**Table 1.** Summary of Investigations Performed, Results, and Their Implications

| Investigation | Finding | Implication |
|---|---|---|
| Ethnic differences in EGFR polymorphisms in CA-SSR1 length | CA-SSR1 was longer in East Asians than in individuals of European descent, both for shorter allele and for combined allele length | For all three polymorphisms (shorter CA-SSR1 length and variant forms of SNPs −216 and −191), the forms associated with increased EGFR protein production are rarer in East Asians |
| Ethnic differences in EGFR polymorphisms in SNP −216 | Variant forms G/T and T/T were more common in individuals of European descent | |
| Ethnic differences in EGFR polymorphisms in SNP −191 | Variant forms C/A and A/A were more common in individuals of European descent | |
| Relationship between CA-SSR1 and SNP polymorphisms | NSCLC patients with rare forms of SNPs −216 and −191 had shorter combined allele length for CA-SSR1 | The forms of the polymorphisms associated with increased protein production tend to co-segregate in lung cancer patients |
| Relationship between SNP −216 variants and EGFR mRNA expression | HBECs that have variant forms tended to make more EGFR mRNA | For SNP −216, data are consistent with higher protein production being associated with the minor form |
| Effect of CA-SSR1 allele length on survival in patients with NSCLC | Patients with longer allele lengths had improved survival | The data are consistent with the concept that patients with less intrinsic protein production have improved survival in the absence of TKI therapy |
| EGFR mutations in NSCLC | Mutations were present in 25% of cases, and more common in East Asians (35.6%) than in individuals of European descent (11.3%) | This finding confirms previous reports that NSCLC tumors in East Asians have a higher incidence of EGFR mutations |
| Relationship between EGFR mutations and CA-SSR1 AI | Mutations were more frequent in tumors with AI, especially those arising in East Asians and those with SAD | Mutations and AI frequently occur together in East Asian NSCLC tumors with SAD |
| Determination of whether AI targets mutant or WT allele | In NSCLC cases having both AI and mutation, the copy number of the mutant allele was preferentially increased compared to that of the WT allele | AI preferentially targets the mutant allele |

three kinds of mutations, in-frame deletions in exon 19, missense mutations (predominantly mutation L858R in exon 21, but also in exons 18 or 20), and in-frame duplications/insertions of one to three codons in exon 20. The resistance-associated T790M mutation in exon 20 [9] was not detected in any tumor.

## Analysis of EGFR Polymorphic Sites

We sequenced genomic DNA encompassing the SNP sites in the promoter region of EGFR −216 and −191 as described previously [21], using a single PCR reaction.

The CA-repeat-containing region of intron one was amplified by PCR. The sequences of the primers were 5'-CCA ACC AAA ATA TTA AAC CTG TCT T-3' (forward) and 5'-CTT GAA CCA GGG ACA GCA AT-3' (reverse). For analysis of repeat allele lengths and relative ratios, instrumentation and reagents from Applied Biosystems (Foster City, California, United States) were utilized. The reverse primer was labeled with TAMRA fluorescent dye (6-FAM) at the 5' end. The 25-µl PCR reaction mixture contained 100 ng of genomic DNA, 10X PCR buffer containing 15 mM $MgCl_2$, 2 mM of each dNTP, 10 pmol of each primer, and 1.25 units of HotStart Taq DNA polymerase (Qiagen, Valencia, California, United States). After an initial denaturalization step at 95 °C for 12 min, samples were cycled 35 times as follows: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The final extension was at 72 °C for 20 min. The size of the products (about 80 bp) was

**Table 2.** Summary of Germline (Blood) and Malignant and Non-Malignant Lung Tissues Examined

| Sample | Ethnicity | Country | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | US | Australia | Japan | Taiwan | Italy | |
| Healthy individuals without cancer | Individuals of European descent | 75 | | | | | 75 |
| | African-Americans | 75 | | | | | 75 |
| | Mexican-Americans | 100 | | | | | 100 |
| | Total | 250 | | | | | 250 |
| Non-malignant lung tissue from NSCLC patients | Individuals of European descent | 133 | 71 | | | 93 | 297 |
| | East Asians | 4 | 1 | 187 | 48 | | 240 |
| | Others | 7 | | | | | 7 |
| | Total | 144 | 72 | 187 | 48 | 93 | 544 |
| Malignant lung tissue from NSCLC patients | Individuals of European descent | 142 | 71 | | | | 213 |
| | East Asians | 4 | 1 | 251 | 80 | | 336 |
| | Others | 7 | | | | | 7 |
| | Total | 153 | 72 | 251 | 80 | | 556 |
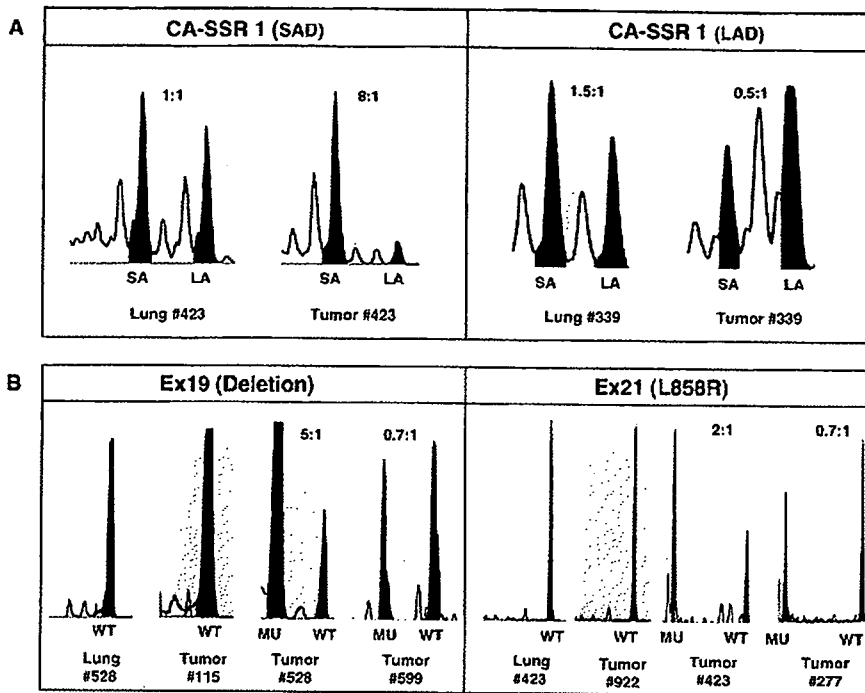
**Figure 1.** Determination of AI for Heterozygous for *CA-SSR1* and for Tumors Having a Deletion Mutation In Exon 19 or the L858R Mutation in Exon 21
Representative wave patterns are illustrated for (A) the *CA-SSR1* allele and (B) the deletion mutation in exon 19 or L858R mutation in exon 21. Both tumors and corresponding lung tissue were analyzed. Note in (A) the ratio of shorter allele to longer allele is actually 1.3:1, as illustrated for lung #423, due to artifactual preferential amplification of the short allele. Thus, an appropriate correction factor is applied.
doi:10.1371/journal.pmed.0040125.g001

confirmed by electrophoresis on 2% agarose gels. After PCR, 1 μl of the product plus 0.5 μl of Genescan 500 ROX molecular weight standard were denatured in 12 μl of Hi-Di Formamide (Applied Biosystems) and separated with a Prism Genetic Analyzer and analyzed by Gene Scan Analysis software 3.1 (Applied Biosystems).

Examination of the resultant traces demonstrated that biallelic (heterozygous) samples showed two sets of waves and two peaks, while the monoallelic (homozygous) samples showed a single set of waves and one peak (Figure 1). The highest peak reflects the repeat number of the *CA-SSR1* allele as determined by the size marker, while the preceding waves (stutter bands) represent PCR-induced artifacts. In samples without AI the shorter peak appears artificially larger as a result of preferential PCR amplification. In non-malignant lung tissue the alleles were presumed to be of equal size, and their ratios were used as a correction factor for this artificial discrepancy.

The degree of the amplification of each allele was indicated by the area under the peak as determined by software provided by the instrument's manufacturer. The relative ratios (AI ratios), termed LOH score in previous reports, of the two peaks (shorter peak area under the curve to longer peak area under the curve) in tumor samples were calculated as previously described [26]. The AI ratio was calculated thus: AI ratio = (T1 × N2)/(T2 × N1), where T indicates tumor, N indicates normal, 1 indicates the area under the peak for the shorter allele, and 2 indicates the area under the peak for the longer allele.

