of the following covariates: analysis batch number and order within batch (both identified in the PCA), and the statistically significant clinical and radiological variables most predictive of ILD identified from the main study, i.e. WHO PS, smoking history, extent of normal lung coverage on CT scan and severity of pre-existing ILD [3]. Peptides were then ranked by the significance of the case-control status term.

To identify and evaluate the best set of peptide predictors, predictive multivariate modeling was then performed in two steps. First the n most significant peptides were selected and a PLS-DA [42] was performed to identify linear combinations of peptides correlating best with case-control status. This was followed by logistic regression modeling with case-control status as the response using the m first PLS components (linear combinations identified) as predictors. To explore the modeling space, the number of peptides, n, and the number of PLS components, m, utilized in the model were varied.

The performance of the statistical analysis and predictive multivariate modeling was assessed using a leave-one-out cross-validation approach to estimate sensitivity, specificity, and overall error rate of the modeling process [43], as follows:

- For each subject in turn, remove this subject as the test set, considering the remaining subjects as the training set.
- Perform the three steps of statistical analysis (ANCOVA and multivariate modeling by PLS-DA and logistic regression) as described above on the training set.
- Use the logistic regression model in the third step to predict the case-control status of the left-out subject based on the other data.
- Repeat, omitting each subject in turn from the training set and predicting the left-out subject and then combining the results to generate a vector of leave-one-out predictions, one for each subject, which can be used to give an estimate of the sensitivity and specificity as well as the overall error rate of the modeling process.

As a robustness check, Random Forests [44] were also used as an alternative to the combination of PLS-DA and logistic regression.

To allow a visualization of the potential choices for the appropriate levels of sensitivity and specificity, the cross-validated results are presented as ROC by varying the probability threshold used for predicting each subject as a case or a control.

Peptide and protein identification

Product ion spectra with at least 10 product ions were converted into peak lists, which were searched with the Mascot algorithm [45] against a Swiss-Prot amino acid sequence database. First mass chromatogram files were generated for every LC-MS/MS measurement. The subsequent data conversion and search process was carried out using Mascot software (Version 2.1.04, Matrix Science Ltd, London, UK). Prior to database search, each product ion spectrum in these files was converted into peak list(s) using an extract_msn.exe program (Thermo Fisher Scientific) without any grouping process. The criterion for the data conversion was at least 10 product ions in a spectrum. These peak lists were searched with the Mascot algorithm (MS/MS Ion Search mode) [45] against a Swiss-Prot amino acid sequence database (http://www.expasy.org/sprot/; Release 55.0; 18,610 entries (*Homo sapiens*); updated on February 26, 2008). The database search parameters were set as follows: tryptic digestion (hydrolysis of the peptide bonds following lysine and arginine residues); fixed modification of cysteine residues (*S*-carboxyamidomethylation, +57.0 Da); variable modification of methionine residues (oxidation, +16.0 Da); ≤ 2 missed cleavages, i.e. assuming at most 2 predicted tryptic digestion sites are not actually digested; peptide tolerance of 2.0 Da; an MS/MS tolerance of ± 0.8 Da (http://www.matrixscience.com).

Bioinformatics

Identification of significant pathways. We utilized the IPA system (Ingenuity® Systems, www.ingenuity.com) to analyze the set of proteins we identified as demonstrating a significant difference in expression levels between cases and controls. The Ingenuity Pathways Analysis Knowledge Base is a large curated database of previously published findings on mammalian biology. The version used was v. 6.5, build 59570, content version 1602, build oqa-kb_enif, 2008-08-20, 21:16:03.

The list of proteins identified was overlaid onto the curated pathways in IPA. The dynamic Canonical Pathways are well-characterized metabolic and cell signaling pathways that have been curated and hand-drawn. The information contained in Canonical Pathways comes from specific journal articles, review articles, text books, and the KEGG (Kyoto Encyclopedia of Genes and Genomes) ligand database (http://www.genome.jp/kegg/ligand.html). The most significant pathway was identified as the pathway with the highest network score. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. The score is the negative logarithm of this p-value. The ratio for an overlay to a given pathway was calculated by the ratio between the number of proteins from the data set found in the pathway and the total number of proteins associated to the pathway.

Gene network analysis. Gene network analysis was performed using IPA Systems web-based software application (http://www.ingenuity.com/products/pathways_analysis.html). In this approach the Ingenuity literature data are used to identify interconnected protein networks based on reported interactions identifying particular proteins as interacting with each other.

Protein level modeling

For each of the proteins identified (through one or more peptides) as having the greatest significant difference between cases and controls, the full set of digested peptides from that protein was also identified, and a summary protein intensity (using the intensities of all component peptides) was calculated using a method based on PCA, as follows:

1. Remove the batch effect from all the peptide intensities by subtracting the batch means.
2. Restrict the set of peptides to those with a positive correlation after removing the batch effect with the peptide showing the greatest evidence of differential expression between the case and control groups (i.e. the smallest p-value).
3. Arrange the remaining batch adjusted peptide intensities in a matrix and apply PCA on the scaled data matrix.
4. The score of the first principal component, i.e. the greatest source of variability, is the protein intensity score.

Where there is strong correlation between all the constituent peptides, this method will produce effectively an average of the peptide intensities, as the loadings given to all the peptides will be similar. Where a peptide has low correlation with the remaining peptides, either because it is also a digestion product and therefore measuring other proteins, or has low intensity making it an unreliable measurement, then it will receive a low loading in the PCA and will contribute little to the protein intensity measure.

A summary pathway intensity score was calculated from the intensities of proteins within a common pathway in a similar manner by taking the scores from the first principal component of the matrix of protein intensities.

Protein and pathway intensities were modeled using logistic regression, with case-control status as the response and optionally the clinical variables as predictive variables, in an analogous way to peptide intensities as described above.

Validation

Reproducibility of the identified differentially expressed peptides was validated by comparing the peptide intensities from 39 repeated samples with duplicate MS/MS analysis, allowing for consistent between-batch differences.

To validate between technologies, protein intensities derived from MS/MS were compared with those derived from Western blots with densitometry for 9 key selected proteins identified by the statistical and bioinformatic analyses. The depleted plasma proteins were reduced and denatured in the presence of 50 mM dithiothreitol and 2% w/v SDS at 95°C for 5 min, and then subjected to SDS-PAGE with 7.5%, 10%, or 12% acrylamide (0.1 or 1 μg protein *per* lane). Separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) with a wet-type transfer system. After transfer, non-specific binding reactions were blocked by rocking the membranes for 1 h with 3% w/v BSA and 3% w/v polyvinylpyrrolidone K30 in TBST (150 mM NaCl, 0.05% v/v Tween 20 and 10 mM Tris-HCl, pH 8.0). The membranes were then washed with TBST, incubated with antibodies in TBST containing 0.1% BSA (TBST-BSA) for 30 min, washed again with TBST, and finally incubated with a HRP-conjugated second antibody in TBST-BSA for 30 min (Table S4). After washing again with TBST, the specific binding was detected using an ECL-Plus system (GE Healthcare) according to the manufacturer's instructions, in combination with a LAS-3000 imaging analyzer (FUJI FILM). For densitometry, the gel image was opened with Image-J software (National Institutes of Health, Research Services Branch, USA; http://rsbweb.nih.gov/), and the density of each band was obtained. After subtraction of a background value, the density data were used for further analyses.

Supporting Information

Acknowledgment S1 Members of the CCS study organization.
(DOC)

Figure S1 Comparison of p-values for all detected peptides from analyses adjusted for and unadjusted for batch. All peptides showing a significant difference in the unadjusted analysis also show a significant difference in the analysis adjusted for batch. The analysis adjusted for batch also identifies additional significantly differentially expressed peptides that were not detected by the unadjusted analysis.
(TIF)

Figure S2 Effect of batch-to-batch variability on the overall intensity difference between cases and controls for peptides. (A) An example peptide which exhibits no discernable between-batch variation and so is significant independent of whether the analysis is adjusted for batch or not. (B) An example peptide with a highly significant variation between batches, which consequently is only significant when batch is accounted for. Overall pooled effect not adjusting for batch on left, followed by batches 1–9 with one study sample each from 180 of the 181 study subjects. Red = cases; blue = controls.
(TIF)

Figure S3 Effect of adjusting for key clinical variables. Left panel shows a comparison of p-values for all detected peptides from analyses adjusted for and unadjusted for 4 key clinical variables. Right panel shows an example of the effect of accounting for the clinical variable WHO PS on the pattern of intensity difference between cases and controls. Overall pooled effect on left, followed by case-control difference (red = cases; blue = controls) plotted by WHO PS.
(TIF)

Figure S4 Networks obtained from entering the identified 29 proteins into the Ingenuity Pathway Analysis system. Highest-scoring (A) and combined highest- and second highest-scoring (B) networks. Panel A represents a more annotated version of Figure 4 in the main manuscript material. In panel B, combining the two networks with the highest scores further adds IL1-beta, HNF1A, HNF4A, HNF6 (ONECUT1), and CEBPB as central components (green shading). In panel A, dark blue shapes and lines = proteins identified as predictors in this study and interactions between them. Grey shapes and lines = proteins identified by Ingenuity to generate the network and interactions between them. Light blue lines = interactions between proteins identified by Ingenuity to generate the network and the proteins identified in the study. **A** Relationship labels: A = Activation; B = Binding; C = Causes/Leads to; CC = Chemical-Chemical interaction; CP = Chemical-Protein interaction; E = Expression (includes metabolism/synthesis for chemicals); EC = Enzyme Catalysis; I = Inhibition; L = ProteoLysis (includes degradation for Chemicals); LO = Localization; M = Biochemical Modification; MB = Group/complex Membership; P = Phosphorylation/Dephosphorylation; PD = Protein-DNA binding; PP = Protein-Protein binding; PR = Protein-RNA binding; RB = Regulation of Binding; RE = Reaction; RR = RNA-RNA Binding; T = Transcription; TR = Translocation. Numbers in brackets = number of observations supporting the interaction. **B** Proteins identified in the study: SERPINA1 = alpha-1-antitrypsin; SERPINA3 = alpha-1-antichymotrypsin; SERPINC1 = antithrombin-III; APOA1 = apolipoprotein A-I; APOB = apolipoprotein B-100; APOC3 = apolipoprotein C-III; C3 = complement C3; C4A, C4B = complement C4-A; complement C4-B; C9 = complement component C9; GSN = gelsolin; HBA2 = hemoglobin alpha; HBB, HBD = hemoglobin beta/delta; HP = haptoglobin; HPR = haptoglobin-related protein; HRG = histidine-rich glycoprotein; KLKB1 = plasma kallikrein; IGKC = Ig kappa chain V-III region Ti; RBP4, Rbp = retinol binding protein 4; APCS = serum amyloid P-component; TF = serotransferrin; TTR = transthyretin; ORM1 = alpha-1-acid glycoprotein 1; A1BG = alpha-1B-glycoprotein; LRG1 = leucine-rich alpha-2-glycoprotein; ARMC2 = armadillo repeat-containing protein 2; AHSG = alpha-2-HS-glycoprotein; ITIH4 = inter-alpha-trypsin inhibitor heavy chain H4.
(TIF)

