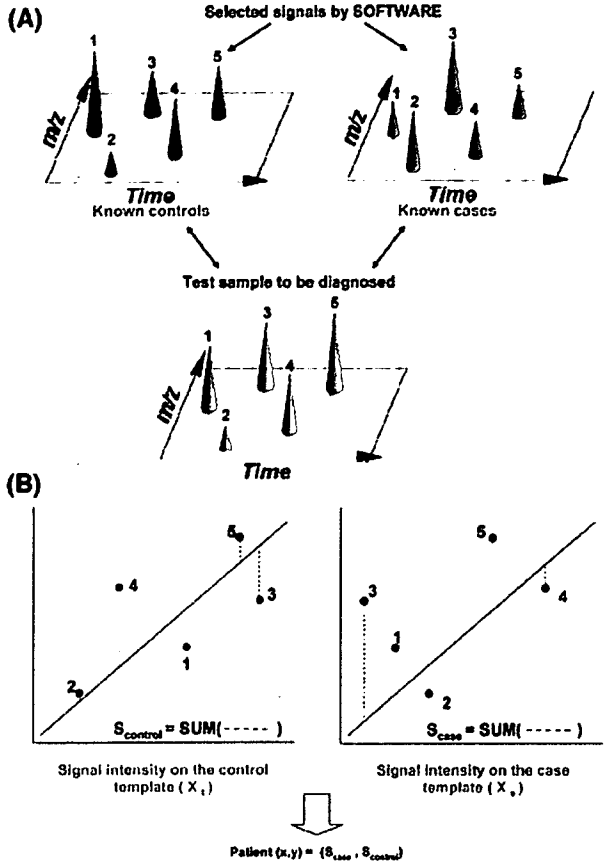

Figure 6. (A) Peptide signal comparison that MSBA (Mass Spectrometric Biomarker Assays) performs of the generated ions from the sample. The comparison is made both with the pattern of the controls and with the pattern of the case group for the corresponding signals. (B) Illustration of the regression model application of the MSBA where control templates and case templates are compared to that of the sample template generated in the analysis process.

then to model it and understand the quality of any models produced. A first step is to perform exploratory data analysis (EDA), for example using principal components analysis (PCA), to understand the major sources of data variation and the covariation between clinical parameters and protein intensity measures. The next step is univariate modeling for each protein marker individually, for example using analysis of covariance (ANCOVA), and an assessment of the effect of clinical parameters across the whole set of protein biomarkers using, for example, the False Discovery Rate as a tool.[75] This provides an understanding of key clinical variables and sources of variation within the data.

The next step is to perform multivariate predictive modeling using the proteins and clinical variables identified as being potentially important. There are a number of mathematical methods described in the literature for performing supervised classification, for example Support Vector Machines,[76] Random Forests,[77] PAM,[78] all of which have been successfully applied to high dimensional genomics data.[79] It remains an important unanswered question which modeling approach, or combination of modeling approaches, will generate the most predictive and robust models for data generated using this technology within a prospective study of this design.

Finally, to confirm that we have a practical prediction, the predictive power of a model must be assessed on a different set of patients from that used to generate the model. There are a number of approaches for external validation given a limited size dataset, for example the sequential approach of building a model based upon currently available data and testing on data from new patients when they become available, or withholding an arbitrary selection of subjects from the modeling as a test set and testing the model on these subjects. Internal validation approaches such as cross-validation or related bootstrapping methods may also be useful to assess the model selection *procedure*, but tend to overestimate the performance of a specific predictive model in subsequent external validation.[80,81] The key properties to consider when selecting an assessment method are to ensure that it will provide both precise and unbiased information regarding the prediction error rate of the potential model to be tested for clinical use. As well as assessing an overall predictive rate, it is also useful to separately assess the predictive rate for both the cases and controls and to consider the relative costs of making these false predictions within a clinical setting. Finally, the prevalence of the condition in question (here IUD) is also a critical factor in estimating what proportion of people predicted to be at risk are truly at risk, and this should also be borne in mind when evaluating a model for potential clinical use. The recently published FDA concept paper on drug-diagnostic co-

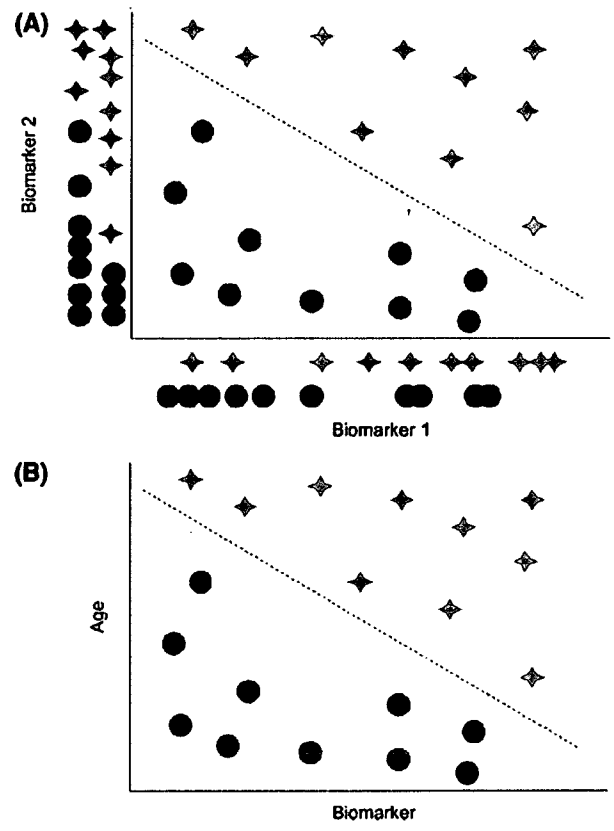

Figure 7. (A) Hypothetical example of the combined disease-linkage effect of two protein biomarkers. (Stars signify affected case individuals, circles non-affected control individuals). (B) Hypothetical example of the combined disease-linkage effect of a biomarker and a clinical variable. (Stars signify affected case individuals, circles non-affected control individuals).