As either peak could be increased in relative size, AI cases were divided into shorter allele dominant (SAD) or longer

allele dominant (LAD) cases. We used the definitions of these two categories as determined previously [26]. SAD cases are defined as cases in which the adjusted AI ratio was greater than 1.27, and LAD cases were those in which the adjusted AI ratio was less than 0.79. For LAD cases, the formula results in ratio values less than unity. Therefore, the ratio was inverted for LAD cases, allowing the AI ratios to reflect the relative size of the longer allele, irrespective of which allele was increased in relative size. We confirmed the previous finding that the ratios of the areas under the curve for the two alleles in constitutional DNA on repeat testing or from different individuals are relatively constant. From an analysis of constitutional DNA from over 500 healthy individuals and cancer patients, we determined that the mean ratio of the two alleles in non-malignant tissues was 1.3, resulting from artificial preferential amplification of the shorter allele (data not shown). For tumor samples lacking corresponding non-malignant tissue, the AI was determined by the formula AI ratio = T1/(T2 × 1.3).

The primers for investigation of selective amplification of the mutant or wild-type (WT) allele of exon 19 in-frame deletions and the exon 21 point mutation L858R were designed as follows: 5′-TCA CAA TTG CCA GTT AAC GTC T-3′ (forward) and 5′-CAG CAA AGC AGA AAC TCA CAT C-3′ (reverse) for exon 19, and 5′-ATG AAC TAC TTG GAG GAC CGT C-3′ (forward) and 5′-TGC CTC CTT CTG CAT GGT ATT C-3′ (reverse) for exon 21. Each forward primer was labeled with TAMRA fluorescent dye (6-FAM) at the 5′ end. The conditions for PCR were the same as for *CA-SSR1* except for the annealing temperature (57 °C for exon 19 and 61 °C for exon 21). The PCR products of exon 21 were cut by
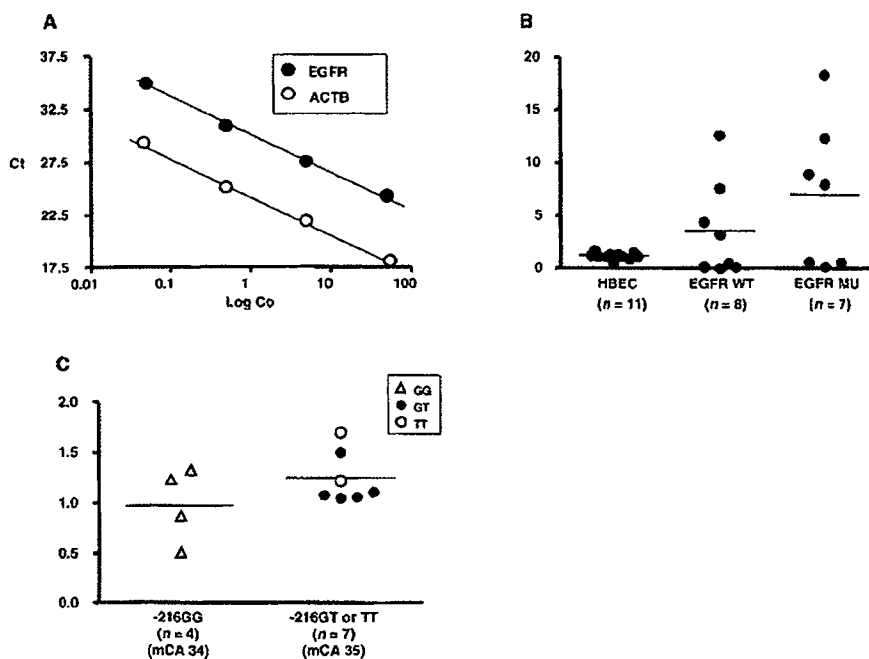
**Figure 2.** Relationship between SNP −216 Variants and *EGFR* mRNA Expression in HBEC Cultures

(A) Standard curves of *EGFR* and *ACTB*. Both slopes of cycle threshold (Ct)/log copies (Log Co) were mostly coincidental.
(B) Comparison of relative ratio of *EGFR/ACTB* among three groups of cultured cells (HBECs, lung cancer cell lines without *EGFR* mutations [WT], and lung cancer cell lines with *EGFR* mutations [MU]).
(C) Comparison of relative ratio of HBECs having SNP −216 G/G versus G/T or T/T. mCA, mean number of *CA-SSR1* repeats.
doi:10.1371/journal.pmed.0040125.g002

the restriction enzyme Sau96I (New England BioLabs, Ipswich, Massachusetts, United States) and analyzed. The size of each product (about 142 bp for mutant alleles of exon 19, 158 bp for the WT allele of exon 19, 100 bp for mutant the allele of exon 21, and 150 bp for the WT allele of exon 21) was also confirmed by electrophoresis in 2% agarose gels.

The ratio (mutant allele/WT allele) to define amplification of each mutant allele, exon 19 in-frame deletion or the L858R point mutation, was determined by ROC (receiver operating characteristics) curves using the definitive value of AI, 1.27 (data not shown). The definitive ratios for exon 19 and 21 were 0.82 (sensitivity 70%, specificity 68%) and 0.2 (sensitivity 90%, specificity 90%), respectively, and the combined definitive ratio was 0.47 (sensitivity 70%, specificity 61%). We used these ratios as cut-off values to determine whether the mutant allele was amplified. Because of the presence of various amounts of non-malignant cells in the tumor samples,

amplifications of the WT allele could not be determined with certainty.

## Real-Time PCR for the Expression of *EGFR* mRNA

cDNA was prepared by reverse transcription of 2 µg of RNA from cell lines using SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, California, United States). Real-time PCR was performed with the Sybro (SYBR) Green 1 method using Power SYBR Green PCR Master Mix (Applied Biosystems). *ACTB* cDNA was used as an internal control. Primer sequences were as follows: 5′-ATA GTC GCC CAA AGT TCC GTG AGT-3′ (forward) and 5′-ACC ACG TCG TCC ATG TCT TCT TCA-3′ (reverse) for *EGFR* and 5′-AGT CCT GTG GCA TCC ACG AAA CTA-3′ (forward) and 5′-ACT GTG TTG GCG TAC AGG TCT TTG-3′ (reverse) for *ACTB*. Standard curves for *EGFR* and *ACTB* were obtained (Figure 2A), and the relative expression ratios of *EGFR:ACTB* were calculated.

**Table 3.** The Distribution of *EGFR* Genotypes by Ethnicity for Lung Cancer Patients

| NSCLC Patients | SNP −216 | | | SNP −191 | | |
| --- | --- | --- | --- | --- | --- | --- |
| | G/G | G/T or T/T | $p$-Value[a] | C/C | C/A or A/A | $p$-Value[a] |
| Individuals of European descent ($n$ = 306) | 39.7% | 60.3% | <0.001[b] | 63.0% | 37.0% | <0.001[b] |
| East Asians ($n$ = 331) | 93.4% | 6.6% | | 99.4% | 0.6% | |

[a]Chi-square test. No significant gender differences were present ($p$ = 0.194, Fisher's exact test).
[b]General linear regression adjusting for gender, age, smoking, histology, and *EGFR* mutations.
doi:10.1371/journal.pmed.0040125.t003

**Table 4.** Ethnic Differences in Distribution of the Allele Lengths of *CA-SSR1* in Lung Cancer Patients

| NSCLC Patients | Shorter Allele Length | | Longer Allele Length | | Combined Allele Length | |
|---|---|---|---|---|---|---|
| | Mean (SD) | p-Value[a] | Mean (SD) | p-Value[a] | Mean (SD) | p-Value[a] |
| Individuals of European descent (n = 306) | 16.6 (1.4) | <0.001[b] | 18.5 (1.8) | <0.001[b] | 35.1 (2.8) | <0.001[b] |
| East Asians (n = 331) | 17.9 (2.0) | | 19.8 (1.2) | | 37.7 (2.7) | |

[a]Two-sample t-test. No significant gender differences were present (p = 0.194, Fisher's exact test). Therefore, gender is not adjusted for in the comparisons.
[b]General linear regression adjusting for gender, age, smoking, histology, and EGFR mutations.
SD, standard deviation.
doi:10.1371/journal.pmed.0040125.t004

## Statistical Analyses

We used the Chi-square test (testing the null hypothesis of equal distributions across study groups) to compare the distributions across study groups when outcomes were discrete such as genotypes of the SNP or SAD frequencies. When events were rare, e.g., where the expected cell counts were less than five, Fisher's exact test was used instead for comparisons. We also used Chi-square for an independent test for the assessment of each ethnic group using the Hardy–Weinberg equilibrium model. When outcomes were continuous, such as *CA-SSR1* repeat numbers, two-sample t-test and analysis of variance were used. In order to control for potential confounding bias in comparisons of SNP and *CA-SSR1* distributions, the multivariate logistic and general linear regression models were used with certain clinicopathological factors such as age, gender, smoking status, and histology as covariates (Tables 3–6). AI ratios of *CA-SSR1* plotted against mutant/WT ratios are shown in Figure 3 with the fitted regression lines. The associations between AI ratios and mutant/WT ratios were tested using Pearson's correlation for exon 19, exon 21, and both combined. To be conservative in case of small sample size and extreme values, the nonparametric Wilcoxon rank sum test was used to compare mutant/WT ratios for those with and without SAD. In this paper, all statistical tests and 95% confidence intervals are two-sided. Because of multiple tests, p-values less than 0.01 were judged to be statistically significant, and p-values less than 0.05 were judged as moderately significant. Both positive and negative results are reported in the tables and in the text.