Figure S5 Scatterplots of intensities from Western blots (densitometry) and MS/MS for 9 selected differentially expressed proteins. Red triangles = cases; green circles = controls. APOA1 = apolipoprotein A–I; C3 = complement C3; C4 = complement C4-A; fetuin = alpha-2-HS-glycoprotein; HPT beta = haptoglobin; ORM1 = alpha-1-acid glycoprotein; serpin A1 = alpha-1-antitrypsin; serpin A3 = alpha-1-antichymotrypsin.
(TIF)

Figure S6 Western blot images from 12 subjects on 9 selected differentially expressed proteins. 1 μg (0.1 μg for serpin A1) of depleted human plasma proteins of study samples (6 ILD cases and 6 controls) were separated by SDS-PAGE, transferred onto PVDF membrane, and detected with Western blotting. alpha-2-HS-glycoprotein = alpha-2-HS-glycoprotein; APOA1 = apolipoprotein A-I; C3 beta-chain = complement C3 beta-chain; C4 beta-chain = complement C4 beta-chain; HPT beta-chain = haptoglobin beta-chain; orsomucoid-1 = alpha-1-acid glycoprotein; serpin A1 = alpha-1-antitrypsin; serpin A3 = alpha-1-antichymotrypsin.
(TIF)

Figure S7 Significance levels from proteins, constituent peptides, and acute phase pathway intensities, adjusted for clinical variables. p-values for the proteins are shown by red stars, p-values for individual peptides are shown by points, and the distribution of these for each protein is shown by a boxplot. In each boxplot, the upper and lower sides of the box represent the higher and lower quartile values (Q3 and Q1), respectively. The black bar in each box represents the median value. The p-value for the acute phase pathway is represented by the dashed line; boxplots for proteins in the acute phase response pathway are shaded. A1AG1 = alpha-1-acid glycoprotein; A1AT = alpha-1-antitrypsin; A1BG = alpha-1-B-glycoprotein; A2GL = leucine-rich alpha-2-glycoprotein; AACT = alpha-1-antichymotrypsin; ANT3 = antithrombin-III; APOA1 = apolipoprotein A–I; APOB = apolipoprotein B-100; APOC3 = apolipoprotein C-III; ARMC2 = armadillo repeat-containing protein 2; CO3 = complement C3; CO4 = complement C4-A, complement C4-B; CO9 = complement component C9; FETUA = alpha-2-HS-glycoprotein; GELS = gelsolin; HBA = hemoglobin alpha; HBB,HBD = hemoglobin beta/delta; HPT = haptoglobin; HPTR = haptoglobin-related protein; HRG = histidine-rich glycoprotein; ITIH4 = inter-alpha-trypsin inhibitor heavy chain H4; KLKB1 = plasma kallikrein; KV3 = Ig kappa chain V-III region Ti; RETBP = retinol binding protein 4; SAMP = serum amyloid P-component; TRFE = serotransferrin; TTHY = transthyretin.
(TIF)

Table S1 Characteristics of study subjects (NSCLC patients treated with gefitinib) included in proteomics analyses.
(DOC)

Table S2 Quality control results of sample preparation for 181 study samples, by LC-MS/MS measurement batches.
(DOC)

Table S3 Protein Identification from the selected peaks.
(DOC)

Table S4 Antibodies used for Western Blot validation.
(DOC)

Acknowledgments

We thank Claire Shewbridge, from Complete Medical Communications, who provided editing assistance funded by AstraZeneca.

We thank the patients, investigators and study personnel who participated in this study (investigators and key study personnel are listed in Acknowledgment S1).

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Conceived and designed the experiments: FN AO CGH TK Y. Kyono Y. Komatsu MK SA TH TN MF K. Nakata YO SK GMV HK. Performed the experiments: TK K. Nagasaka ST Y. Kyono TD Y. Komatsu MK. Analyzed the data: FN AO CGH TK ST KW HKT MO Y. Komatsu SA ISP MCS IGC TN GMV. Contributed reagents/materials/analysis tools: AO TK K. Nagasaka ST KW HKT MO Y. Kyono TD Y. Komatsu MK SA MF K. Nakata YO SK HK. Wrote the paper: FN AO CGH TK Y. Komatsu IGC TN GMV. Supervised clinical and epidemiological aspects of the study: FN TH MF K. Nakata YO SK HK. Supervised proteomic aspects of the study: AO TN GMV. Critical review and revision of the manuscript: FN AO CGH TK K. Nagasaka ST KW HKT MO Y. Kyono TD Y. Komatsu MK SA ISP MCS TH MF K. Nakata YO SK IGC TN GMV HK.

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Interstitial lung disease in gefitinib-treated Japanese patients with non-small-cell lung cancer: genome-wide analysis of genetic data

Aim: To investigate potential relationships between SNPs and acute interstitial lung disease (ILD) events in Japanese non-small-cell lung cancer patients receiving gefitinib. **Materials & methods:** Japanese non-small-cell lung cancer patients treated with gefitinib from a prospective pharmacoepidemiological cohort with a nested case–control study component ('CCS'; 52 ILD cases, 139 controls) and a retrospective study (28 ILD cases, 55 controls) were genotyped for nearly 500,000 SNPs. Associations between genotype and ILD were evaluated using Fisher's exact test and logistic regression modeling, and false discovery rate analysis was used to adjust for the large number of statistical tests. **Results:** The CCS data provided some false discovery rate evidence that the significance of top-ranking SNPs exceeded levels expected by chance, suggesting some genuine associations. However, replication analyses using retrospective study data were not supportive and there was little evidence of strong genetic associations from a combined analysis. Adjustment of CCS analyses for clinical variables provided little additional convincing evidence. Significant gene–gene interactions between SNP pairs using CCS data were not confirmed in retrospective study replication analyses. **Conclusion:** Although it is not possible to exclude genetic influences in ILD etiology, common sequence variation is unlikely to explain a major component of ILD risk. Our top results may provide a useful hypothesis-generating starting point for further research.

Presented, in part, at the 26th ICPE: International Conference on Pharmacoepidemiology & Therapeutic Risk Management, Brighton, UK, 19–22 August 2010.

Original submitted 1 December 2010; Revision submitted 22 February 2011

KEYWORDS: gefitinib genome-wide association interstitial lung disease non-small-cell lung cancer pharmacogenomics

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The EGF receptor tyrosine kinase inhibitor (EGFR-TKI), gefitinib, was first approved for the treatment of advanced non-small-cell lung cancer (NSCLC) in July 2002 in Japan. Following approval, an unexpected number of spontaneous reports of acute interstitial lung disease (ILD) events were seen [1,2]. ILD is a general term for a group of conditions that cause fibrosis or inflammation of the interstitium, leading to breathing difficulties [3]. Approximately two-thirds of cases have no known cause (idiopathic), while a third result from known factors, including environmental factors, infections, drugs (including chemotherapy) and radiation [4,5].

At the time when acute ILD-type events were first reported in gefitinib-treated patients in Japan, understanding of ILD risk factors and mechanisms in patients with NSCLC was quite limited. Therefore, a pharmacoepidemiological study was designed to determine risk factors for, and the relative risk of, ILD in a cohort of patients with advanced/recurrent NSCLC in Japan who received gefitinib or other treatment (chemotherapy of choice) [6]. This nonrandomized cohort study included 3166 Japanese patients with advanced/recurrent NSCLC who were followed for 12 weeks after initiating gefitinib (n = 1872 treatment periods) or chemotherapy (n = 2551). A nested case–control study component (hereafter referred to as the CCS) included patients who developed acute ILD (n = 122) and randomly selected control subjects (n = 574). In this study, the observed (unadjusted) incidence rate of ILD over 12 weeks was 4.5 (95% CI: 3.5–5.4) per 1000 person-weeks for gefitinib versus 1.7 (95% CI: 1.2–2.2) for chemotherapy. The odds ratio (OR) of developing acute ILD with gefitinib versus chemotherapy, adjusted for imbalances in risk factors in the nested case–control analysis, was 3.2 (95% CI: 1.9–5.4). Other ILD risk factors identified were older age, poor WHO performance status (PS), smoking, recent NSCLC diagnosis, reduced normal lung on CT scan, pre-existing ILD and concurrent cardiac disease [6].

The present article reports the results of exploratory genetic analyses of data on gefitinib-treated patients in the CCS, designed to further

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investigate the mechanisms and risk factors associated with ILD.

Data from an earlier, smaller retrospective case–control study (hereafter referred to as the retrospective study) of genetic influences on ILD in Japanese patients with NSCLC are also included here. This study identified in a more *ad hoc* fashion, based on adverse drug reaction reports, patients who developed ILD during treatment with gefitinib and a control sample of similar patients who did not develop ILD within 3 months of starting treatment with gefitinib.

The objectives of the exploratory genetic analyses reported here were to identify SNPs associated with the development of ILD in gefitinib-treated NSCLC patients in Japan and to investigate possible interrelations between the clinical ILD risk factors identified from the main analyses of the CCS and such associations.

Patients & methods

Study design

The nonrandomized, prospective, pharmacoepidemiological cohort study (V-15–33; clinicaltrials.gov identifier NCT00252759 [101]) was conducted between November 2003 and February 2006 in 50 centers across Japan; the main clinical results have been published previously [6]. Patients with advanced or recurring NSCLC who had received at least one previous chemotherapy regimen were followed for up to 12 weeks on gefitinib 250 mg/day or chemotherapy. Patients who developed acute ILD during follow-up were registered to the case–control study nested within the cohort, as clinically diagnosed potential cases. For each potential case, four patients were randomly selected as control subjects from patients registered to the cohort at that time who had not developed ILD. This resulted in somewhat longer time on average to index date (i.e., ILD date in case or selection date in control) for controls (37.1 days) than cases (25.6 days). Exploratory genetic analyses were only performed for gefitinib-treated patients from the CCS. Participation in the genetic analyses of the CCS was voluntary and required separate consent.