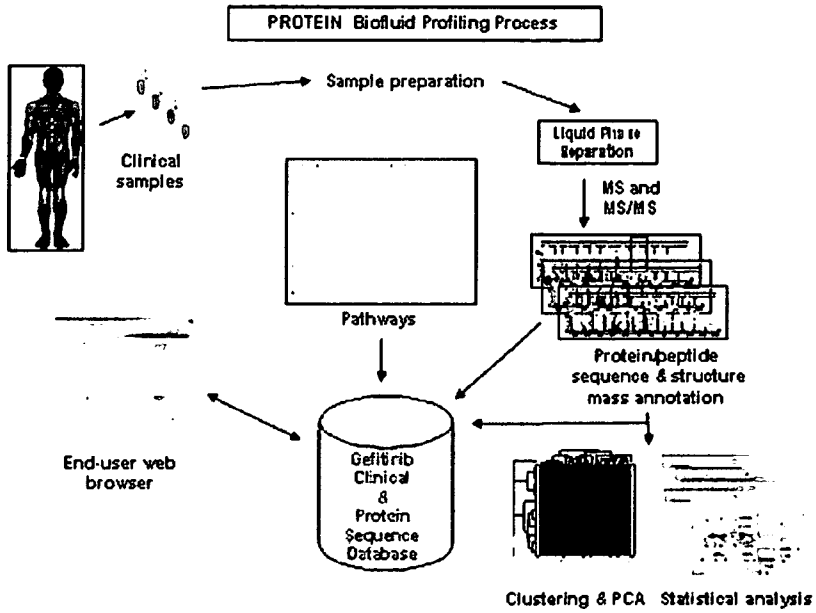

Figure 8. Illustration of the bioinformatics and data processing structure within which MSBA (Mass Spectrometric Biomarker Assays) data are captured, modified and analyzed.

development discusses many of the issues around validating predictive biomarkers.[62]

Finally, it is preferable to be able to assign a biological rationale to the biomarkers. Confidence in the reliability of a biomarker is greatly enhanced if we can correctly understand how it relates to the mechanism and progression of the disease of interest. Figure 8 illustrates a bioinformatics and data processing structure that we have developed to allow us to both conduct interactive exploratory and statistical analyses, and also investigate the disease and pathway linkage of discovered biomarker proteins through direct access to reference databases.

Future Perspectives

Within this paper we have discussed many of the issues that need to considered in developing a personalized medicine approach. A key starting point is that rigorous steps are taken to ensure accurate diagnosis and the careful gathering of both clinical and proteomic data to facilitate the search for peptide patterns.

There are many challenges in performing protein analysis in blood, but mass spectrometry equipment and methods can now be used to generate peptide data with high sensitivity, high scanning speed, and improved quantification. Data handling and processing techniques for steps such as peak alignment and the subsequent methodologies for statistical modeling and analysis are now far enough developed to generate high quality data and robustly analyze these data with confidence.

We have provided details of the MSBA method that can be used to easily translate protein intensities into a practical multiplex assay which can be exploited in the clinic without the need to develop anti-bodies for ELISA. We have also described how an expanded statistical analysis can be used to allow for the individual variance of protein expression to enable us to focus on the proteomic patterns that are actually related to ILD. Finally, we have emphasized the importance of validating the predictive power of a biomarker tool in a way that reflects the real-life setting of intended clinical use.

Hopefully, this combination of developments over a range of different areas brings us one step closer to a practical personalized medicine.

IRESSA is a trademark of the AstraZeneca group of companies.

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Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway

Kazuko Sakai,[1,3] Hideyuki Yokote,[1,4] Kimiko Murakami-Murofushi,[3] Tomohide Tamura,[2] Nagahiro Saijo[2] and Kazuto Nishio[1,4,5]

[1]Shien-Laboratory,[2]Medical Oncology, National Cancer Center Hospital; Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045; [3]Department of Biology, Faculty of Science, Ochanomizu University, Ohtsuka 2-1-1, Bunkyo-ku, Tokyo 112-8610; [4]Depatment of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan

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A humanized anti-HER2 monoclonal antibody pertuzumab (Omnitarg, 2C4), binding to a different HER2 epitope than trastuzumab, is known as an inhibitor of heterodimerization of the HER receptors. Potent antitumor activity against HER2-expressing breast and prostate cancer cell lines has been clarified, but this potential is not clear against lung cancers. The authors investigated the *in vitro* anti-tumor activity of pertuzumab against eight non-small cell lung cancer cells expressing various members of the HER receptors. A lung cancer 11_18 cell line expressed a large amount of HER2 and HER3, and its cell growth was stimulated by an HER3 ligand, heregulin (HRG)-α. Pertuzumab significantly inhibited the HRG-α-stimulated cellular growth of the 11_18 cells. Pertuzumab blocked HRG-α-stimulated phosphorylation of HER3, mitogen-activated protein kinase (MAPK), and Akt. In contrast, pertuzumab failed to block epidermal growth factor (EGF)-stimulated phosphorylation of EGF receptor (EGFR) and MAPK. Immunoprecipitation showed that pertuzumab inhibited HRG-α-stimulated HER2/HER3 heterodimer formation. HRG-α-stimulated HER3 phosphorylation was also observed in the PC-9 cells co-overexpressing EGFR, HER2, and HER3, but the cell growth was neither stimulated by HRG-α nor inhibited by pertuzumab. The present results suggest that pertuzumab is effective against HRG-α-dependent cell growth in lung cancer cells through inhibition of HRG-α-stimulated HER2/HER3 signaling. (*Cancer Sci* 2007; 98: 1498–1503)

The HER family of receptor tyrosine kinases consists of four members: EGFR (also termed HER1/ErbB-1), HER2/ErbB-2/Neu, HER3/ErbB-3, and HER4/ErbB-4.[1] Binding of ligands leads to the homo- and heterodimer formation of the receptor tyrosine kinase.[2] There are numerous HER-specific ligands that generate signaling diversity within the cell.[3] EGF, amphiregulin, and TGF-α are known as a specific ligand of EGFR. HB-EGF, β-cellulin, and epiregulin have dual specificity for binding to EGFR and HER4. HRG-α binds HER3 and HER4.[4] No direct ligand for HER2 has been discovered. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of receptors, and activates the downstream-signaling molecules such as MAPK, Akt, JAK, and STAT.[5,6]

Pertuzumab is a humanized monoclonal antibody and binds to the dimerization domain of HER2 distinct from the domain that trastuzumab binds to.[7] Therefore, pertuzumab is known as a dimerization inhibiter between HER2 and the other HER family receptors. A phase I trial of pertuzumab has been performed for advanced tumors,[8] and phase II studies of pertuzumab are underway. Two members of the HER family, HER2 and HER3, act as key oncogenes in breast cancer cells.[9,10] *In vitro* and *in vivo* anti-tumor activities of pertuzumab have been reported in breast tumors through the inhibition of the HER2/HER3 heterodimer formation.[11,12] In lung cancer cells, EGFR plays a crucial role in their biological behavior, but it is unclear whether pertuzumab inhibits the growth of the lung cancer cells mediated by HER family receptors.

The authors have focused on the growth inhibitory effect of pertuzumab against NSCLC cells expressing different types of HER receptors, and analyzed the mechanism of action of pertuzumab in response to the HER receptor ligand.

Materials and Methods

Reagents. Pertuzumab (Omnitarg, 2C4) was provided in sterile water at 25 mg/mL by Genentech, Inc. (South San Francisco, CA, USA) before use. All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless noted otherwise.

Cell lines. The human NSCLC cell lines PC-7, PC-9, and PC-14 (Tokyo Medical University, Tokyo, Japan),[13,14] A549 (American Type Culture Collection, Manassas, VA, USA), and PC-3, Ma-1, Ma-24, and 11_18,[15] were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD, USA).

Cell stimulation and lysis. Cells were starved in serum free RPMI 1640 medium for 24 h and treated with EGF, TGF-α, HB-EGF, and HRG-α at 100 ng/mL for 10 min. Cells were washed twice with ice-cold PBS, and lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium vanadate, 4 mg/mL leupeptin, 4 mg/mL apoprotein, 1 mM PMSF). Protein concentration of the supernatants was determined by the BCA protein assay (Pierce, Rockford, IL, USA).

Immunoprecipitation. Cell lysates (1000 μg) were incubated with the anti-HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Protein G magnetic beads (New England BioLabs, Beverly, MA, USA) were added for 2 h. Beads were washed three times with lysis buffer, resuspended in SDS sample buffer with 2% β-mercaptoethanol, boiled, and separated using SDS-PAGE.

Western blotting. Cell lysates were electrophoretically separated on SDS-PAGE and transferred to a polyvinylidene difluoride

[5]To whom correspondence should be addressed. E-mail: knishio@med.kindai.ac.jp

Abbreviations: BCA, bicinchoninic acid; ECL, electrochemiluminescence; EDTA, ethylene diamine tetra-acetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HB-EGF, heparin-binding epidermal growth factor; HRG-α, heregulin-α; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RPMI, Roswell Park Memorial Institute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT, signal transducer and activator of transcription; TGF-α, transforming growth factor-α.

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membrane (Millipore, Bedford, MA, USA). The membrane was probed with each antibody against EGFR and HER2 (Transduction Laboratory, San Diego, CA, USA), HER3 (Santa Cruz Biotechnology), phospho-EGFR (Tyr1068), phospho-HER3 (Tyr1289), MAPK, phospho-MAPK (Thr202/204), Akt, phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA), phosphotyrosine (PY-20, Transduction Laboratory), and β-actin (Sigma) as the first antibody, followed by detection using a horseradish peroxidase-conjugated secondary antibody. The bands were visualized with ECL (Amersham, Piscataway, NJ, USA), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD, USA).