## Results

Because of the complex nature of the findings and their interrelationships, a tabular summary of our major findings is presented in Table 1.

## Ethnic Differences in Distribution of Polymorphisms

We examined ethnic differences in the distribution of the minor alleles of the two SNPs −216 and −191 in the promoter region of the *EGFR* gene and mean *CA-SSR1* repeat numbers. A summary of the samples studied from healthy individuals and cancer patients is presented in Table 2. For healthy US individuals, the frequencies of the −216 genotypes showed a borderline statistically significant difference between individuals of European descent, African-Americans, and Mexican-Americans (p = 0.08) (Dataset S1). The G/G genotype was present in 46.7% (95% confidence interval: 35.4%–58.0%) of individuals of European descent compared to 60% (48.9%–71.1%) and 63% (53.5%–72.5%) of African-Americans and Mexican-Americans, respectively. The frequencies of the minor forms of the −191 polymorphism were significantly lower (p < 0.001) in African-Americans (10.7%, 3.7%–17.7%) than in individuals of European descent (36%, 25.1%–46.9%) and Mexican-Americans (43%, 33.3%–52.7%). Also, the mean *CA-SSR1* repeat number was significantly shorter in individuals of European descent (for the shorter, longer, or combined allele lengths) than in African-Americans and Mexican-Americans (combined allele length for individuals of European descent, 35.3, 34.7–35.9, for African-Americans, 36.2, 35.6–36.8, and for Mexican-Americans, 36.8, 36.3–37.3; p = 0.001). The differences between African-Americans and Mexican-Americans were relatively modest and only reached significance for the shorter allele length (Dataset S1).

Among US European-descent individuals in this study, there were no significant differences in the frequency of the three polymorphisms between the healthy individuals (DNA from PBMCs) and those with NSCLC (DNA from nonmalignant tissue). As shown in Table 3 and Dataset S1, the −216 G/G form was present in 46.7% (35.4%–58.0%) of the healthy individuals and 39.7% (30.8%–47.4%) of the patients with lung cancer (p = 0.321), and the −191 C/C genotype was

**Table 5.** The Relationship between Repeat Length of *CA-SSR1* and SNPs

| CA-SSR1 Allele | SNP −216 | | | SNP −191 | | |
|---|---|---|---|---|---|---|
| | G/G | G/T or T/T | p-Value[a] | C/C | C/A or A/A | p-Value[a] |
| Shorter | 17.7 (1.9) | 16.3 (1.0) | <0.001 | 17.3 (1.9) | 16.8 (1.2) | 0.084 |
| Longer | 19.6 (1.3) | 18.2 (1.8) | <0.001 | 19.2 (1.7) | 18.8 (1.3) | 0.011 |
| Combined | 37.3 (2.8) | 34.5 (2.4) | <0.001 | 36.6 (3.1) | 35.6 (2.3) | 0.011 |

Data are given as mean repeat length (standard deviation).
[a]Two-sample t-test after adjustment for ethnicity.
doi:10.1371/journal.pmed.0040125.t005

**Table 6.** Ethnic Differences in the Relationship between the Length of CA-SSR1 and SNPs −191 and −216

| SNP | Genotype | East Asians | | Individuals of European Descent | |
|---|---|---|---|---|---|
| | | Percentage with Shorter Combined CA-SSR1[a] | p-Value[b] | Percentage with Shorter Combined CA-SSR1[a] | p-Value[b] |
| −216 | G/G | 36.3% | 0.001 | 53.7% | <0.001 |
| | G/T or T/T | 72.7% | | 81.2% | |
| −191 | C/C | 38.3% | 0.149 | 76.2% | 0.017 |
| | C/A or A/A | 100.0% | | 59.5% | |
| Both | C/C + G/G | 35.8% | <0.001 | 59.0% | 0.119 |
| | Others | 75.0% | | 72.7% | |

[a]Having a shorter combined allele is defined as having 36 or fewer combined CA-SSR1 repeats. (This cut-off is based on the overall mean length of the combined allele for both ethnic groups. Using the individual group means for East Asians and Individuals of European descent as the cut-offs shows the similar results.)
[b]p-Values are from Fisher's exact tests.
doi:10.1371/journal.pmed.0040125.t006

present in 64% (53.1%−74.9%) of the healthy individuals and 63% (54.8%−71.2%) of the patients with cancer ($p$ = 0.941). Also, the mean CA-SSR1 repeat numbers for the short allele, long allele, and combined alleles of healthy European-descent individuals were not significantly different from those of European-descent patients with cancer ($p$ = 0.492, 0.604, and 0.495, respectively) (Table 4; Dataset S1). These data permitted us to presume that the polymorphism frequencies in patients with lung cancer follow the pattern of the general population, and we can combine the data from healthy individuals and patients with NSCLC for individuals of European descent, which is the dominant ethnicity of the US, Italy, and Australia populations in this study. Furthermore, no significant differences were observed in this study for the frequencies of all three polymorphisms between individuals of European descent in the US versus in Italy, nor between East Asians in Japan versus in Taiwan (data not shown). Thus, we pooled the data from these two groups and labeled them as "individuals of European descent" and "East Asians," which were then used for further analyses.

Comparing individuals of European descent and East Asians, the frequency of the minor forms of the −216 polymorphism was significantly higher ($p$ < 0.001) in individuals of European descent (60.3%, 54.8%−65.8%) than in East Asians (6.6%, 3.9%−9.3%). This was also true for the minor forms of the −191 polymorphism (individuals of European descent, 37.0%, 31.6%−42.4%; East Asians, 0.6%, 0%−1.4%; $p$ < 0.001), as shown in Table 3. In addition, Table 4 shows that both alleles of CA-SSR1 (and the combined allele length) were significantly shorter in individuals of European descent than in East Asians ($p$ < 0.001). The comparisons were controlled for potential confounders such as gender, age, and smoking.

### Relationship between CA-SSR1 Allele Lengths and SNPs

We first examined the concordance of the SNP −216, SNP −191, and CA-SSR repeat polymorphisms. As shown in Table 5, individuals who were homo- or heterozygous for the variant forms of SNP −216 (G/T or T/T) had significantly lower mean CA-SSR repeat numbers in short, long, and combined allele lengths than those who were homozygous for the common form −216 G/G after adjustment for ethnicity. In similar comparisons for the variant forms of SNP −191, there was significant concordance with the longer and combined allele lengths, but not for the shorter allele.

We next investigated the relationship between the combined allele length and the SNPs for different ethnicities. For convenience, since the overall mean CA-SSR1 repeat number for shorter and longer allele combined was 36, we dichotomized the combined allele length as "longer" for those with greater than 36 repeats and as "shorter" for those with 36 repeats or fewer. As shown in Table 6, the frequency of the "shorter" combined allele was significantly higher in individuals with the minor forms of −216 (East Asians, 72.7%, 54.1%−91.3%; individuals of European descent, 81.2%, 76.1%−86.3%) than in those with the common form (East Asians, 36.6%, 30.9%−41.7%; individuals of European descent, 53.7%, 45.8%−61.6%). A similar pattern for SNP −191 was noted in East Asians but not in individuals of European descent. Also, for individuals carrying both variant genotypes of the two SNPs, the frequency of the "shorter" combined allele was observed to be higher than in those with the common forms of the SNPs in both individuals of European descent and East Asians, although the difference was statistically significant only in East Asians (Dataset S2).