The earlier retrospective study was a joint research study of the Institute of Medical Science, University of Tokyo, and AstraZeneca, involving nine centers in Japan, with DNA samples collected between July 2003 and June 2004. This study included retrospectively identified patients with NSCLC who developed ILD during treatment with gefitinib and a sample of similar patients with no ILD within 3 months after starting treatment with gefitinib (approximate 1:3 ratio). Candidate subjects for the ILD group were ILD cases in spontaneous postmarketing adverse drug reaction reports from physicians to AstraZeneca. In this study, baseline clinical data from treatment initiation were not collected.

In the CCS, stringent design features were employed to maximize detection and accurate diagnosis of ILD [6]. The diagnosis of ILD was confirmed in a blinded review by an independent case review board (SUPPLEMENTARY MATERIAL, www.futuremedicine.com/doi/suppl/10.2217/pgs.11.38) of radiologists, pulmonologists and oncologists, based on radiological evidence (including high-resolution CT and CT scans) and clinical data collected for all potential cases in a structured way [6]. In the retrospective study, the diagnosis of ILD was confirmed using available x-ray or CT images by three external reviewers (SUPPLEMENTARY MATERIAL).

DNA samples & genotyping

Of the 79 confirmed ILD cases and 252 control subjects from gefitinib-treated patients in the CCS, 52 and 139 consented to the genetic analyses, respectively. From the retrospective study, there were 28 confirmed ILD cases and 74 control subjects, of whom 55 were available for the final full (main) analysis; 19 controls had sufficient DNA available only for earlier genotyping in the block sequential analysis (see below).

Blood samples (7 ml ethylenediaminetetraacetic acid) were taken from patients consenting to the genetic analyses and DNA was extracted by SRL Inc. (Tokyo, Japan). Genotyping was performed using hypothesis-free, genome-wide tag SNPs. A staged approach was employed as the number of samples accumulated and genotyping technology improved in throughput and decreased cost during the study. See the SUPPLEMENTARY MATERIAL for more information.

Statistical analyses

Two main approaches were employed to analyze the data, a block sequential analysis in the data collection phase, and a regular case–control association analysis on the full data after study completion.

Block sequential design (cumulative staged approach in parallel with data collection)

Initially, a block sequential design was used during the data collection phase of the studies to identify any strong associations between SNPs and ILD as early in the study as possible. Starting with some of the data from the earlier

retrospective study, blocks of data were subsequently added as acquired from the CCS, and the association between SNPs and ILD, and potential predictive SNP models, were evaluated at each step. Data were analyzed to identify predictive SNPs (separate locus approach) and multiple potentially predictive SNPs to build a predictive score (scoring approach). More detailed methodology for this approach is summarized in the SUPPLEMENTARY MATERIAL.

Case–control design (main association analysis)

The main analysis was a case–control design using all available subjects at the completion of data collection, with the robust prospective CCS as the discovery cohort and the retrospective study as a replication dataset, with various subanalyses.

Following exclusion of SNPs with exact Hardy–Weinberg equilibrium p-values $<1 \times 10^{-4}$, SNPs with assay results monomorphic in the study population, and SNPs with genotype call rates of <90% in the combined sample of 274 patients, 465,816 autosomal SNPs, of the 561,466 SNPs genotyped, were included in the association analyses. Four separate association analyses were conducted.

Analysis one: case–control analyses of CCS (exploratory) & retrospective study (confirmatory/replication)

In analysis one, the subjects from the prospective CCS were treated as a dataset for initial discovery and the retrospective study subjects used to test for independent replication for the top-ranking (most significant) results.

Case–control associations were evaluated in the CCS subjects using two-sided Fisher's exact tests based on allele counts (2 × 2 tables). The distribution of observed results was compared with the expected distribution of results under the hypothesis of no association using false discovery rate (FDR) methodology [7]. The expected distribution of results was generated by randomly permuting ILD case–control status 100 times to generate 100 datasets of 465,816 SNPs, each with 52 subjects designated as cases and 139 designated as controls. Each of the 100 datasets was then analyzed for association and the resulting 46,581,600 Fisher's exact p-values (two-sided) recorded. A smoothing algorithm [7] was applied to the raw FDR values to generate q-values, and both the q-values and raw FDR values were used to determine an appropriate cut-off point for the extent to which replication should be tested.

Top-ranking SNPs above this cut-off were then selected from the exploratory analysis of the CCS data and tested for replication with the retrospective data using one-sided Fisher's exact tests.

Analysis two: combined case–control analyses of CCS & retrospective study

In analysis two, the CCS and retrospective data were combined in order to maximize the sample size. The analysis process was as for the CCS-only association and FDR analysis of analysis one.

Analysis three: case–control analyses adjusting for effects of covariates

Using the eight clinical variables identified as potentially associated with ILD from the larger main clinical CCS dataset [6], a forward selection process using logistic regression-based likelihood ratio tests was performed, in order to evaluate their association with ILD in the subjects selected for the genetic study and to determine which were the most important clinical variables to include as covariates in the genetic analyses, given the more limited sample size. The most significant individual variable was entered into the model first, and a significance of $p < 0.1$ was used as the inclusion criterion for addition of further variables.

The selected clinical variables were then incorporated into the genetic analyses. Adjusted ORs with 95% CIs and p-values were estimated using logistic regression, and models with and without and with the clinical covariates included (considered individually or a number simultaneously) were compared for all 465,816 SNPs.

Analysis four: SNP–SNP interaction analysis

Using the 100 top-ranking SNPs in the CCS obtained in analysis one (Fisher's exact test [unadjusted] analyses), all pairwise interactions (n = 4950) were tested by logistic regression. Owing to sample size limitations, a simple allelic model (genotypes coded 0, 1 and 2 for the number of copies of the minor allele) was used for the parameterization of the interaction. The p-values for interactions were generated by likelihood ratio tests based on logistic regression, where the model for the null hypothesis included one variable for each of the pairs of SNPs and the alternative hypothesis model included the SNP variables and an interaction variable (the product of counts of allele 2). Replication of the 50 top-ranking (most significant) interactions in the CCS was tested using data from the retrospective study.

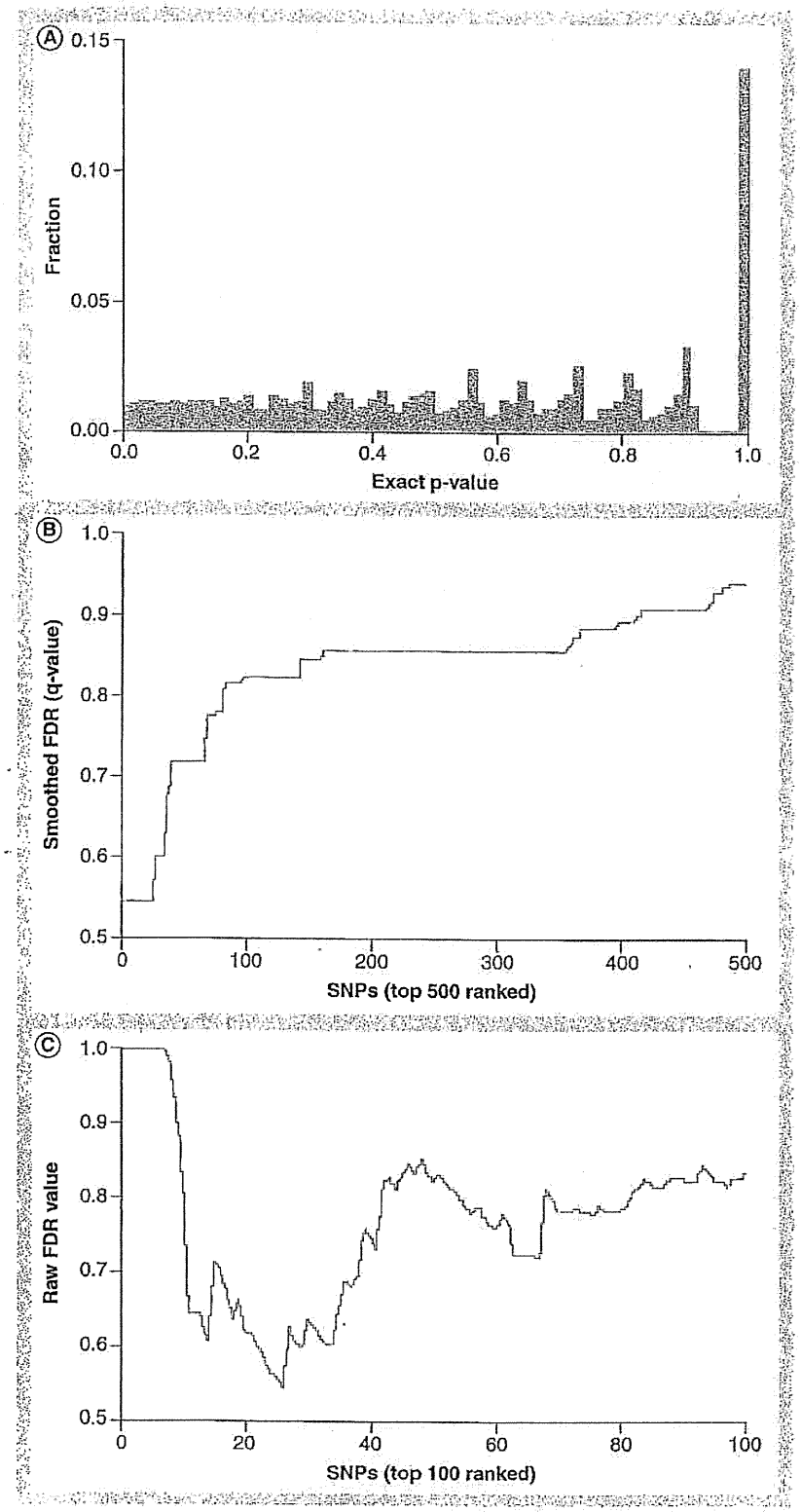

Figure 1. Genetic association analysis without covariates (prospective nested case–control study data only). (A) Expected distribution of p-values generated under the hypothesis of no association. **(B)** Smoothed FDR (q-values) for the top 500 ranked SNPs from the observed results. **(C)** Raw FDR values for the top 100 ranked SNPs.
FDR: False discovery rate.