Growth inhibition assay. A 100-μL volume of cell suspension (5000 cells/well) in serum-free RPMI 1640 medium was seeded into a 96-well plate and 50 μL of each drug at various concentrations and 50 μL of EGF, TGF-α, HB-EGF, and HRG-α, at 100 ng/mL was added. Human IgG1 (Calbiochem, Cambridge, MA, USA) was used as isotype control. After incubation for 72 h at 37°C, 20 μL of MTS solution (Promega, Madison, WI, USA) was added to each well and the plates were incubated for a further 2 h at 37°C. The absorbance readings for each well were determined at 490 nm with a Delta-soft on a Macintosh computer (Apple, Cupertino, CA, USA) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ, USA). For ligand-stimulated growth of cells, the experiment was performed in six replicate wells for each ligand and carried out independently three times. For growth inhibition of pertuzumab, the experiment was performed in three replicate wells for each drug concentration and carried out independently three times as described elsewhere.[16]

Results

HRG-α dependent cell growth in lung cancer cells. Ligand-dependent cell growth of lung cancer cells was examined (Fig. 1). The addition of EGF, TGF-α, and HB-EGF increased the cell growth of the PC-3, 11_18, and A549 cells, but not that of the PC-7, PC-9, PC-14, Ma-1, and Ma-24 cells. HRG-α addition significantly increased the growth of the 11_18 cells (390% of control, $P < 0.01$ by t-test) and Ma-24 cells (204% of control, $P < 0.01$ by t-test), but did not influence the growth of any other cells. These findings suggest that the growth of the 11_18 and Ma-24 cells is depending upon HRG-α.

Pertuzumab inhibits HRG-α-dependent cell growth of the 11_18 and Ma-24 cells. Pertuzumab inhibited cell growth stimulated by HRG-α ($IC_{50} = 0.12$ μg/mL) but not stimulated by EGF, TGF-α, and HB-EGF in the 11_18 cells ($IC_{50} > 100$ μg/mL; Fig. 2). Pertuzumab also inhibited HRG-α dependent cell growth in the Ma-24 cells ($IC_{50} = 39.8$ μg/mL). Isotype control human IgG1 had no effect on ligand-dependent growth in the 11_18 and Ma-24 cells (data not shown). The growth of the other cells was not affected by exposure to pertuzumab (data not shown). This finding suggests that pertuzumab selectively inhibits HRG-α-dependent cell growth.

Ligand-stimulated phosphorylation of HER receptors. The expression levels of the HER receptors in the pertuzumab-sensitive (11_18 and Ma-24 cells) and pertuzumab-resistant cell (PC-9 cells) lines were determined using western blotting (Fig. 3a). Comparison of the protein expression levels of EGFR revealed high to moderate expression in the PC-9 and Ma-24 cells. EGFR was also detected in the 11_18 cells, although the expression in this