### Relationship between EGFR Expression and the −216 Polymorphism

The polymorphism genotype of the 11 HBEC cultures was determined as previously described. The lines, derived from American individuals of European descent, showed little variation in the repeat length of the shorter CA-SSR1 allele (mean length 16.2, range 16−17). Similarly, for the −191 polymorphism, ten of the cases had the common C/C genotype and only one case demonstrated the C/A genotype. Thus, we were unable to study the effects of these two polymorphisms on gene expression in the HBEC cultures. However, for the −216 polymorphism, four of the cases had the common form, G/G, while the remaining seven cases expressed the variant forms G/T ($n$ = 5) or T/T ($n$ = 2). Thus, we limited our examination of the relationship of SNPs to EGFR expression to the −216 polymorphism (Figure 2B and 2C).

The standard curves for ACTB and EGFR mRNA expression were straight lines nearly parallel to each other (Figure 2A), permitting us to use the expression ratio of these two genes for comparisons. To further validate our assays, we determined the ratios for the HBECs as well as for eight NSCLC cell lines having the WT form and for seven cell lines having a mutant form of the EGFR gene. As expression in normal

1040
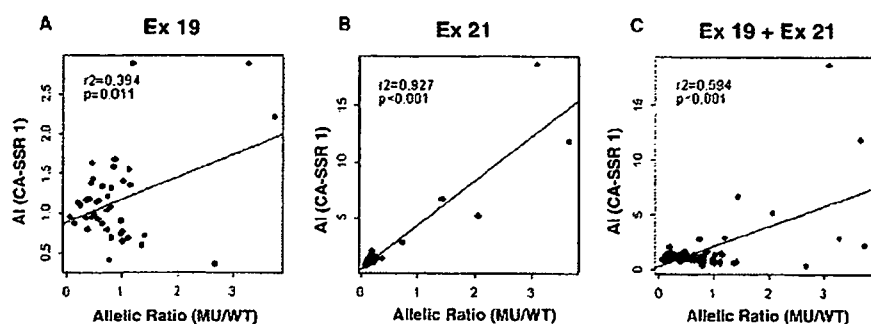
| | Mean (N) of AR (MU/WT) | | |
|---|---|---|---|
| mutant | With SAD | Without SAD | p-value* |
| Ex19 + | 1.24 (13) | 0.74 (28) | 0.050 |
| Ex21 + | 1.13 (11) | 0.13 (21) | <0.001 |
| Ex19 +/ Ex21 + | 1.19 (24) | 0.48 (49) | 0.001 |

* Wilcoxon Rank Sum test.

**Figure 3.** The Correlation between AI and Allelic Ratio

The correlation between allelic ratio of *CA-SSR1* (shorter allele/longer allele) and the allelic ratio (AR) of mutant (MU) to WT allele of (A) the exon 19 in-frame deletion ($r^2 = 0.394$, $p = 0.011$), (B) the exon 21 L858R point mutation ($r^2 = 0.927$, $p < 0.001$), or (C) both ($r^2 = 0.594$, $p < 0.001$) in the same mutant cases.
doi:10.1371/journal.pmed.0040125.g003

epithelial cells is low or not detectable in the absence of ligand, the HBECs were cultured in EGF-containing medium (5 ng/ml). Expression in the HBECs was relatively low, with a narrow range (Figure 2B). The lung cancer lines, grown in the absence of added ligand, showed considerable variability of expression. Four WT lines had low expression, while four lines, all having *EGFR* copy number of four or greater, had considerably higher expression levels. Four of the mutant lines, all highly amplified for copy number and lacking the secondary resistance-associated T790M mutation [27,28], had high expression ratios. However three mutant lines had low expression ratios. Two of these lines had the secondary T790M mutation as well as an activating mutation, while the third line had a relatively low copy number.

While the range of expression in the HBECs was modest, we correlated expression with the −216 genotype (Figure 2C). The four lines having the G/G phenotype had a mean expression ratio of 1.0 (range 0.5–1.3). The seven lines having one of the two variant forms had a mean expression ratio of 1.2 (range 1.0–1.7). The two lines homozygous for the variant form T/T were among the three highest expressing lines. While these differences were not significant, they may represent a trend towards higher expression being associated with the variant forms.

The range of relative expression of *EGFR* compared to *ACTB* of lung cancer cell lines was variable. The two high values were observed in the cell lines with *EGFR* mutation. The mean value of cell lines having the common SNP −216 G/ G ($n = 4$) was 0.97, compared to 1.24 for the lines with the minor forms SNP −216 G/T or T/T ($n = 7$) (Figure 2C). The range of the number of *CA-SSR1* repeats in the cell lines, all from individuals of European descent, was from 16 to 17 for the shorter allele, 16 to 19 for the longer allele, and 32 to 38 for the combined length. The highest value was observed in the group with the shortest combined number of *CA-SSR1* repeats (32) and one of the minor SNP −216 forms.

## The Relationship between Polymorphisms and Survival

We also investigated the relationship between the SNP −216, SNP −191, and *CA-SSR* repeat polymorphisms and patient overall survival (Figure S1). We did not observe a relationship between survival and either SNP form or any combination of SNP forms after adjusting for age, gender, ethnicity, smoking, and histology. For the shorter allele of *CA-SSR1* in the tumor cases, the mean length was 17.5. We divided the cases into those having shorter alleles, with mean lengths of 17 or fewer repeats, and those having a mean length of 18 or more repeats. We found that cases having a mean length of 18 or more repeats had improved survival compared to those having shorter allele lengths of 17 or fewer repeats ($p = 0.017$). These findings suggest that patients (in the absence of TKI therapy) whose tumor cells are predicted to make less *EGFR* protein have an improved survival compared to those whose cells are predicted to have higher intrinsic protein production. Similar data have been reported recently from another group [29]. For cases with AI of *CA-SSR1* (see below) or of the mutant allele, no differences in patient survival were noted (data not shown).

## AI of the *CA-SSR1* Alleles

The degree of amplification of each allele was reflected by the relative area under the peak (Figure 1), and the AI was determined by the ratio of shorter to longer *CA-SSR1* alleles in informative cases where two alleles were of different length. Among 450 tumor cases where the corresponding non-malignant lung tissues were available, there was no difference in the presence of homo- or heterozygosity of allele length or in the repeat length of each allele between tumor and non-malignant tissues (data not shown). These findings permitted us to analyze all 556 cases using the tumor tissues alone. For the *CA-SSR1* alleles, 376 (68%) of 556 cases were informative. The informative rate was similar to that in other previous studies [16,26]. However, in our study the

**Table 7.** Frequencies of AI of Either Allele of *CA-SSR1* by Ethnicity

| NSCLC Patients | AI | *p*-Value[a] | Mutant or WT *EGFR* Allele[b] | AI | *p*-Value[a] |
|---|---|---|---|---|---|
| All cases (*n* = 356) | 109/356 (30.6%) | | MT (*n* = 90) | 40/90 (44.4%) | 0.002 |
| | | | WT (*n* = 266) | 69/266 (25.9%) | |
| East Asians (*n* = 205) | 73/205 (35.6%) | 0.019 | MT (*n* = 73) | 34/73 (46.6%) | 0.022 |
| | | | WT (*n* = 132) | 39/132 (29.5%) | |
| Individuals of European descent (*n* = 151) | 36/151 (23.8%) | | MT (*n* = 17) | 6/17 (35.3%) | 0.24 |
| | | | WT (*n* = 134) | 30/134 (22.4%) | |

These analyses were limited to informative cases of East Asians from Japan or Taiwan and individuals of European descent from the US and Australia.
[a]Chi-square test with continuity adjustment.
[b]Mutant (MT) *EGFR* alleles are limited to exon 19 deletions and exon 21 L858R.
doi:10.1371/journal.pmed.0040125.t007

informative rate was not consistent across ethnicities: there was an informative rate of 62.8 % (211/336) in East Asians and 75.0% (165/220) in other ethnicities. Of the 376 informative cases, we excluded cases with mutations other than deletions in exon 19 or the L858R mutation in exon 21 (*n* = 12) as well as patients of ethnicities other than East Asians and individuals of European descent (*n* = 5) and Asians in the US (*n* = 3). Of the remaining 356 NSCLC cases of East Asian or European descent, 263 had the WT *EGFR* gene and 95 had the mutations in exon 19 or exon 21.