Results

Block sequential design

No variations that showed an effect of sufficient magnitude to adequately predict ILD status in a subsequent blinded block were identified. At each step, none of the SNPs were significantly (corresponding to $p < 0.05$ nominally) associated with ILD when corrected for multiple comparisons. As additional blocks were added, the top-ranked SNPs also changed, suggesting that genuinely associated SNPs had not been identified.

Case–control design

Analysis one: case–control analyses of CCS (exploratory) & retrospective study (confirmatory/replication)

The smoothed FDR (q-values) and underlying raw FDR values for the top-ranking SNPs provided some indication that there were more small significant p-values than would be expected by chance. The deviation from the expected distribution of p-values was mainly restricted to the top 26 SNPs (corresponding to a p-value threshold of $\leq 4.3 \times 10{-}1^{-5}$); however, given the raw FDR results, the majority were likely to be false discoveries (Figure 1).

The ORs were consistent (i.e., the risk allele was the same) between the CCS and retrospective study for 11 of the 26 top-ranking SNPs identified from the CCS data (Table 1).

None of the 26 SNPs evaluated in the retrospective study demonstrated strong significant evidence of association with ILD (Table 1). In the retrospective study, the SNP rs4233356 (ranked 26 in the CCS data) demonstrated evidence for association consistent with the CCS data at a nominal p-value of <0.05 ($p = 0.0478$). However, considering the 26 tests conducted in the retrospective data at this stage, and the previous weak evidence for the hypothesized SNPs based on the CCS hypothesis-free results only, this does not provide convincing statistical evidence for genuine association. The rs4233356 SNP is situated on chromosome 1, in the 3′ flank of *DUSP23*, and has no known functional effect.

Given a lack of convincing statistical evidence to support a genuine association at this stage of analysis, SNPs ranking 27–67 in the CCS data (with a somewhat lower FDR support) were also assessed for evidence of their association in the retrospective study. The identification of the risk allele was consistent between the two studies for 21 of the 41 additional SNPs but none of these demonstrated strongly significant evidence of association with ILD (Supplementary Table 1). The

Table 1. Top-ranking (most significant) 26 SNP associations with acute interstitial lung disease events in the prospective nested case–control study with their corresponding odds ratios and p-values (for interstitial lung disease vs noninterstitial lung disease; i.e., case vs control), and the odds ratios and p-values for the SNPs with consistent risk alleles in the retrospective study (i.e., same allele associated with increased risk in the prospective nested case–control study and retrospective study).

Rank in CCS	SNP ID	Risk allele in CCS	Risk allele in retrospective study	Minor allele frequency (all controls)	CCS		Retrospective study	
					p-value[†]	*OR*[‡] *(95% CI)*	*p-value*[§]	*OR*[‡] *(95% CI)*
1	rs2607391	T	T	0.389	1.02×10^{-5}	2.85 (1.74–4.69)	0.556	1.01 (0.49–2.05)
2	rs2607374	T	C	0.369	1.36×10^{-5}	2.80 (1.72–4.57)	NA	NA
3	rs2249790	C	T	0.374	1.37×10^{-5}	3.39 (1.85–6.51)	NA	NA
4	rs2384130	T	G	0.482	1.66×10^{-5}	2.89 (1.72–4.95)	NA	NA
5	rs1880521	C	T	0.180	1.69×10^{-5}	6.59 (2.34–25.60)	NA	NA
6	rs2842880	C	C	0.361	1.83×10^{-5}	3.44 (1.85–6.73)	0.448	1.11 (0.53–2.36)
7	rs298380	G	A	0.319	2.39×10^{-5}	2.76 (1.69–4.51)	NA	NA
8	rs1510936	G	A	0.314	2.39×10^{-5}	2.73 (1.67–4.46)	NA	NA
9	rs2072409	T	T	0.232	2.49×10^{-5}	2.87 (1.73–4.74)	0.0731	1.79 (0.83–3.83)
10	rs6745588	A	A	0.242	2.51×10^{-5}	4.66 (2.04–12.43)	0.105	1.95 (0.74–5.75)
11	rs12658051	G	A	0.119	2.54×10^{-5}	3.48 (1.88–6.42)	NA	NA
12	rs12145722	G	G	0.111	2.54×10^{-5}	3.48 (1.88–6.42)	0.357	1.31 (0.46–3.53)
13	rs10799754	T	T	0.111	2.54×10^{-5}	3.48 (1.88–6.42)	0.357	1.31 (0.46–3.53)
14	rs13396021	A	G	0.302	2.59×10^{-5}	3.67 (1.87–7.73)	NA	NA
15	rs11033354	T	T	0.314	3.18×10^{-5}	2.68 (1.64–4.36)	0.0706	1.75 (0.85–3.59)
16	rs781296	C	C	0.415	3.34×10^{-5}	2.67 (1.63–4.42)	0.120	1.56 (0.77–3.14)
17	rs11128917	T	T	0.049	3.35×10^{-5}	4.95 (2.17–11.63)	0.147	2.1 (0.59–7.42)
18	rs558034	G	A	0.338	3.37×10^{-5}	3.46 (1.80–7.09)	NA	NA
19	rs7074044	A	G	0.214	3.68×10^{-5}	2.86 (1.70–4.79)	NA	NA
20	rs33355	C	T	0.374	3.92×10^{-5}	2.65 (1.63–4.32)	NA	NA
21	rs33352	G	T	0.371	3.92×10^{-5}	2.65 (1.63–4.32)	NA	NA
22	rs9567265	C	T	0.338	4.01×10^{-5}	3.39 (1.80–6.78)	NA	NA
23	rs1962474	C	T	0.459	4.12×10^{-5}	2.79 (1.65–4.81)	NA	NA
24	rs11689980	G	G	0.240	4.12×10^{-5}	4.57 (2.00–12.20)	0.105	1.95 (0.74–5.75)
25	rs6578067	C	T	0.384	4.25×10^{-5}	2.63 (1.61–4.29)	NA	NA
26	rs4233356	T	T	0.201	4.30×10^{-5}	2.84 (1.69–4.75)	0.0478	2.01 (0.89–4.45)

[†]*Exact p-value (two-sided).*
[‡]*ORs are given for the risk allele only and are, therefore, all greater than 1.*
[§]*Exact p-value (one-sided).*
CCS: Prospective nested case–control study; ID: Identification; NA: Not applicable (result not listed because risk allele not concordant with CCS result); OR: Odds ratio.

SNP rs170020 (ranked 27 in the CCS data) demonstrated evidence for association consistent with the CCS data at a nominal p-value of <0.05 in the retrospective study ($p = 0.0214$). However, considering the 67 tests conducted in the retrospective data, this does not provide convincing statistical evidence to support a genuine association. The rs170020 SNP is situated on chromosome 5 in an intron of a human cDNA of unknown function.

Analysis two: combined case–control analyses of CCS & retrospective study

There was little or no indication that, within the top-ranking results, p-values were more significant than would be expected by chance (SUPPLEMENTARY FIGURE 1). As might be expected, the top two ranked SNPs in the combined data were the two SNPs showing the best consistency between the CCS results and retrospective data in analysis one (CCS rank 26 and 27). The 30 top-ranking SNPs in the combined data are shown in TABLE 2.

Table 2. Top-ranking SNPs in the combined prospective-nested case–control study and retrospective data, with their corresponding odds ratios and p-values (for interstitial lung disease versus noninterstitial lung disease [i.e. case vs control]).

SNP	Risk allele	OR (95% CI)	p-value
rs170020†	C	2.96 (1.80–5.00)	3.97×10^{-6}
rs4233356†	T	2.51 (1.64–3.84)	9.10×10^{-6}
rs2072409†	T	2.45 (1.62–3.69)	1.13×10^{-5}
rs6745588†	A	3.33 (1.81–6.54)	1.74×10^{-5}
rs11033354†	T	2.29 (1.54–3.40)	1.78×10^{-5}
rs2822567	A	2.41 (1.57–3.68)	2.42×10^{-5}
rs11689980†	G	3.29 (1.78–6.45)	2.70×10^{-5}
rs9983082	T	2.44 (1.58–3.74)	2.80×10^{-5}
rs11033348†	C	2.24 (1.51–3.32)	2.91×10^{-5}
rs6863880	C	2.22 (1.49–3.30)	3.40×10^{-5}
rs8131875	C	2.37 (1.55–3.62)	3.87×10^{-5}
rs1297152	C	2.37 (1.55–3.62)	3.87×10^{-5}
rs1874252	T	5.34 (2.21–13.77)	3.89×10^{-5}
rs10872468	A	3.35 (1.84–6.09)	4.03×10^{-5}
rs2822572	C	2.41 (1.56–3.71)	4.11×10^{-5}
rs219472	C	2.39 (1.53–3.79)	4.51×10^{-5}
rs11128917†	T	3.77 (1.93–7.44)	4.64×10^{-5}
rs4805985	A	2.63 (1.60–4.46)	4.94×10^{-5}
rs514457	T	2.29 (1.51–3.44)	4.99×10^{-5}
rs7000897†	A	2.19 (1.47–3.28)	5.01×10^{-5}
rs781296†	C	2.17 (1.47–3.22)	5.03×10^{-5}
rs4075046	C	2.65 (1.61–4.33)	6.11×10^{-5}
rs9879164	T	2.14 (1.44–3.17)	7.55×10^{-5}
rs1354379	C	2.13 (1.43–3.16)	7.89×10^{-5}
rs549190	C	2.22 (1.47–3.34)	8.33×10^{-5}
rs998091	C	2.70 (1.58–4.79)	8.99×10^{-5}
rs8006948	T	2.25 (1.46–3.49)	9.33×10^{-5}
rs6995394	C	2.24 (1.48–3.38)	9.61×10^{-5}
rs731028	C	2.92 (1.63–5.50)	9.80×10^{-5}
rs6471970	A	2.51 (1.55–4.05)	1.03×10^{-4}

†SNP included also among the top 67 results from analysis one.
OR: Odds ratio.

Analysis three: case–control analyses adjusting for effects of covariates

In order of significance, WHO PS, smoking status, severity of pre-existing ILD and concurrent cardiac disease were selected as covariates in the subsequent adjusted analyses of SNP data. In addition, since the extent of normal lung on the CT scan had been identified as a key, strongly associated risk factor in the main CCS analyses [6], the genetic analyses were adjusted for these five clinical variables.