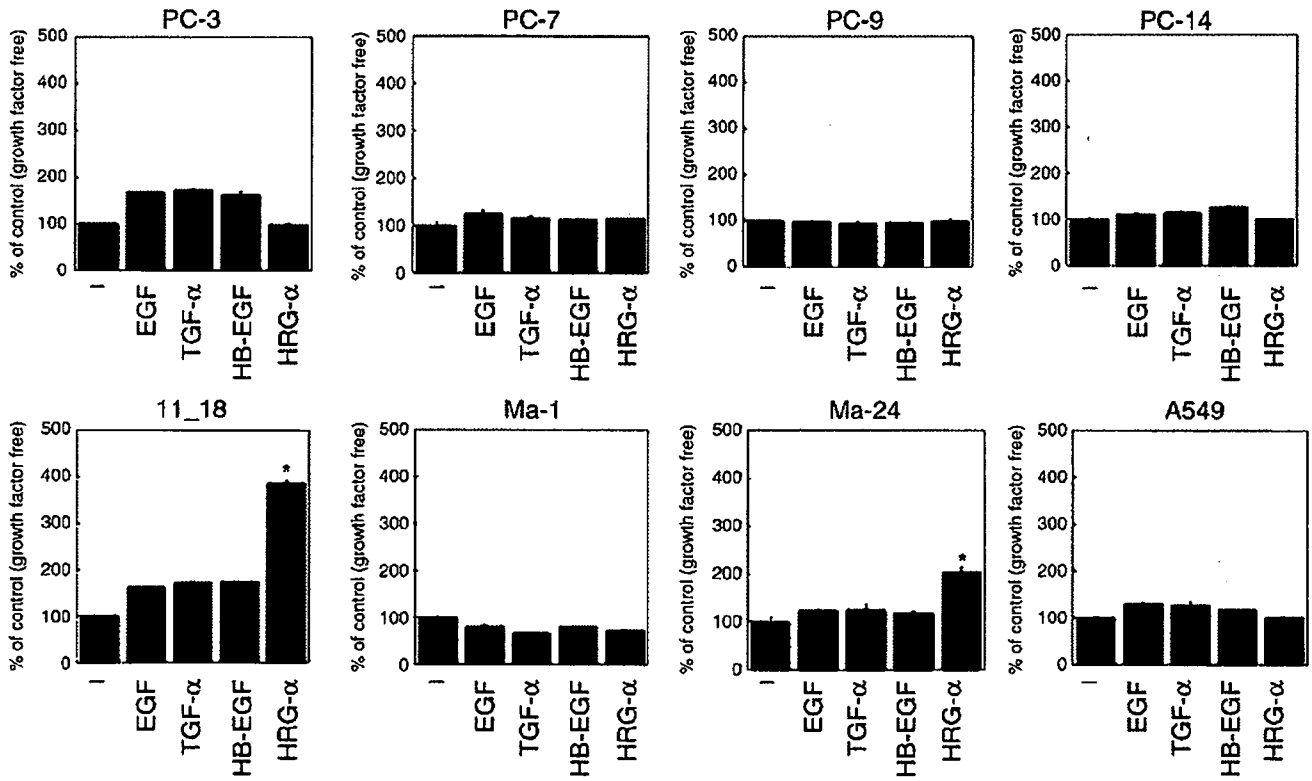

Fig. 1. Ligand-dependent cell growth in the lung cancer cells. Non-small cell lung cancer cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α. After incubation for 72 h, cell growth was determined using the MTS assay. The growth of cells was presented as the percentage of absorbance compared with ligand-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; t-test) compared to the ligand-non-stimulated cells. Data shown are representative of at least three independent experiments with similar results.

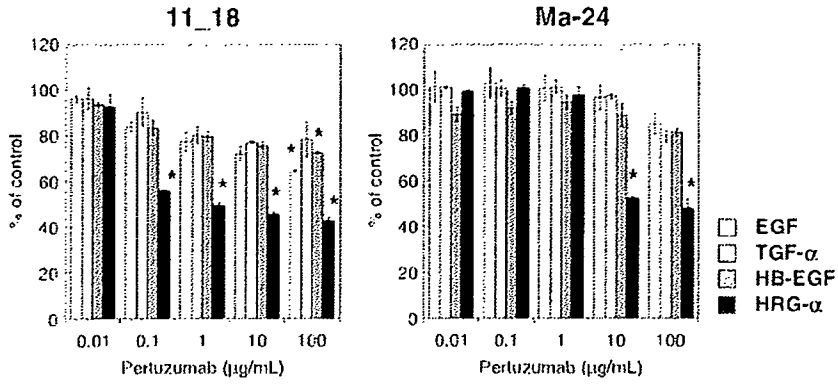

Fig. 2. Growth inhibitory effect of pertuzumab in the lung cancer cells. The lung cells were exposed to pertuzumab (0.01–100 μg/mL) for 72 h in serum free medium with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), or heregulin (HRG)-α. The viability was determined using the MTS assay. Result are presented as the percentage of absorbance compared with pertuzumab-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; t-test) compared to pertuzumab-untreated cells. Data shown are representative of at least three independent experiments with similar results.

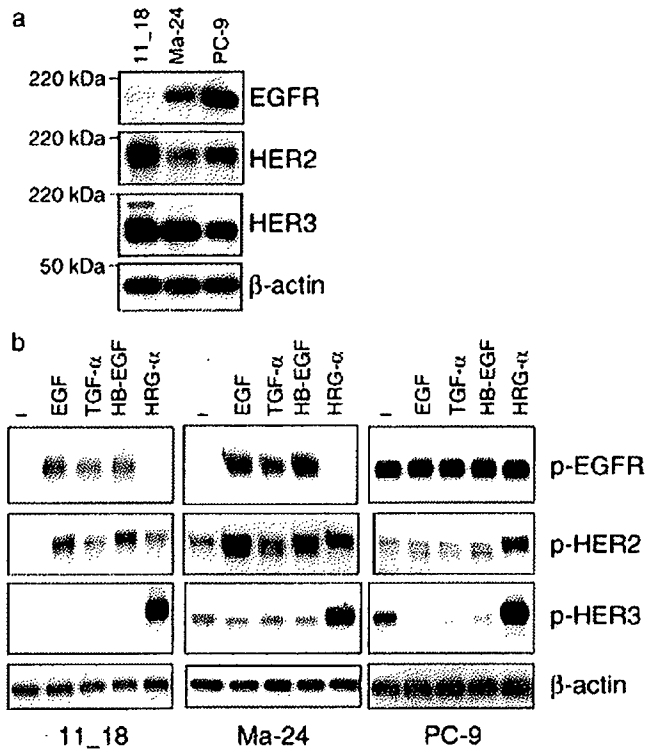

Fig. 3. Expression and phosphorylation of HER receptors in non-small cell lung cancer cells. (a) Expression of epidermal growth factor receptor (EGFR), HER2, and HER3 was detected using western blot analysis. Each lane contained 20 μg protein. β-Actin was used as a loading control. (b) The cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α for 10 min. Phosphorylation of EGFR and HER3 was detected using western blot analysis. Phosphorylation of HER2 was detected using immunoprecipitation followed by western blotting. β-Actin was used as a loading control. Data shown are representative of at least two independent experiments with similar results.

cell line was weak. The expression levels of HER2 were higher in the PC-9 and 11_18 cells than in the Ma-24 cells, which only expressed moderate levels of this receptor. All three cell lines showed strong expression of HER3. HER4 could not be detected in any of the three cell lines (data not shown). In contrast, these lung cancer cell lines expressed different types of EGFR mutations; the PC-9 cells had a 15-base deletion mutant (delE746-A750, exon 19), the 11_18 cells had a L858R point mutation (exon 21) of EGFR, and the Ma-24 cells had a E709G point mutation (exon 18) of EGFR. No mutations were detected in exons 19–21 of HER2 (data not shown).