For these 356 cases, we determined the ratios of the *CA-SSR1* alleles as previously described in the Methods section. AI, defined by an allelic ratio greater than 1.27 or less than 0.79, was present in 109 (30.6 %) of the cases but was significantly more frequent (*p* = 0.002) in cases with mutant tumors (44.4%, 34.1%–54.7%) than in those with WT tumors (25.9%, 20.6%–31.2%), and in East Asians (35.6%, 29.0%–42.2%) than in individuals of European descent (23.8%, 17.0%–30.6%) (*p* = 0.019) (Table 7; Dataset S3).

The 109 cases with AI were also divided into SAD or LAD. As shown in Table 8 (and Dataset S3), the overall frequency of SAD was 60.3% (49.1%–71.5%) in East Asians and 44.4% (28.2%–60.6%) in individuals of European descent. Also, in East Asians the SAD frequency was significantly higher (*p* = 0.001) in tumors with the exon 19 or exon 21 mutation than in those without mutations (82.4%, 69.6%–95.2%, versus 41.0%, 25.6%–56.4%). This difference, however, was not observed in patients of European descent.

## AI of Mutant to WT Allele

For cases with the deletions in exon 19 or the L858R mutation in exon 21, the AI of the mutant allele was determined by the mutant/WT allele ratio. A flow chart describing the process of case selection and exclusion is presented in Figure 4. These mutant cases gave us an opportunity to examine the association between AI in amplification of *CA-SSR1* repeats and AI in the ratio of mutant to WT alleles. Specifically, we wished to determine, in cases having both forms of AI, whether the mutant form was selectively amplified in association with selective amplification of the shorter allele of *CA-SSR1*. As described in the Methods section, we devised methods to determine the ratios of mutant to WT alleles for the two most frequent mutations, deletions in exon 19 and the L858R mutation in exon 21, which together account for ~85% of *EGFR* mutations in NSCLC [9]. Of the 109 cases with mutations (in exon 19 or L858R), sufficient DNA was available from 76. Of these 76 samples, 32 (42.1%) tumors had selective imbalance involving the mutant allele. The ratio of *CA-SSR1* alleles was utilized to determine whether AI was present and, if present, which of the two alleles was preferentially overrepresented. Of these 32 samples having AI of the mutant allele, 26 (81.3%) also had AI of *CA-SSR1*. In addition, a positive correlation between AI ratios of *CA-SSR1* and mutant/WT ratios was observed in tumors having either form of mutation (Figure 3). The linear correlation was tested using Pearson's correlation and found to be significant. However, because of the possibility that the observed strong correlation might be driven by extreme

**Table 8.** Frequencies of AI of *CA-SSR1* by Ethnicity

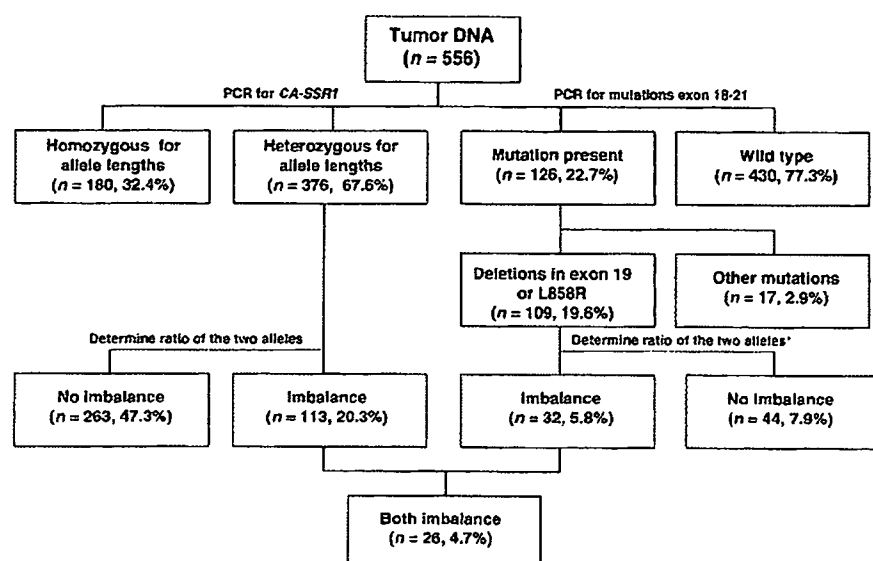| Cases with AI of *CA-SSR1* | SAD[a] | *p*-Value[b] | Mutant or WT *EGFR* Allele[c] | SAD[a] | *p*-Value[b] |
|---|---|---|---|---|---|
| All cases (*n* = 109) | 60/109 (55.0%) | | MT (*n* = 40) | 30/40 (75.0%) | 0.003 |
| | | | WT (*n* = 69) | 30/69 (43.5%) | |
| East Asians (*n* = 73) | 44/73 (60.3%) | 0.214 | MT (*n* = 34) | 28/34 (82.4%) | 0.001 |
| | | | WT (*n* = 39) | 16/39 (41.0%) | |
| Individuals of European descent (*n* = 36) | 16/36 (44.4%) | | MT (*n* = 6) | 2/6 (33.3%) | 0.672 |
| | | | WT (*n* = 30) | 14/30 (46.7%) | |

These analyses were limited to informative cases of East Asians from Japan or Taiwan and individuals of European descent from the US and Australia.
[a]Number of SAD cases (analyses limited to cases with AI).
[b]Chi-square test with continuity adjustment.
[c]Mutant (MT) *EGFR* alleles are limited to exon 19 deletions and exon 21 L858R.
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**Figure 4.** Flow Chart for Examination of the Relationship between AIs of *CA-SSR1* Length and *EGFR* Mutations
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values given the small sample size of the available cases, we used a nonparametric test instead to compare mutant/WT ratios between those with SAD and those without. As expected, for all the mutations under study, the cases with SAD had higher mean mutant/WT ratios than those without SAD. These findings agreed with our hypothesis that in cases demonstrating *CA-SSR1* imbalance, the mutant allele was more frequently increased in relative copy number compared to the WT allele.

## Discussion

In this report we examined the frequency of three germline polymorphisms in the *EGFR* gene in healthy individuals of different ethnicities, and in non-malignant and malignant lung tissue from patients with NSCLC. We found ethnic-related differences in polymorphism frequencies consistent with previous reports, indicating that the shorter allele of *CA-SSR1* and the minor forms of SNPs −191 (C/A or A/A) and −216 (G/T or T/T) are significantly less frequent in East Asians than in individuals of European descent [21]. In addition, we noted a relationship between the presence of the short form of *CA-SSR1* and the minor forms of the SNPs. The published data [13,19,21,26] and our observations regarding *EGFR* mRNA expression in HBECs suggest that the shorter *CA-SSR1* allele lengths and the variant forms of the −191 and −216 polymorphisms are associated with increased intrinsic gene expression. However, most of the data in the literature are from the results of transfection studies or tumor cell lines, and thus may not reflect the state of normal epithelial cells. As sections of non-malignant lung contain only a small minority of epithelial cells, a study of adjacent non-malignant lung tissues from resected cases or peripheral blood cells would not yield meaningful data. In an attempt to overcome these limitations, we studied 11 cultures of immortalized HBECs. These cultures show minimal genetic changes. In the

presence of ligand stimulation, we demonstrated a trend for increased mRNA expression in lines having the SNP −216 G/T or T/T genotypes, consistent with published data. The published reports and our results are consistent with the hypothesis that cells of individuals of East Asian ethnicity express less *EGFR* protein constitutively than cells of individuals of other ethnicities. However, final experimental proof for this hypothesis is still lacking.

Amplification of the *EGFR* gene is relatively common in lung and other cancers, and may be associated with mutations of the TK domain in lung cancers [12] or of the extracellular domain in glioblastomas [30]. Two recent reports describe a correlation between copy numbers of the *EGFR* gene as measured by fluorescence in situ hybridization (FISH) and response to TKIs [11,31]. In this study we used allelic size differences in the *CA-SSR1* repeat polymorphism to determine AI of the gene. AI was observed in 30.2% of informative cases, a frequency comparable to increased copy number as detected by FISH analyses [32]. AI was significantly more frequent in East Asians and occurred nearly twice as frequently in mutant cases than in WT cases. A relationship between increased copy number by FISH analysis and mutation has also been described previously [12]. While there were no significant differences in the frequencies of either the shorter or longer allele being involved in the imbalance for all of the cases or for all of the mutant cases, in mutant cases arising in East Asians, the shorter allele was twice as likely to be preferentially amplified as the longer allele.