For the comparison of unadjusted ORs to ORs adjusted for the effect of these five clinical variables, the top 200 SNPs ranked by p-value were selected from each set of results, with 93 markers common to both top-ranking sets. The ratios of adjusted to unadjusted ORs for the 307 top-ranking SNPs ranged from 0.59 to 3.68 (mean 1.24) (FIGURE 2), reflecting some substantial changes in ORs when adjusting for the combined effects of the five clinical covariates, that is, suggestive of confounding. The 30 top-ranking SNPs adjusted for all five clinical covariates are listed in TABLE 3.

To further investigate which covariates might be contributing most to the potential confounding observed for the full set, the ORs were estimated in analyses adjusted for each clinical covariate individually in the same subset of 307 SNPs. The mean ratios of adjusted to unadjusted ORs were 1.07 (range: 0.70–1.83) for adjustment with WHO PS; 1.06 (range: 0.88–2.27) for severity of pre-existing ILD; 1.06 (range: 0.82–1.76) for concurrent cardiac disease; and less for adjustment with the other two variables.

Analyses of all SNPs adjusted for each covariate individually were also performed. This was considered prudent, as each clinical covariate appeared to have a confounding effect (i.e., >10% change) on the ORs for at least a proportion of the 307 SNPs evaluated above; and due to caveats regarding the reliability of results when adjusting for all five covariates at the same time (the models may be too saturated, especially for rare alleles, possibly leading to upward bias in some cases). An additional set of analyses adjusting for gender was also performed. A summary of the top 30 ranking SNPs from all CCS-based adjusted and unadjusted analyses is shown in

FIGURE 3 (and listed in SUPPLEMENTARY TABLE 2). Several regions with one or more strongly associated SNPs that are relatively insensitive to adjustment (have low p-values in all or most of the models) can be noted on chromosomes 1 (two regions), 2, 3, 5, 11 and 13, within or closest to *ZBTB40*, *SMG5/CCT3*, *STK39*, *MAGI1*, *ENC1*, *RNF26* and *CCDC122*, respectively (SUPPLEMENTARY TABLES 3–5). Of the 50 lowest p-values in this combined set, the majority come from analyses adjusted for all covariates simultaneously, WHO PS or concurrent cardiac disease individually; suggesting that adjustment for these variables may better model disease risk and increase the power to detect genetic association.

Of the top-ranking SNPs common to both the unadjusted and adjusted results, only rs10799754 and rs12145722 on chromosome 1 were among those with consistent risk alleles between the CCS and the retrospective study (although the p-values in the retrospective study were not significant). In the National Center for Biotechnology Information Build 37.1, these SNPs are approximately 4 kb apart on chromosome 1, and are not within any known transcript; the closest gene being *ZBTB40*, approximately 30–35 kb 5′ of the two SNPs (SUPPLEMENTARY TABLE 5).

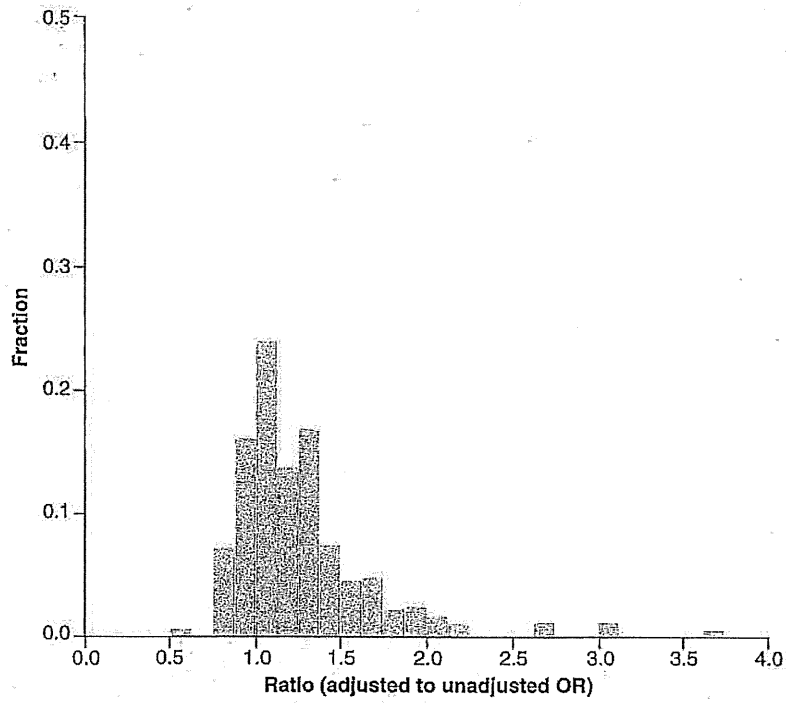

Figure 2. Distribution of ratios of adjusted to unadjusted odds ratios for the union of the top 200 ranked SNPs from association analyses without covariates and top 200 ranked SNPs from analyses with covariates (307 SNPs in total). Prospective nested case–control study data only.
OR: Odds ratio.

Analysis four: SNP–SNP interaction analysis

The 50 top-ranked interaction p-values of the 4950 possible tested combinations in the CCS data ranged from 0.0117 to 0.000322. In the corresponding tests using data from the retrospective study, interaction p-values ranged from 0.941 to 0.0705. Given the number of tests performed in the initial round of testing in the CCS data, such that the smallest p-value observed would not withstand Bonferroni correction, and the sparse nature of the data, the significance of nominal p-values obtained from standard logistic regression and likelihood ratio test is likely to be inflated. The 50 top-ranked interaction tests using the retrospective data compared with the corresponding CCS p-values are shown in SUPPLEMENTARY FIGURE 2. No evidence to support the interactions observed in the CCS data was observed in the results from the retrospective data analyses.

Discussion

A block sequential analysis approach was first used in an attempt to identify any strong genetic risk factors for ILD as early as possible during the study data collection phase. No SNPs with striking effect and predictivity were identified using this approach. Therefore, a regular case–control association analysis design using all of the data was employed at the end of data collection. There was some limited evidence from the CCS subjects that a proportion of top-ranking SNPs exceeded the levels of significance that might be expected by chance and these findings may, therefore, include some genuine associations between genotype and ILD status. There were several chromosome regions where one or more SNPs showed low p-values that were insensitive to adjustment for clinical risk factors for ILD, suggesting that these may be more likely to represent true associations, assuming independence of genes and environment. However, supporting evidence for these associations in the retrospective samples failed to reach a convincing level of statistical significance in the overall context of the number and range of statistical tests performed.

Explanations for the absence of significant associations in the retrospective study data at the top-ranking SNPs for the CCS subjects may include the smaller overall sample size of the retrospective study and a less clear identification of ILD cases and appropriate controls, due to its *ad hoc* retrospective design and less stringent ILD case diagnosis and review. The

Table 3. Top-ranking SNPs from analyses adjusted for clinical variables (prospective nested case–control study data only) with their corresponding odds ratios and p-values (for interstitial lung disease versus normal/minimal lung disease [ILD cases vs controls]).

SNP	Risk allele	$OR_{adjusted}$ (95% CI)	p-value$_{adjusted}$
rs1880521	C	11.17 (3.34–37.37)	7.48×10^{-7}
rs1002486	A	4.38 (2.19–8.75)	2.79×10^{-6}
rs1402236	T	4.38 (2.19–8.75)	2.79×10^{-6}
rs9567265	C	5.08 (2.30–11.21)	2.94×10^{-6}
rs298380	G	3.52 (1.98–6.25)	4.51×10^{-6}
rs7833659	C	16.88 (3.03–94.12)	5.99×10^{-6}
rs6469481	A	16.88 (3.03–94.12)	5.99×10^{-6}
rs7853023	T	3.81 (2.00–7.25)	6.71×10^{-6}
rs726568	G	5.68 (2.31–13.99)	7.06×10^{-6}
rs13326823	T	11.33 (2.88–44.57)	7.87×10^{-6}
rs1266824	T	7.24 (2.51–20.86)	9.51×10^{-6}
rs16973104	C	8.61 (2.61–28.45)	1.12×10^{-5}
rs1510936	G	3.22 (1.85–5.61)	1.16×10^{-5}
rs669441	A	4.54 (2.13–9.68)	1.31×10^{-5}
rs12145722	G	4.66 (2.26–9.63)	1.33×10^{-5}
rs10799754	T	4.66 (2.26–9.63)	1.33×10^{-5}
rs8030304	A	18.01 (2.36–137.67)	1.99×10^{-5}
rs4591498	G	3.74 (1.96–7.14)	2.26×10^{-5}
rs823114	T	3.21 (1.78–5.78)	2.61×10^{-5}
rs4611674	T	3.42 (1.84–6.36)	2.66×10^{-5}
rs2159415	A	5.28 (2.17–12.83)	2.67×10^{-5}
rs1066398	T	3.20 (1.81–5.67)	2.81×10^{-5}
rs1524966	T	10.30 (2.58–41.17)	2.84×10^{-5}
rs4688577	A	10.37 (2.60–41.43)	2.88×10^{-5}
rs298389	A	3.11 (1.79–5.43)	2.89×10^{-5}
rs4314161	C	3.79 (1.96–7.34)	3.02×10^{-5}
rs390755	G	2.97 (1.73–5.10)	3.23×10^{-5}
rs12632242	C	3.57 (1.89–6.72)	3.30×10^{-5}
rs11046589	A	3.15 (1.74–5.70)	3.97×10^{-5}
rs4808849	T	3.23 (1.80–5.79)	4.04×10^{-5}

[†]*The five clinical variables were WHO PS, severity of pre-existing ILD, concurrent cardiac disease, extent of normal lung on CT scan and smoking status.*
ILD: Interstitial lung disease; OR: Odds ratio; PS: Performance status.

CCS, by contrast, has a cohort with prospective follow-up as a clear source population for both ILD cases and appropriate controls, in combination with several design features to ensure full and accurate identification of all ILD cases in the cohort. Indeed, the even more disappointing FDR results for the combined analysis than for the CCS-only analysis may suggest that the retrospective study does not provide a sufficiently strictly defined and homogeneous sample for gene identification or validation purposes.