Next, the ligand-stimulated phosphorylation of the HER receptors in the lung cancer cells after serum starvation was examined (Fig. 3b). While the ligands for EGFR (EGF, TGF-α, and HB-EGF) phosphorylated cellular EGFR in the 11_18 and Ma-24 cells, the EGFR in the PC-9 cells was hyperphosphorylated even under the non-stimulated condition, because PC-9 cells express an active mutant of EGFR. These results suggest that the EGF/TGF-α or HB-EGF–EGFR signals are active in lung cancer cells. The ligands for HER3 (HRG-α) specifically phosphorylated HER3 in the 11_18, Ma-24, and PC-9 cells. Phosphorylation of HER2 was analyzed by immunoprecipitation using an anti-HER2 antibody followed by western blotting for phosphotyrosine. The ligands for EGFR and HER3 phosphorylated HER2 in the 11_18 and Ma-24 cells, whereas only HRG-α but not the other ligands specifically phosphorylated HER2 in the PC-9 cells. These findings also suggest that the HRG-α–HER3 signal is active in lung cancer cells.

Pertuzumab blocks HRG-α but not EGF-stimulated signals. An inhibitory effect of pertuzumab on HRG-α-dependent cell growth in the 11_18 cells was demonstrated. To examine the effect of pertuzumab on signal transduction of both EGFR and HER3 in this cell line, the 11_18 cells were exposed to pertuzumab (0.2–200 μg/mL for 6 h) (Fig. 4a,b). HRG-α-stimulated phosphorylation of HER3 was dose-dependently inhibited by exposure to pertuzumab in the 11_18 cells, whereas EGFR phosphorylation was not stimulated by HRG-α stimulation (data not shown). MAPK and Akt were phosphorylated by HRG-α stimulation and these were inhibited by pertuzumab dose-dependently in the 11_18 cells. In contrast, EGF-stimulated phosphorylation of EGFR and MAPK was not inhibited by pertuzumab in the 11_18 cells. Phosphorylation of Akt was not detected by addition of EGF in the 11_18 cells. EGF did not phosphorylate HER3 and pertuzumab did not affect it (data not shown). Taken together, these results showed that pertuzumab inhibited HRG-α-stimulated phosphorylation of HER3, MAPK, and Akt, but not EGF-stimulated EGFR phosphorylation signaling.

HER3 is phosphorylated in response to HRG-α in the PC-9 cells as observed in the 11_18 cells, but the growth of the PC-9 cells was not increased by HRG-α (Figs 1,3b). To clarify the phosphorylation-inhibitory potential of pertuzumab, the effect of pertuzumab on signal transduction of the PC-9 cells was examined (Fig. 4c). When the PC-9 cells were stimulated by the addition of HRG-α, HER3 was phosphorylated in the PC-9 cells, but phosphorylation of HER3 was not inhibited by pertuzumab (20 and 200 μg/mL for 6 h). EGFR expressed in the PC-9 cells is constitutively active and pertuzumab failed to affect

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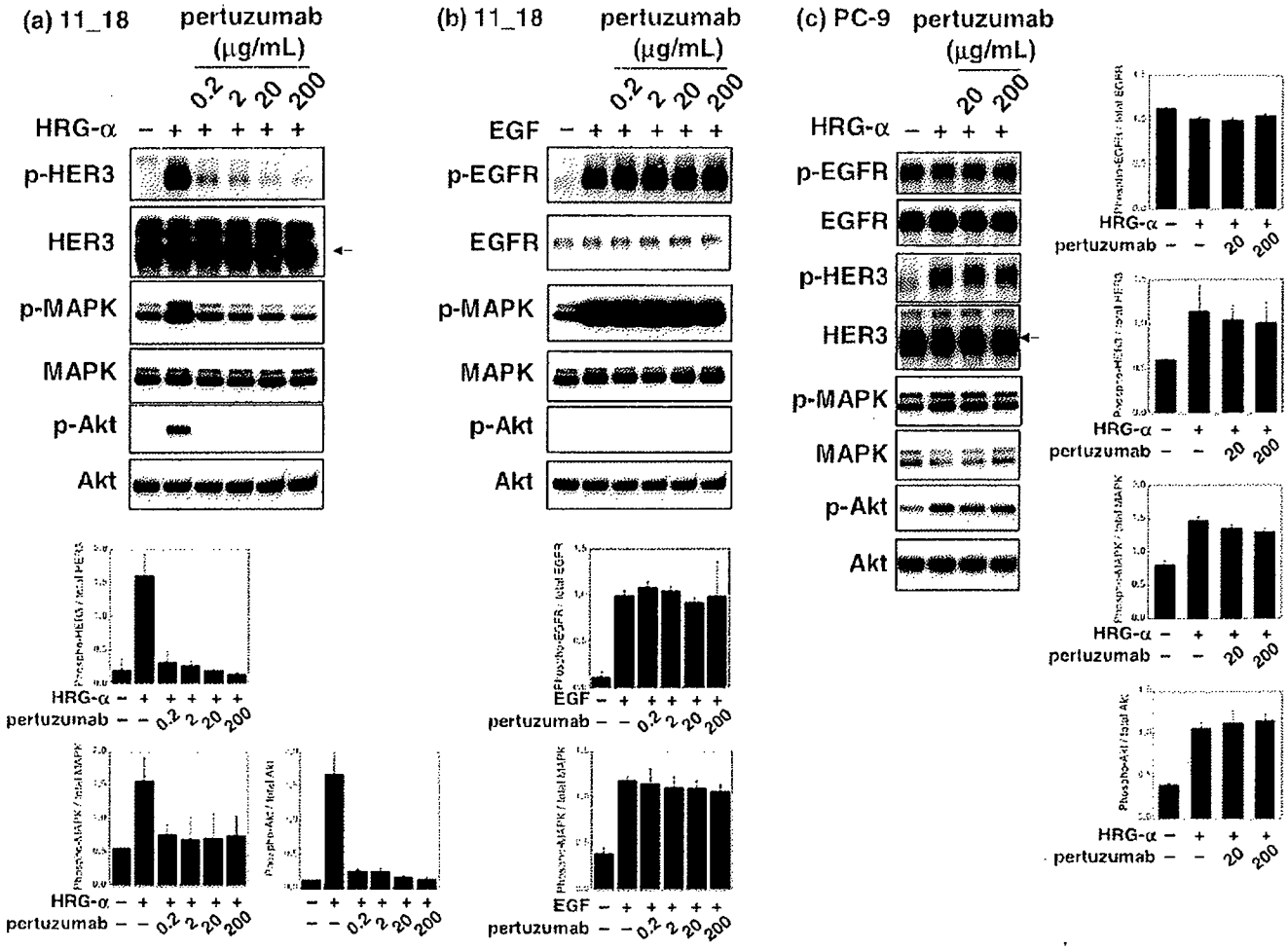