Finally we determined whether the mutant allele was selectively amplified in tumors having both mutation and imbalance. For tumors having deletion mutations in exon 19 or the L858R point mutation in exon 21 (together accounting for 86.5% of all mutations) we devised methods for determining the ratio of mutant to WT alleles. Of 76 cases examined, 42.1% demonstrated imbalance of the mutant allele. This figure is consistent with our finding of an overall
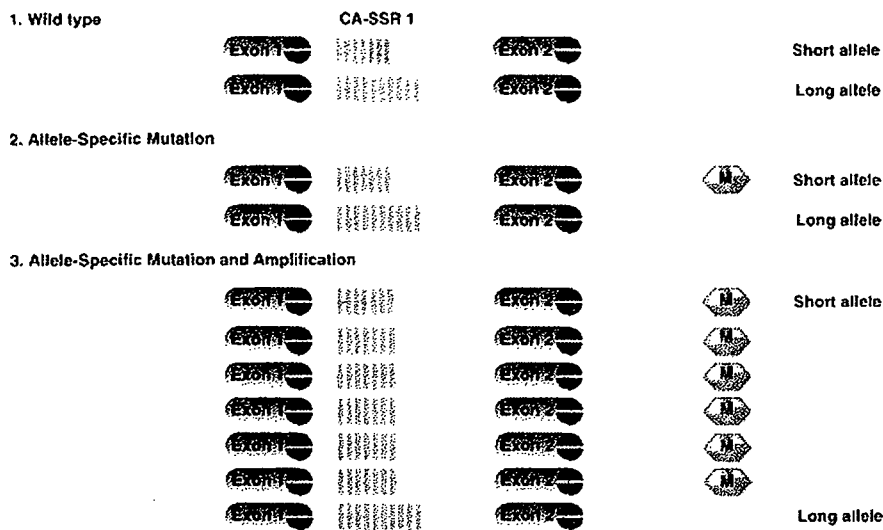
**Figure 5.** Hypothesized Allele-Specific Mutation and Amplification of *EGFR* in Lung Cancers
We hypothesized that CA-SSR1 polymorphism occurs, mutations (M) target the EGFR allele with the shorter CA-SSR1 repeat number, and then there is allele-specific amplification. These three events, targeting the same allele, would be predicted to result in greater protein production than random allelic occurrence.
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AI (from analysis of the *CA-SSR1* alleles) percentage of 45.3% in mutant cases, and suggests that in mutation-containing tumors having AI, the mutant allele is the one that is usually amplified. Having found, by separate analyses in mutant cases, that both the shorter *CA-SSR1* allele and the mutant allele were selectively amplified, we performed a correlation of these two forms of imbalance and demonstrated a strong positive association.

Incorporation of our findings and previously published data form the basis of a hypothesis suggesting a close relationship between *CA-SSR1* length, SNP −191 polymorphism, and SNP −216 polymorphism and *EGFR* gene amplification. As mentioned above, all three of these polymorphisms (shorter *CA-SSR1* length and the variant forms of the two SNPs) are reported to be associated with increased *EGFR* production, and they were rarely observed in East Asians. These findings suggest that the cells of most East Asians make less *EGFR* protein than do the cells of individuals of other ethnicities. If a certain critical level of *EGFR* is required to drive the cell toward a malignant phenotype, mutations of the TK domain and autonomous activation of downstream signaling may target East Asians, the subgroup with possibly lower intrinsic protein production. Also, we found in East Asians (but not in individuals of European descent) that mutations target the shorter *CA-SSR1* allele (suggestive of greater protein production) followed by allele-specific amplification of the mutant allele. As illustrated in Figure 5, three events target the same allele: (a) shorter *CA-SSR1* repeat length, (b) activating mutation, and (c) selective amplification of the mutant allele. These interactions favor greater protein production in mutant tumors. A similar observation was made in glioblastomas, which frequently contain a mutation or splicing variant resulting in loss of much of the extracellular domain of *EGFR*. The variant form of the allele frequently demonstrated allele-specific amplification [33]. As previously mentioned, FISH technology has

been used to demonstrate that *EGFR* amplification and mutation often, but not invariably, occur together [12].

## Conclusions

The three polymorphisms associated with increased EGFR protein production (shorter *CA-SSR1* length and the variant forms of SNPs −216 and −191) were found to be rare in East Asians as compared to individuals of other ethnicities, suggesting that the cells of East Asians may make relatively less intrinsic EGFR protein. Interestingly, especially in tumors from patients of East Asian ethnicity, *EGFR* mutations were found to favor the shorter allele of *CA-SSR1*, and selective amplification of the shorter allele of *CA-SSR1* occurred frequently in tumors harboring a mutation. These distinct molecular events targeting the same allele would both be predicted to result in greater EGFR protein production and/ or activity. These findings may reveal what underlies some of the ethnic differences observed in mutational frequencies and responses to TKIs.

## Supporting Information

## Acknowledgments

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### Editors' Summary

**Background.** Most cases of lung cancer—the leading cause of cancer deaths worldwide—are "non-small cell lung cancer" (NSCLC), which has a very low cure rate. Recently, however, "targeted" therapies have brought new hope to patients with NSCLC. Like all cancers, NSCLC occurs when cells begin to divide uncontrollably because of changes (mutations) in their genetic material. Chemotherapy drugs treat cancer by killing these rapidly dividing cells but, because some normal tissues are sensitive to these agents, it is hard to kill the cancer completely without causing serious side effects. Targeted therapies specifically attack the changes in cancer cells that allow them to divide uncontrollably so it might be possible to kill the cancer cells selectively without damaging normal tissues. Epidermal growth factor receptor (EGFR) was one of the first molecules for which a targeted therapy was developed. In normal cells, messenger proteins bind to EGFR and activate its "tyrosine kinase," an enzyme that sticks phosphate groups on tyrosine (an amino acid) in other proteins. These proteins then tell the cell to divide. Alterations to this signaling system drive the uncontrolled growth of some cancers, including NSCLC.

**Why Was This Study Done?** Molecules that inhibit the tyrosine kinase activity of EGFR (for example, gefitinib) dramatically shrink some NSCLCs, particularly those in East Asian patients. Tumors shrunk by tyrosine kinase inhibitors (TKIs) often (but not always) have mutations in EGFR's tyrosine kinase. However, not all tumors with these mutations respond to TKIs, and other genetic changes—for example, amplification (multiple copies) of the EGFR gene—also affect tumor responses to TKIs. It would be useful to know which genetic changes predict these responses when planning treatments for NSCLC and to understand why the frequency of these changes varies between ethnic groups. In this study, the researchers have examined three polymorphisms—differences in DNA sequences that occur between individuals—in the EGFR gene in people with and without NSCLC. In addition, they have looked for associations between these polymorphisms, which are present in every cell of the body, and the EGFR gene mutations and allelic imbalances (genes occur in pairs but amplification or loss of one copy of allele often causes allelic imbalance in tumors) that occur in NSCLCs.

**What Did the Researchers Do and Find?** The researchers measured how often three EGFR polymorphisms (the length of a repeat sequence called CA-SSR1 and two single nucleotide variations [SNPs]—all of which probably affect how much protein is made from the EGFR gene—occurred in normal tissue and NSCLC tissue from East Asians and

individuals of European descent. They also looked for mutations in the EGFR tyrosine kinase and allelic imbalance in the tumors, and then determined which genetic variations and alterations tended to occur together in people with the same ethnicity. Among many associations, the researchers found that shorter alleles of CA-SSR1 and the minor forms of the two SNPs occurred less often in East Asians than in individuals of European descent. They also confirmed that EGFR kinase mutations were more common in NSCLCs in East Asians than in European-descent individuals. Furthermore, mutations occurred more often in tumors with allelic imbalance, and in tumors where there was allelic imbalance and an EGFR mutation, the mutant allele was amplified more often than the wild-type allele.