However, the presence of genuine associations in the top-ranking SNPs cannot be excluded. There is a paucity of published literature on genetic determinants of ILD. A possible association between ILD related to prescribed medication and genetic variants in cytochrome P450 (CYP)2D6, 2C9 and 2C19 was reported in a case–control study in The Netherlands [8]; although the wide range of potentially causative drugs included in that study (patients often receiving multiple agents) makes the link between individual causative drugs and CYP variants unclear. In our study, neither CYP3A4 (via which gefitinib is largely metabolized) nor other CYP enzymes ranked among the top findings. On the other hand, using a hypothesis-free approach, our analyses have identified the strongest candidates from this dataset (Supplementary Tables 2–5), and these may be regarded as potential mechanistic candidates for the development of ILD, whether drug-induced or not, but will require further supporting genetic or other evidence from other datasets. In principle, even the strongest signal can be made nonsignificant in a multiple-testing framework after inclusion of enough tests for false hypotheses, particularly when the sample size is limited, thus the final evaluation will always rest on overall plausibility given the present data and any prior or emerging evidence. Nevertheless, our analyses suggest that any true genetic signals for ILD risk are not particularly strong.

Analyses adjusted for the effects on ILD status of key clinical variables previously identified from the CCS data also failed to provide convincing evidence for genetic associations. Given the sample size, for many SNPs it is possible that there are serious limitations on the reliability and interpretation of the results when adjusting for all five covariates simultaneously and, indeed, even when adjusting for the effect of each covariate individually. It is not clear whether the shifts in ORs observed at top-ranking SNPs might not be expected by chance given the nature of the dataset and the number of tests performed. In additional attempts to identify interaction effects of pairs of SNP on ILD status, the retrospective data analyses failed to confirm CCS-based top findings. Overall, substantial confounding by other variables seems unlikely.

Despite the rigorous nature of the analyses, no common SNPs have been identified as clear candidates for follow-up studies such as functional analysis, although it is not possible to exclude genetic influences in ILD etiology. There may be multiple small genetic effects and/or interactive effects that we did not identify due to insufficient genetic coverage, low

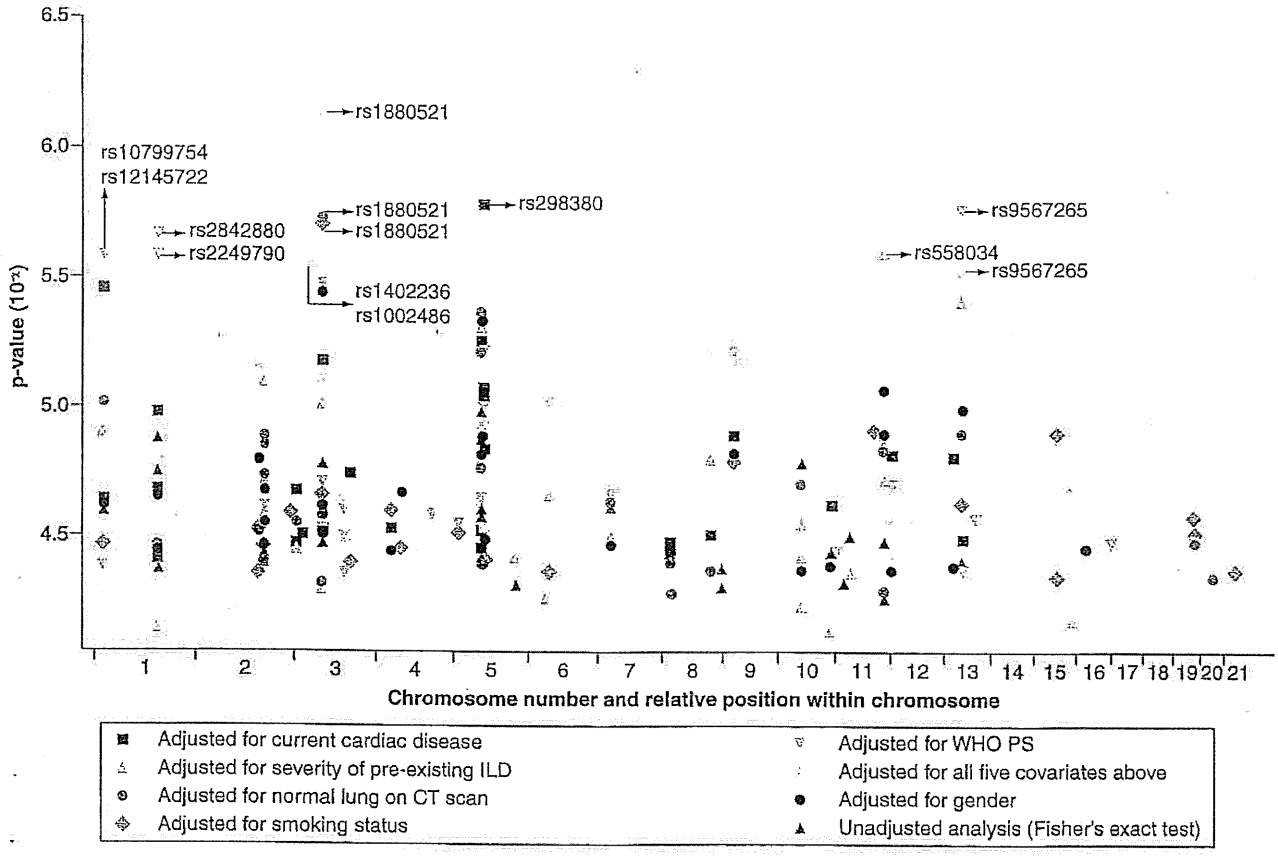

Figure 3. Plot of prospective nested case–control study-based analyses for top-ranking (most significant) 30 SNPs for each analysis performed: unadjusted, adjusted for single clinical covariates, and adjusted for the full selected set of the most important clinical variables.
The labels show SNP identification (rs number)
ILD: Interstitial lung disease; PS: Performance status.

power or simple inability to statistically model the data. The list of genes relating to top-ranking SNPs from this study (Supplementary Tables 3–5) may have utility when considering results from other studies relating to ILD etiology. This will require the assembly of new cohorts and datasets with well-characterized ILD cases, which will represent an important avenue for future work to better understand this disease. As the term 'ILD' describes heterogeneous clinical manifestations [3], it is possible that different subtypes are predicted by specific risk factors, suggesting that a subtype genetic analysis may yield candidate biomarkers not identified here. We did not take this approach for several reasons: the clinical utility of a biomarker predicting any type of ILD would be much higher than one predicting a single subtype; the diagnosis of ILD subtypes is uncertain and generally not very reproducible, particularly without biopsies; and, finally, our statistical power was too low for such an analysis.

In mid-2004, somatic mutations in the *EGFR* gene were identified in NSCLC and subsequently shown to be a key predictive factor for the efficacy of gefitinib in patients with advanced NSCLC. Since our study investigated host (germline) genetics as a potential susceptibility risk factor for ILD (and also since it was initiated prior to the identification of activating *EGFR* mutations), tumor material for *EGFR* mutation analysis was not collected in the present study. Furthermore, to date, literature has not suggested an association between tumor *EGFR* mutations and ILD. Indeed, in reporting the clinical results of the overall study in which the present CCS genetic analysis is nested [6], we commented that "evidence to date suggests that subgroups less at risk of ILD tend to be those that respond well to gefitinib treatment" [9], and "The factors associated with risk of acute ILD observed in this Japanese NSCLC population are largely different or even complementary to factors that predict better response to gefitinib" [9].

Further research is indicated on both genetic and other possible influences on ILD etiology, such as environmental factors. Increase in sample size and the use of stringent methods to diagnose ILD and associated subtypes are recommendations for the future when additional studies are designed to search for genetic factors related to acute ILD events, whether related to gefitinib or not.

Conclusion

In this study, it was not possible to identify genetic associations between common sequence variation and diagnosis of ILD that could have clinical utility for prediction of ILD risk, despite genotyping of approximately 0.5 million SNP markers in 80 ILD cases and 194 non-ILD controls from two studies, and the rigorous and comprehensive nature of the statistical analyses. Although it is not possible to exclude genetic influences in ILD etiology, these results suggest that common sequence variation is unlikely to explain a major component of ILD risk. The highest-ranked SNPs identified in this study may provide a useful hypothesis-generating starting point for further research to increase mechanistic understanding of the development of acute ILD events.

Acknowledgements

The authors thank the patients, investigators and study personnel who participated in these studies (investigators and key study personnel are listed under Study Organization in the SUPPLEMENTARY MATERIAL*); Iain McKendrick and Catriona Tate of Discovery Information, AstraZeneca R&D, for bioinformatics support to the genetic analyses; and Annette Smith, from Complete Medical Communications, who provided medical writing support funded by AstraZeneca.*

Financial & competing interests disclosure

The prospective study was sponsored and funded by AstraZeneca. AstraZeneca also provided funding for the retrospective study, which was a collaborative research effort between AstraZeneca and Tokyo University. Fredrik Nyberg, Ansar Jawaid, Shuji Hada, Takaaki Umemura, Ruth March and Bryan J Barratt are employees of AstraZeneca. Fredrik Nyberg and Bryan J Barratt have stock ownership of AstraZeneca. Masahiro Fukuoka has received honoraria from AstraZeneca. The other coauthors have no potential conflicts of interest to disclose. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Medical writing assistance was provided by Annette Smith, Complete Medical Communications, funded by AstraZeneca.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Interstitial lung disease (ILD) events have been reported in Japanese patients with non-small-cell lung cancer (NSCLC) receiving gefitinib. This study aimed to investigate whether genetics could potentially provide mechanistic insights and help in the prediction of ILD.
- Genotype data for 465,816 SNPs were obtained from Japanese patients with NSCLC treated with gefitinib as part of both a nested case–control study within a patient cohort with prospective follow-up (52 ILD cases, 139 controls [non-ILD patients]) and a retrospective case–control study (28 ILD cases, 55 controls).
- Despite the rigorous and comprehensive nature of the statistical analyses, it was not possible to identify genetic associations between common sequence variation and diagnosis of ILD that could have clinical utility for prediction of ILD risk.
- Although it is not possible to exclude genetic influences in ILD etiology, these results suggest that common sequence variation is unlikely to explain a major component of ILD risk.
- The highest-ranked SNPs identified in this study may provide a useful hypothesis-generation starting point for further research.