Fig. 4. Effect of pertuzumab on epidermal growth factor receptor (EGFR) and HER3 phosphorylation and their downstream signaling pathways. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysate were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results. MAPK, mitogen-activated protein kinase.

the phosphorylation level of the EGFR. Phosphorylation of MAPK and Akt was detected by the addition of HRG-α, but these were not inhibited by pertuzumab. These results suggest that pertuzumab is unable to affect HRG-α-stimulated phosphorylation of HER3 in the PC-9 cells.

To clarify the effect of pertuzumab on HER2 phosphorylation and HER2/HER3 heterodimer formation, cell lysates were immunoprecipitated with anti-HER2 antibody (Fig. 5a,b). HRG-α stimulation increased HER2/HER3 heterodimer formation in the 11_18 cells, and pertuzumab decreased HRG-α-stimulated heterodimer formation. EGFR/HER2 heterodimer formation could be barely detected by HRG-α stimulation because of slight expression of EGFR in the 11_18 cells. In the case of EGF stimulation, HER2/HER3 heterodimer was not increased in the 11_18 cells. These findings suggest that pertuzumab inhibits HER2/HER3 heterodimerization by HRG-α stimulation. The HRG-α-stimulated phosphorylation of HER2 was inhibited by pertuzumab in the 11_18 cells. In contrast, the EGF-stimulated phosphorylation of HER2 was not inhibited. These data suggest that pertuzumab inhibits HRG-α stimulated phosphorylation in 11_18 cells. In the PC-9 cells, HRG-α stimulated HER2/HER3 heterodimer formation could be detected without any ligand stimulation, and pertuzumab diminished HRG-α-stimulated heterodimer formation (Fig. 5c). Phosphorylation of HER2 was increased by HRG-α stimulation, but not inhibited by pertuzumab in PC-9 cells. EGFR/HER2 heterodimer formation could be detected without any ligand stimulation, but pertuzumab did not affect it. Based on these results, it is speculated that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling, and phosphorylation of HER3 is maintained by active mutant EGFR.

Discussion

Overexpression of HER3 was observed in the lung cancer cell lines and the HER3 was phosphorylated by the HER3 ligand in these cells. These results suggest that HER3 signaling is active in some types of lung cancer cells. Recently it was reported that high HER3 expression was associated with decreased survival.[17] A relationship between lung cancer metastasis and the expression of HER3 as well as EGFR and HER2 has been reported.[18] These bodies of evidence suggest that HER2/HER3 signaling is activated in a subpopulation of lung cancers and that HER2 and HER3 play an important role in the biological behavior of these lung cancers. Both HER2 and HER3 are therefore considered as a possible important target in the therapeutic strategy against lung cancer, just as they are in breast cancers.