**What Do These Findings Mean?** The researchers use these associations between gene variants and tumor-associated alterations to propose a model to explain the ethnic differences in mutational frequencies and responses to TKIs seen in NSCLC. They suggest that because of the polymorphisms in the EGFR gene commonly seen in East Asians, people from this ethnic group make less EGFR protein than people from other ethnic groups. This would explain why, if a threshold level of EGFR is needed to drive cells towards malignancy, East Asians have a high frequency of amplified EGFR tyrosine kinase mutations in their tumors—mutation followed by amplification would be needed to activate EGFR signaling. This model, though speculative, helps to explain some clinical findings, such as the frequency of EGFR mutations and of TKI sensitivity in NSCLCs in East Asians. Further studies of this type in different ethnic groups and in different tumors, as well as with other genes for which targeted therapies are available, should help oncologists provide personalized cancer therapies for their patients.

**Additional Information.** Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0040125.

- US National Cancer Institute information on lung cancer and on cancer treatment for patients and professionals
- MedlinePlus encyclopedia entries on NSCLC
- Cancer Research UK information for patients about all aspects of lung cancer, including treatment with TKIs
- Wikipedia pages on lung cancer, EGFR, and gefitinib (note that Wikipedia is a free online encyclopedia that anyone can edit)

# Predictive factors associated with prolonged survival in patients with advanced non-small-cell lung cancer (NSCLC) treated with gefitinib

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This study aimed to identify predictive factors associated with prognostic benefits of gefitinib. A total of 221 Japanese patients who received gefitinib (250 mg day$^{-1}$) were examined retrospectively and potential predictive factors analysed. Overall response rate (ORR) was 24.4% and median survival time (MST) was 8.0 months. In a log-rank test, survival was significantly better in females, patients with adenocarcinoma, never-smokers, favourable performance status (PS) and patients with epidermal growth factor receptor (EGFR) mutation. The lower the smoking exposure (Brinkman Index (BI) = cigarettes per day × years smoked), the better the MST (BI 0: 14.5 months, BI <500: 9.5 months, BI 500 to <1000: 6.9 months, BI ≥1000: 4.0 months). Positive-EGFR mutation status and PS 0–1 were independent predictors of favourable prognosis by multivariate analysis. Prognosis was significantly different according to EGFR mutation status (with the same smoking status), but not according to smoking status (with the same EGFR mutation status). EGFR mutation status is the most important independent predictor of survival benefit with gefitinib treatment. Although differences in prognosis were observed according to relative smoking status and smoking exposure, the results suggested that smoking is not a direct predictor of prognosis, yet is a surrogate marker of EGFR mutation status.
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**Keywords:** epidermal growth factor receptor (EGFR) inhibitor; EGFR mutations; gefitinib; IRESSA; non-small-cell lung cancer; smoking

Gefitinib (IRESSA) is an orally active small-molecule compound that inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) by competing with adenosine triphosphate (ATP) at the ATP-binding site. In two large Phase II trials (IDEAL: IRESSA Dose Evaluation in Advanced Lung cancer 1 and 2) gefitinib-induced tumour regression and provided symptom relief in previously treated patients with non-small-cell lung cancer (NSCLC) (Fukuoka et al, 2003; Kris et al, 2003). Although a placebo-controlled Phase III study (ISEL) in previously-treated patients with NSCLC has not shown a statistically significant improvement in survival associated with gefitinib, preplanned subgroup analysis suggested survival benefits in patients of Asian origin and never-smokers (Thatcher et al, 2005). Patient selection criteria were not incorporated in this comparative study, which most likely contributed to the absence of a positive survival benefit in the overall population. In fact, a Phase II study in which gefitinib was used as first-line therapy for NSCLC in a subgroup of never-smokers with adenocarcinoma reported favourable outcomes, with an overall response rate (ORR) of 61% (Lee et al, 2005).

In 2004, mutations in the EGFR gene conferring increased sensitivity to gefitinib were reported (Lynch et al, 2004; Paez et al, 2004). Recently, very favourable outcomes (response rate (RR) 75%) in a Phase II study of gefitinib as first-line therapy for patients with NSCLC with EGFR gene mutations has been reported (Inoue et al, 2006).

Therefore, it is important to conduct patient selection before using gefitinib and, in particular, it is vital to identify the predictive factors that may contribute to survival. To aid future selection of patient groups for gefitinib treatment we conducted a retrospective analysis of patients who had been treated with gefitinib, assessing the relationship between clinical characteristics, the EGFR mutation status, antitumour activity and patient survival.

## PATIENTS AND METHODS

### Patients

A total of 221 patients who had been initiated on gefitinib monotherapy (250 mg day$^{-1}$) during a 3-year span from July 2002 (gefitinib was launched in Japan) to June 2005 at the Hyogo Medical Center for Adults in Japan were retrospectively examined.

## Clinical assessments

Clinical parameters studied were gender, age, smoking history (Brinkman Index (BI) = number of cigarettes per day × number of years smoked), Eastern Cooperative Oncology Group performance status (PS) and previous lines of chemotherapy.

Assessment of tumour regression was conducted according to the response evaluation criteria in solid tumours (RECIST) guideline. The National Cancer Institute Common Toxicity Criteria, version 3.0, was used to evaluate toxicity.

## EGFR gene analysis

EGFR gene mutation detection was performed on samples from 106 patients: surgical specimens were obtained from 34 patients and a transbronchial lung biopsy (TBLB) was performed on 72 patients. EGFR mutation analysis was successfully performed in 91 of the 106 samples. EGFR mutation was analysed at Aichi Cancer Center Hospital in Japan. A cycleave PCR technique for codon 858 of EGFR gene was used on a SmartCycler system (SC-100, Cepheid, Sunnyvale, CA, USA). Deletion in exon 19 of the EGFR gene was detected with fragment analysis using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) (Yatabe et al, 2006). Many of the cases began treatment on gefitinib before it had been reported that EGFR mutation detection was important when treating with the drug. Moreover, many of those cases had already died before our plans to undergo EGFR mutation detection, effectively preventing us from obtaining informed consent in this regard. Accordingly, our Institutional Review Board approved our study plan, provided that samples would be processed anonymously, that samples would be analysed only for somatic mutations and not germline mutations, and that the presence of the study be publicly disclosed, strictly according to the 'Ethical Guidelines for Human Genome Research' published by The Ministry of Education, Culture, Sports, Science and Technology, The Ministry of Health, Labour and Welfare and The Ministry of Economy, Trade and Industry, Japan. (http://www.mext.go.jp/a_menu/shinkou/seimei/genome/04122801.htm).

## Statistical analysis

OS analysis was conducted on all 221 and 91 patients in which EGFR mutation analysis could be successfully performed.

The differences in responders (complete response; CR + partial response; PR) by each factor (gender, PS, histology, prior chemotherapy, smoking status and mutation status) were examined with the Fisher's exact test. The difference in mutation rate among groups categorised by smoking exposure was examined with $\chi^2$ test.

An OS curve was plotted using the Kaplan–Meier method and survival curve comparisons were conducted with the log-rank test. Univariate analysis and multivariate analysis of the impact of the factors, including gender (male vs female), smoking history (ever-smokers vs never-smokers), histology (adenocarcinoma vs others), PS (PS 0–1 vs 2–4) and EGFR mutation (positive vs negative) were conducted using the Cox regression model. All analysis was determined to be statistically significant where the P-value was < 0.05.

Analyses were conducted using the SPSS 11.0.1.

## RESULTS

### Patient characteristics

The clinical characteristics of the patients are shown in Table 1. The majority of patients (89%) had adenocarcinoma histology. One hundred and thirty-one patients (59%) were ever-smokers.

## EGFR mutation analysis and clinical response

TBLB or surgical samples were available from 106 patients for EGFR-mutation detection, but actual analysis was only possible for 103 patients because tumour cells were not found in three post-treatment specimens.

DNA could not be amplified in 12 cases. Analysis of the remaining 91 samples showed EGFR mutations in 28 patients (30.8%) and wild type in 63 patients (69.2%). EGFR mutation rate was high in females, never-smokers and patients with adenocarcinoma. Among ever-smokers, EGFR mutation rate was higher in patients with BI < 500 and BI 500 to 1000 than BI > 1000 (Table 2). Of the 28 EGFR mutations, 19 (67.9%) were exon 19 in-frame deletions and nine (32.1%) were exon 21-point mutations (L858R). Seven (36.8%) of the exon 19 deletions and four (44.4%) of the L858R cases were smokers. Significantly high mutation rates were observed in females and never-smokers.

In the overall population, RR was 24.4% (95% confidence interval (CI) 18.0–30.6%) (Table 1). Response rate was significantly higher among females, patients with adenocarcinoma histology, never-smokers and patients with the EGFR mutation. Disease control rate (DCR: CR + PR + stable disease; SD) was 51.1% (95% CI 44.3–57.9%) and among those with EGFR mutation, 100%.