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DOI 10.1007/s10637-011-9715-4

PHASE I STUDIES

Figitumumab combined with carboplatin and paclitaxel in treatment-naïve Japanese patients with advanced non-small cell lung cancer

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Received: 11 May 2011 / Accepted: 29 June 2011

Summary *Objectives* The insulin-like growth factor (IGF) signaling pathway has been implicated in the pathogenesis of numerous tumor types, including non-small cell lung cancer (NSCLC). Figitumumab is a fully human IgG2 monoclonal antibody against IGF-1 receptor (IGF-1R). *Methods* This phase I, open-label, dose-escalation study (ClinicalTrials.gov: NCT00603538) assessed the safety and tolerability of figitumumab (6, 10 and 20 mg/kg) in combination with carboplatin (area under the curve: 6 mg·min/mL) and paclitaxel (200 mg/m^2) in Japanese patients (N=19) with chemotherapy-naïve, advanced NSCLC. Treatments were administered intravenously on day 1 of a 21-day cycle for four to six cycles. Pharmacokinetics, biomarkers, and antitumor activity were also evaluated. *Results* Figitumumab in combination with carboplatin and paclitaxel was well tolerated at doses up to 20 mg/kg; no dose-limiting toxicities were observed at this dose level. When given in combination, figitumumab plasma exposure increased in an approximately dose-proportional manner. The approximate 2-fold accumulation following repeated administration supported the 21-day regimen as appropriate for figitumumab administration. Serum total IGF-1 and IGF binding protein-3 concentrations increased following figitumumab dosing, but a clear dose-dependent relationship was not demonstrated. Seven of 18 evaluable patients experienced a partial response. *Conclusions* Figitumumab 20 mg/kg in combination with carboplatin and paclitaxel was well tolerated in chemotherapy-naïve Japanese patients with NSCLC. Further analysis of biomarker data is necessary for the development of figitumumab therapy.

Keywords Carboplatin · Figitumumab · Non-small cell lung cancer · Paclitaxel

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Introduction

The insulin-like growth factor (IGF) signaling pathway comprises IGF ligands (IGF-1 and IGF-2), IGF binding proteins (IGFBP1–6) which regulate ligand bioavailability, and IGF receptors (IGF-1R and IGF-2R) [1–3]. IGF signaling has been implicated in the development of a variety of tumors, including breast, colorectal, prostate, and lung cancers [2, 3]. IGF-1R is a receptor tyrosine kinase involved in the regulation of various biological processes, including cell growth, proliferation, and inhibition of apoptosis. In non-small cell lung cancer (NSCLC), IGF-1R is frequently over-expressed in tumor tissue and also mediates the proliferation of lung cancer cell lines [3–6].

Figitumumab (CP-751,871; Pfizer Inc, La Jolla, USA), a fully human IgG2 monoclonal antibody (mAb) against IGF-1R, is one of several agents currently in development which target the IGF pathway [7]. Figitumumab monotherapy has been well tolerated in phase I studies of patients with refractory solid tumors or multiple myeloma [8–12]. The safety and efficacy of figitumumab in combination

Published online: 13 July 2011

with carboplatin and paclitaxel were investigated previously in a Western phase Ib/II study in patients with chemotherapy-naïve, locally advanced or metastatic NSCLC [13]. Results suggested that figitumumab in combination with chemotherapy was safe and effective in this patient population.

The aim of this phase I, open-label, dose-escalation study was to assess the safety and tolerability of figitumumab in combination with carboplatin and paclitaxel in Japanese chemotherapy-naïve patients with advanced NSCLC. Secondary objectives were to evaluate pharmacokinetics, biomarkers, and antitumor activity.

Materials and methods

Study population

Patients eligible for inclusion in the study were aged 20–74 years, had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and had previously untreated, measurable, stage IIIB/IV NSCLC. All patients had adequate organ function assessed by hemoglobin (≥10 g/dL), platelet (≥100 000 cells/μL), and absolute neutrophil (≥2,000 cells/μL) counts; serum creatinine (≤1.5 mg/dL), albumin (≤3.0 g/dL), total bilirubin (≤1.8 mg/dL), and alanine aminotranferase and aspartate aminotransferase (≤80 IU/L) levels; circulating glycosylated hemoglobin (HbA_{1c}) <7% and fasting plasma glucose levels <126 mg/dL.

Exclusion criteria included prior anticancer therapy for advanced NSCLC, presence of symptomatic brain metastases or central nervous system metastases, history of active malignancy other than NSCLC within the previous 5 years (skin cancer other than malignant melanoma and in situ cervical, gastric, and colorectal cancers were permitted), treatment for pleural effusions and/or pericardial effusions, gastrointestinal bleeding within the previous 3 months, treatment with systemic corticosteroids within the previous 2 weeks, or neuropathy ≥grade 2 within the past 2 weeks. Subjects with diabetes and significant cardiac disease, including myocardial infarction, angina, uncompensated congestive heart failure, and serious cardiac ventricular arrhythmia, and uncontrolled hypertension within the past 6 months were also excluded.

The study protocol was approved by the Institutional Review Board at the National Cancer Center, Tokyo, Japan, and the study conformed to the provisions of the Declaration of Helsinki (1996). All patients provided written, informed consent.

Study design and dosing

This was a phase I, single-center, open-label, dose-escalation study to evaluate the safety and tolerability of figitumumab in combination with carboplatin (area under the curve [AUC] 6 mg·min/mL) and paclitaxel (200 mg/m^2).

Treatments were administered intravenously on day 1 of a 21-day cycle for four to six cycles, unless disease progression or unacceptable toxicity was observed. Carboplatin was administered following completion of the paclitaxel infusion, and figitumumab was administered following completion of the carboplatin infusion. A standard 3+3 dose-escalation scheme was used to escalate the dose of figitumumab. The first cohort of patients received figitumumab 6 mg/kg, and the second and third cohorts received figitumumab at doses of 10 mg/kg and 20 mg/kg, respectively. To minimize the risk of hypersensitivity, patients received prophylactic anti-allergy medication prior to paclitaxel administration, per the prescribing information for paclitaxel. The 20 mg/kg dose was judged effective and tolerable in phase I/II studies in Western patients [8–11, 13] and therefore no dose-expansion cohort was enrolled in this study.

Dose-limiting toxicities (DLTs) were figitumumab-related grade 3 or 4 toxicities assessed during the first treatment cycle according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) v3.0. DLTs included: grade 4 neutropenia lasting ≥7 days or complicated by fever (body temperature >38.0°C); and grade 4 thrombocytopenia or grade ≥3 thrombocytopenia necessitating a blood transfusion. Grade ≥3 non-hematologic adverse events (AEs; including gastrointestinal events, hyperglycemia, and/or fatigue despite the use of adequate medical intervention), and other clinically significant treatment-related AEs identified by the investigator, were also considered as DLTs.

Assessments and analyses

All patients who received at least one dose of figitumumab, carboplatin or paclitaxel were assessed for safety. AEs were graded according to the NCI CTCAE v3.0. Laboratory tests were performed at regular intervals throughout the study (including measurement of hematology, blood chemistry, coagulation, and urinalysis parameters). Vital signs and electrocardiograms (ECGs) were also assessed during screening and at regular intervals throughout the study. Blood samples for the measurement of circulating total IGF-1 and IGFBP3 by radio-immunoassay were collected prior to chemotherapy dosing and 168 h post-figitumumab infusion in cycles 1–4, prior to dosing in cycles 5 and 6, and at the end of the study. Circulating levels of human growth hormone (hGH) and insulin (fasting) were assessed at screening, day 1 (prior to dosing with study medication), and day 22 of each cycle, and at the end of the study. Glycosylated hemoglobin (HbA_{1c}) levels were assessed at screening only. Circulating glucose (fasting) and other

blood chemistry parameters were assessed on days 8 and 15 of each cycle. Tumor assessments were performed at baseline, during cycles 2, 4, and 6, and at end of study treatment. Objective response was determined according to Response Evaluation Criteria In Solid Tumors (RECIST v1.0) [14].

Pharmacokinetic profiles of figitumumab were obtained during cycles 1 and 4: blood samples were collected prior to figitumumab infusion, and 1, 24, 72, and 168 h after figitumumab infusion. In other cycles, samples were collected prior to figitumumab infusion, and 1 h after figitumumab infusion. An additional pharmacokinetic blood sample was collected at the end of the study. Plasma concentrations of figitumumab were determined by a validated enzyme-linked immunosorbent assay. Briefly, an IGF-1-soluble receptor was utilized to capture figitumumab. Figitumumab bound to the receptor was detected using a biotinylated mouse anti-human IgG2, followed by Streptavidin–Horseradish Peroxidase conjugate, and visualized using SureBlue™ peroxidase substrate. The lower limit of quantitation for the assay was 120 ng/mL. Pharmacokinetic parameters, which were calculated using non-compartmental methods, included C_{max} (maximum observed plasma concentration after the end of figitumumab infusion), $AUC_{(0-day22)}$ (area under the plasma concentration–time curve from time zero to day 22 [the nominal time of the pre-dose sample for the next cycle]), AUC_{tau} (AUC from time zero to tau [the actual time of the pre-dose sample for the next cycle]), and $t_{½}$ (apparent disposition half-life). The accumulation ratio was calculated as: cycle 4 AUC_{tau}/cycle 1 AUC_{tau}.

Due to the exploratory nature of this study, enrollment was dependent upon the observed safety profile and confirmatory inferential analyses were not planned. Descriptive statistics were used to summarize patient characteristics, safety, antitumor activity, and pharmacokinetic parameters. Analyses of the relationships between antitumor activity and circulating levels of total IGF-1, IGFBP3, hGH, and insulin were conducted. Summary statistical data are shown; statistical associations between biomarker levels and clinical outcome were not investigated due to the exploratory nature of these analyses and the small patient numbers.

Results

Baseline characteristics

Nineteen patients were enrolled across three figitumumab dose levels: 6 mg/kg, n=6; 10 mg/kg, n=7; 20 mg/kg, n=6. All patients were Japanese, and demographic and baseline characteristics are summarized in Table 1. Most patients (n=15; 78.9%) presented with stage IV NSCLC. Fourteen patients (73.7%) had adenocarcinoma, four patients (21.1%) had squamous cell carcinoma, and one patient had their histology classified as 'NSCLC not otherwise specified'.

Eighteen patients completed the first treatment cycle. One patient in the 10-mg/kg cohort discontinued the study due to a serious paclitaxel-related AE (hypersensitivity); this patient did not receive figitumumab. The median number of treatment cycles started for the 6-, 10-, and 20-mg/kg figitumumab dose levels was 4 (range 2–6), 4 (range 1–6), and 4 (range 3–6), respectively.