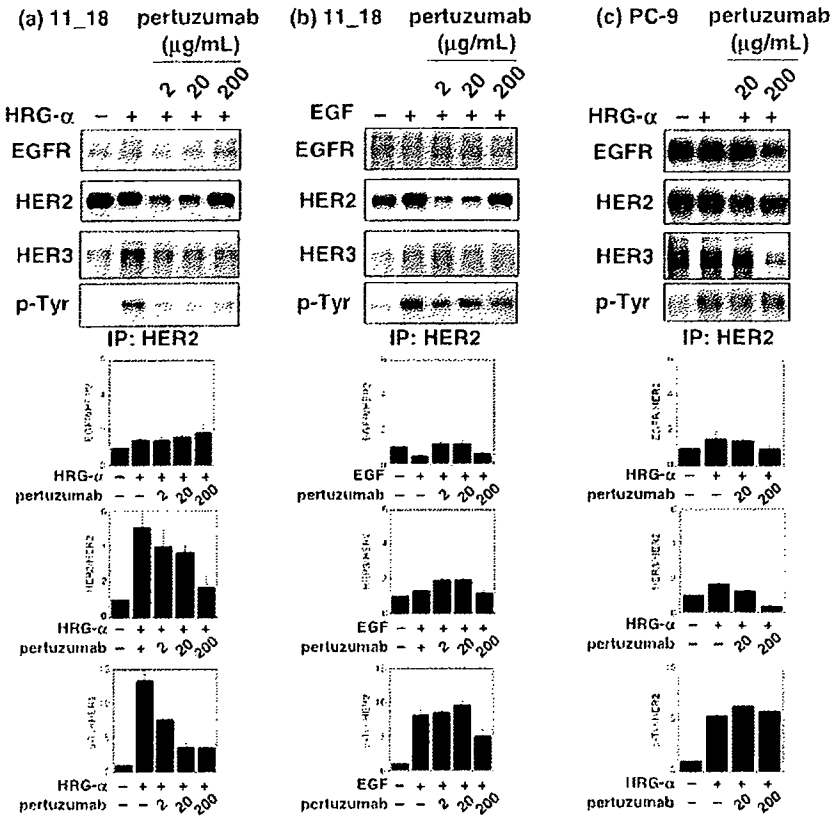

Fig. 5. Effect of pertuzumab on heterodimer formation. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysates were immunoprecipitated with anti-HER2 antibody, separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results.

HER3 lacks kinase activity because of several base substitutions in motifs that are essential to tyrosine kinase and heterodimerization with HER2 or EGFR is essential for its signal transduction. Therefore co-expression of HER3 and its partners are determinants for the cellular sensitivity against pertuzumab in cancer cells. The present results showed that HER2/HER3 heterodimers are detected by HRG-α stimulation and these data are consistent with previous reports.[19] In contrast, the authors monitored the downstream phosphorylation signal, and demonstrated that HRG-α, but not EGF, phosphorylated Akt in the 11_18 cells. This finding allows us to speculate that HRG-α stimulation leads to Akt phosphorylation through HER2/HER3 heterodimerization.[20–22]

Recently, EGFR mutations have been reported in lung cancers and it was of great interest to clarify the relationship between the EGFR mutation and sensitivity to EGFR-targeted tyrosine kinase inhibitors.[23–25] The PC-9 cells express the deletional mutant EGFR (delE746-A750 in exon 19 of EGFR),[16,23,26,27] and their EGFR was constitutively phosphorylated under non-stimulated conditions (Fig. 3a). The authors speculate that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling. In Fig. 3b, treatment with EGF and TGF-α seemed to decrease the phosphorylation of HER3 in PC-9 cells. Unfortunately, we could not conclusively explain this phenomenon. PC-9 cells express deletional EGFR and form EGFR homodimers in the absence of ligand stimulation. At the same time, phospho-HER3 was also detected under these conditions, suggesting that heterodimers of EGFR–HER3 were also formed. Ligand stimulation may alter the balance between homodimers and heterodimers, causing a reduction in HER3 phosphorylation, although there is not any evidence to support this hypothesis. In contrast, the phosphorylation of EGFR in the 11_18 cells that express a different type of mutant EGFR (L858R in exon 21 of EGFR),[26] was not constitutive. This finding may be explained by the differences between deletion mutant EGFR and L858R; constitutive active in the deletion mutant versus hyper-response to ligand stimulation in L858R.[28] Engelman *et al.* suggested that the mutant EGFR is used to couple HER3 in gefitinib-sensitive NSCLC cell lines.[29] The expression level of EGFR in the 11_18 cells was much lower than in the PC-9 cells, and a similar extent of HER3 expression was observed in these cell lines (Fig. 3a). The authors have demonstrated the differential inhibitory effect of pertuzumab against 11_18 and the PC-9 cells. Pertuzumab inhibited HER2/HER3 heterodimer formation and phosphorylation in the 11_18 cells, considering that mutant EGFR do not influence HER3 signals in the 11_18 cells. HER3 phosphorylation in the PC-9 cells was also increased by HRG-α stimulation. Although pertuzumab decreased HER2/HER3 heterodimer formation, it failed to inhibit HRG-α-stimulated HER3 phosphorylation, speculating that an active mutant EGFR transactivates HER3 in the PC-9 cells.

Several EGFR-targeted small inhibitors and antibodies have been under clinical evaluation in the treatment of lung cancer. An EGFR-targeted tyrosine kinase inhibitor, erlotinib, has been clinically applied as a second or third-line single agent therapy in NSCLC patients who have failed standard chemotherapy.[30] Anti-EGFR monoclonal antibodies such as cetuximab and ABX-EGF have been examined in a clinical study.[31] In addition to EGFR, HER2 and HER3 are also considered as important targeting molecules in lung cancers. The present results indicated that pertuzumab effectively inhibited signaling within HER2 and HER3, and may thus be effective in lung cancers expressing HER2 and HER3. To confirm the pertuzumab-sensitive population of lung cancer cells, experiments using small interfering RNA for mutant EGFR will be necessary in future studies.

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In conclusion, the authors have demonstrated that pertuzumab inhibits HRG-α-stimulated cell growth in lung cancer cells through the inhibition of HRG-α-stimulated HER3 signaling. It was further demonstrated that pertuzumab exerts an antiproliferative activity against lung cancer cells expressing HER2 and HER3. The next step will be to examine the clinical relevance of the occurrence of heterodimer formation between HER2 and the other HER receptors in lung cancer.

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