Safety and tolerability

DLTs were experienced by one patient at the figitumumab 6-mg/kg dose level (grade 4 thrombocytopenia) and by two patients at the 10-mg/kg dose level (one patient had grade 4 hyperuricemia, grade 3 hypermagnesemia, grade 3 hyponatremia, and grade 3 hyperkalemia, and another patient had grade 4 thrombocytopenia). No DLTs occurred in

Table 1 Patient characteristics at baseline

Characteristic	Figitumumab dose level[a]		
	6 mg/kg	10 mg/kg	20 mg/kg
Enrolled patients, *n*	6	7	6
Median age (years)	54	40	63
Range	45–69	21–61	37–74
Gender, *n*			
Male	3	4	5
Female	3	3	1
ECOG performance status, *n*			
0	5	7	4
1	1	0	2
NSCLC histologic subtype, *n*			
Adenocarcinoma	4	6	4
Squamous cell carcinoma	1	1	2
Not otherwise specified	1	0	0
Disease stage, *n*			
IIIB	0	1	3
IV	6	6	3
Smoking history, *n*			
Never smoker	4	3	0
Smoker	2	2	2
Ex-smoker	0	2	4

AUC area under the curve, *ECOG* Eastern Cooperative Oncology Group, *NSCLC* non-small cell lung cancer

[a] Figitumumab in combination with carboplatin (AUC 6 mg·min/mL) and paclitaxel (200 mg/m^2)

patients treated at the highest figitumumab dose level of 20 mg/kg (Table 2).

The most common all-causality, non-hematologic AEs of all grades across all dose levels and cycles were peripheral sensory neuropathy (n=16 [84.2%]), anorexia (n=14 [73.7%]), and diarrhea (n=9 [47.4%]). Grade 3 and 4 treatment-related hematologic and non-hematologic AEs occurring at each figitumumab dose level are presented in Table 3. The only grade ≥3 non-hematologic AEs to occur in two or more patients across all dose levels and cycles were hyponatremia/blood sodium decrease (grade 3, n=3 [15.8%]), anorexia (grade 3, n=2 [10.5%]), and hyperuricemia (grade 4, n=2 [10.5%]); hyperuricemia was the only grade 4 non-hematologic AE reported. There were no cases of grade 3 or 4 hypoglycemia or hyperglycemia. Most grade 3 or 4 non-hematologic AEs were observed during cycle 1, and figitumumab dose level did not appear to influence the frequency of grade 3 or 4 AEs.

Grade 3 treatment-related hematologic AEs (across all dose levels and cycles) were neutropenia (n=5 [26.3%]), leukopenia (n=4 [21.1%]), anemia (n=2 [10.5%]), and thrombocytopenia (n=1 [5.3%]), and grade 4 treatment-related hematologic AEs were neutropenia (n=11 [57.9%]) and thrombocytopenia (n=3 [15.8%]; Table 3). No treatment-related deaths occurred at any figitumumab dose level.

Pharmacokinetics

When given in combination with carboplatin and paclitaxel, plasma concentrations of figitumumab declined in a multi-exponential manner (Fig. 1a, b). Both C_{max} and AUC within the first cycle increased in an approximately dose-proportional manner (Table 4). As indicated by the accumulation ratio, repeated administration of figitumumab every 21 days resulted in moderate accumulation (an approximate 2-fold increase in plasma exposure in the limited number of patients with sufficient data in cycle 4; Table 4). The $t_{½}$ of figitumumab was at least 248 h (more than 10 days) for all three dose levels, and achieved the $t_{½}$ of endogenous IgG2 (approximately 21 days) in some patients.

Biomarkers

Serum total IGFBP3, IGF-1 and hGH concentrations increased following figitumumab dosing compared with baseline values for each dose level (Fig. 2a–c). However, a clear dose-dependent relationship was not demonstrated, and the levels of insulin levels were not changed clearly prior and after figitumumab administration (data not shown).

Antitumor activity

Of 18 evaluable patients, seven experienced a partial response (PR; 38.9%). One PR was observed at the figitumumab 6-mg/kg dose level, and three PRs were observed at each of the 10- and 20-mg/kg dose levels. PRs were observed in patients with adenocarcinoma (five of 14 patients [35.7%]) and in patients with squamous cell carcinoma (two of four patients [50.0%]). No patient had a complete response. Stable disease (SD) was observed in eight patients (44.4%; three at each of the 6- and 20-mg/kg dose levels, and two at the 10-mg/kg dose level), and progressive disease (PD) was observed in two patients (11.1%; both at the 6-mg/kg dose level).

Relationship between antitumor activity and biomarkers

Serum total IGFBP3 and IGF-1 concentration–time profiles were stratified by best response (PR, SD, and PD, respectively) to evaluate the relationship between concentrations of biomarkers and clinical response. No clear

Table 2 Planned dose levels and observed DLTs

Dose level	Figitumumab[a]	Paclitaxel (mg/m^2)	Carboplatin (mg·min/mL)	N	DLTs
1	6 mg/kg	200	6	6	Grade 4 thrombocytopenia (n=1)
2	10 mg/kg	200	6	7	Grade 3: hyperkalemia, hypermagnesemia, hyponatremia; grade 4 hyperuricemia (n=1) Grade 4 thrombocytopenia (n=1)
3	20 mg/kg[b]	200	6	6	None

DLT dose-limiting toxicity

[a] If none of the three patients in the 6 mg/kg cohort experienced a DLT during cycle 1, subjects were enrolled onto the next dose level. If one DLT was observed, the cohort was to be expanded to six patients. If none of the three or two or less of the six patients experienced a DLT, then dose escalation was to be continued and three patients were enrolled to the 10 mg/kg cohort. In a similar manner depending on observed DLTs, the 10 mg/kg cohort could be expanded to six patients and dose escalation continued to a 20 mg/kg cohort of six patients. If two or more of the three, or three or more of the six patients experienced a DLT, dose escalation would be stopped

[b] Six patients dosed, and 20 mg/kg deemed tolerable if two or fewer of the six patients experienced a DLT

Table 3 Treatment-related AEs with maximum CTC grade ≥3, in cycle 1 and all cycles. The numbers of patients are shown for grades 3 and 4 AEs separately, and for all grades

	Figitumumab dose level																	
	6 mg/kg (n=6)						10 mg/kg (n=7)						20 mg/kg (n=6)					
	Cycle 1			All cycles			Cycle 1			All cycles			Cycle 1			All cycles		
	All	G3	G4	All	G3	G4	All	G3	G4	All	G3	G4	All	G3	G4	All	G3	G4
AEs, hematologic																		
Anemia	4	0	0	5	0	0	3	0	0	3	1	0	4	0	0	5	1	0
Leukopenia	6	0	0	6	1	0	6	1	0	6	1	0	5	0	0	6	2	0
Neutropenia	6	2[a]	3	6	0	5	6	1	2	6	3	2	5	2	1	6	2	4
Thrombocytopenia	4	0	1	5	0	1	5	0	1	5	0	1	4	1	0	6	1	1
AEs, non-hematologic																		
Anorexia	2	1	0	3	2	0	5	0	0	6	0	0	5	0	0	5	0	0
Diarrhea	3	0	0	3	0	0	1	0	0	2	1	0	3	0	0	4	0	0
Hyperkalemia	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
Hypermagnesemia	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
Hypersensitivity	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
Hyperuricemia	0	0	0	0	0	0	2	0	1	2	0	1	2	0	1	2	0	1
Hyponatremia	2	1	0	2	1	0	1	1	0	1	1	0	1	0	0	3	1	0
Peripheral sensory neuropathy	3	0	0	5	0	0	4	0	0	5	0	0	2	0	0	6	1	0
Vomiting	1	0	0	2	1	0	2	0	0	2	0	0	1	0	0	1	0	0

AE adverse event, *All* all grades, *CTC* Common Terminology Criteria, *G* grade

[a] Two patients with grade 3 neutropenia during cycle 1 experienced worsening of symptoms to grade 4 after cycle 2

differences were observed in the IGFBP3 concentration–time profiles according to clinical response, or when baseline IGFBP3 concentration was stratified by best response (Fig. 3a, b). However, the serum total IGF-1 concentration–time profile in patients with PR as their best response was higher than the profiles in both SD and PD patients (Fig. 3c). Higher baseline serum total IGF-1 concentrations were also observed for patients with PR compared with patients with SD/PD as best response (Fig. 3d).

Discussion

Figitumumab in combination with carboplatin and paclitaxel was well tolerated at doses up to 20 mg/kg in chemotherapy-naïve Japanese patients with advanced NSCLC in this phase I study. No DLTs were observed at the highest figitumumab dose level of 20 mg/kg. In addition, no grade 3 or 4 AEs appeared to show dose dependency, and there was no apparent tendency towards cumulative toxicity.

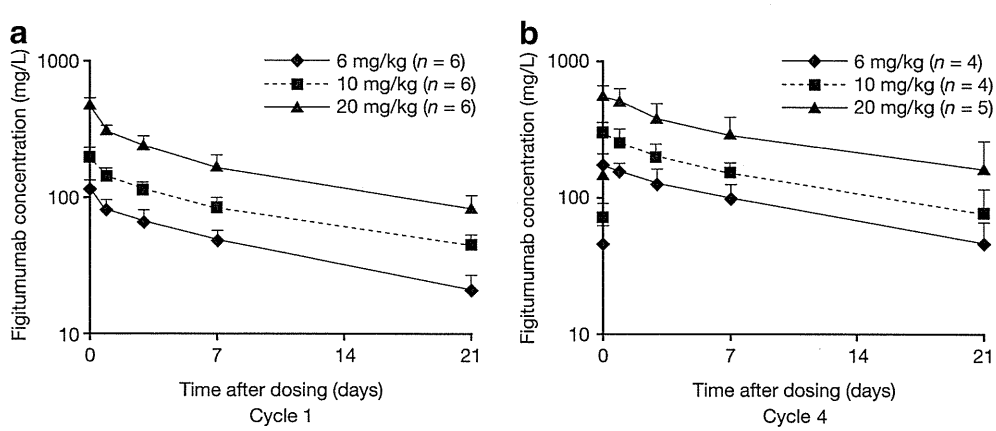

Fig. 1 Concentration–time profiles of plasma figitumumab. Data shown are mean + standard deviation