## Survival analyses

Median survival time (MST) in the overall population was 8 months, with 34.8% surviving 1 year. MST among patients showing PR was significantly longer than that in the SD cases (P = 0.003) and MST of the SD cases was also shown to be significantly longer than that of the PD cases (P < 0.0001) (Table 1).

Kaplan–Meier curves indicated significantly longer survival in patients with favourable PS (P < 0.0001), in patients with adenocarcinoma histology (P < 0.0001), in never-smokers (P < 0.0001), and in patients with EGFR mutations (P < 0.0001) (Table 1, Figure 1). L858R patients tend to survive longer than those with deletions at exon 19 (P = 0.0539). Multivariate analysis was conducted to identify factors contributing to survival. When all patients were analysed considering of the clinical characteristics (gender, adenocarcinoma histology, smoking status and PS), adenocarcinoma, never-smoker status and PS 0–1 were found to be prognostic factors of survival (Table 3a). However, analysis (including that on EGFR mutation status and clinical characteristics) of the patients for whom EGFR mutation results were obtained showed PS 0–1 and EGFR gene mutation status to be the independent prognostic factors, and the relationship between smoking status and survival did not reach statistical significance (Table 3b).

Further analysis of smoking exposure and survival indicated that the higher the exposure, the shorter the MST (Figure 2). The presence of EGFR mutation was associated with significantly prolonged survival in both never-smokers (P = 0.014) and ever-smokers (P = 0.012). Furthermore, among EGFR mutation-positive patients, there was no statistically significant difference in median survival between never-smokers and ever-smokers (P = 0.864), although patient numbers were small (Figure 3).

## Tolerability

Adverse events were observed in 165 out of 221 (75%) patients. Common adverse events were rash/dry skin (51%), diarrhoea (22%), liver dysfunctions (20% (2.3% were Grade 3)) and paronychia (14%). Sixteen (7%) of the patients developed interstitial lung disease (ILD) and three (1.4%) died. As three out of 14 (21%) patients with PS 3 developed ILD, patients with poorer PS were more likely to develop ILD. There were no differences in ILD incidence by gender, smoking history, age or

**Table I** Demographics and relationship between clinical variables and antitumor response/overall survival in patients treated with gefitinib

| Characteristic | No. of patients (%) | PR (n) | RR (%) | (95% CI) | P-value[a] | MST (months) | (95% CI) | P-value |
|---|---|---|---|---|---|---|---|---|
| All | 221 | 54 | 24.4 | (18.9–30.6) | | 8 | (6.66–9.34) | |
| *Gender* | | | | | | | | |
| Male | 142 (64) | 20 | 14.1 | (8.8–20.9) | <0.001 | 6.8 | (5.04–8.56) | 0.036 |
| Female | 79 (36) | 34 | 43 | (31.9–54.7) | | 13.3 | (8.84–17.76) | |
| *Age* | | | | | | | | |
| 65< | 100 | 20 | 20 | (12.7–29.2) | 0.208 | 9 | (6.41–11.59) | 0.2852 |
| <65 | 121 | 34 | 28.1 | (20.3–37.0) | | 7.3 | (5.88–8.72) | |
| *ECOG PS* | | | | | | | | |
| 0–1 | 160 (72) | 44 | 27.5 | (20.7–35.1) | 0.114 | 11.1 | (8.30–13.90) | <0.001 |
| 2–4 | 61 (28) | 10 | 16.4 | (8.2–28.1) | | 2.1 | (1.26–2.94) | |
| *Histology* | | | | | | | | |
| Adenocarcinoma | 196 (89) | 52 | 26.5 | (20.5–33.3) | 0.048 | 9.3 | (7.66–10.94) | 0.137 |
| Others | 25 (9) | 2 | 8 | (1.0–26.0) | | 3.6 | (2.13–5.07) | |
| *Prior chemotherapy* | | | | | | | | |
| Yes | 188 (85) | 45 | 24.6 | (18.5–43.3) | 1 | 8.1 | (6.67–9.53) | 1 |
| No | 33 (15) | 9 | 25.7 | (12.5–43.3) | | 8.4 | (5.72–11.08) | |
| *Smoking history (n = 220)* | | | | | | | | |
| No | 89 (40) | 37 | 41.6 | (31.2–52.5) | <0.001 | 14.5 | (10.87–18.13) | <0.001 |
| Yes | 131 (15) | 17 | 13 | (7.7–20.0) | | 6.5 | (4.36–8.64) | |
| BI I <500 | 25 (11) | 5 | 20 | (6.8–40.7) | | 9.5 | (6.41–12.59) | |
| BI 500 to <1000 | 59 (27) | 9 | 15.3 | (7.2–27.0) | | 6.9 | (5.64–8.16) | |
| BI≥1000 | 45 (21) | 2 | 4.4 | (0.5–15.1) | | 4 | (3.00–5.00) | |
| *EGFR gene status (n = 91)* | | | | | | | | |
| Wild type | 63 (69) | 7 | 11.1 | (4.6–21.6) | <0.001 | 7.4 | (4.84–9.96) | <0.001 |
| Mutation positive | 28 (31) | 20 | 71.4 | (51.3–86.8) | | 24.9 | (14.27–35.53) | |
| Exon 19 deletion | 19 (21) | 15 | 78.9 | (54.4–93.9) | | 16.1 | (6.22–25.98) | |
| Exon 21 (L858R) | 9 (10) | 5 | 55.6 | (21.2–86.3) | | >34.5 | — | |
| *Tumor response (n = 191)* | | | | | | | | |
| PR | 54 | .... | ... | | | 26.2 | (15.76–36.64) | 0.003 |
| SD | 59 | — | — | | | 11.9 | (7.47–16.33) | <0.0001 |
| PD | 78 | — | — | | | 5.6 | (3.20–8.00) | |

Abbreviations: BI = Brinkman Index: BI = defined as number of cigarettes per day × number of years smoking: CI = confidence interval; EGFR = epidermal growth factor receptor; ECOG PS = Eastern Cooperative Oncology Group performance status; MST = median survival time; PD = progressive disease; PR = partial response; RR = response rate; SD = stable disease.[a]Fisher's exact test.

**Table 2** Mutation rate by patient background

| Population | N | Mutation (%) | 95%CI | P-value[a] |
|---|---|---|---|---|
| All samples | 91 | 28 (30.8) | | |
| Male | 59 | 12 (20.3) | 11.0–32.8 | 0.005 |
| Female | 32 | 16 (50.0) | 31.9–68.1 | |
| Never-smoker | 38 | 17 (44.7) | 28.6–61.7 | 0.014 |
| Ever-smoker | 53 | 11 (20.8) | 10.8–34.1 | |
| Adeno | 81 | 27 (33.3) | 23.2–44.7 | 0.166 |
| Non-adeno | 10 | 1 (10.0) | 0.3–44.5 | |
| *Brinkman Index* | | | | |
| 0 | 38 | 17 (44.7) | 28.6–61.7 | 0.055[b] |
| 1≪500 | 9 | 2 (22.2) | 2.8–60.0 | |
| 500≪1000 | 25 | 7 (28.0) | 12.1–49.4 | |
| 1000< | 18 | 2 (11.1) | 1.4–34.7 | |

[a]Fisher's exact test (two-sided). [b]$\chi^2$-test (likelihood ratio).

histology. ILD was experienced by four out of 63 patients with wild type and two out of 28 patients with EGFR mutation (both with an exon 19 deletion).

## DISCUSSION

The data from this retrospective study suggest that in a practical setting

(1) A favourable PS, adenocarcinoma histology, never-smoking and presence of an EGFR mutation are predictive of increased antitumour activity with gefitinib,

(2) Although PR cases showed longer median survival than SD cases, SD cases also displayed significantly longer median survival than PD cases,

(3) Although, in terms of clinical characteristics, PS 0–1, adenocarcinoma histology and never-smoking status are predictive factors of survival with gefitinib in the overall population, PS 0–1 and EGFR mutation status were identified as independent predictive factors in patients in which EGFR mutation status has been detected,

(4) The relationships between smoking/EGFR mutation status and survival suggest that the latter is more related to prognosis. Conceivably, smoking has a strong confounding relationship with EGFR mutation status and smoking exposure can result in a different prognosis.

IDEAL 1 reported favourable antitumour activity in females and patients with adenocarcinoma histology (Fukuoka et al, 2003).
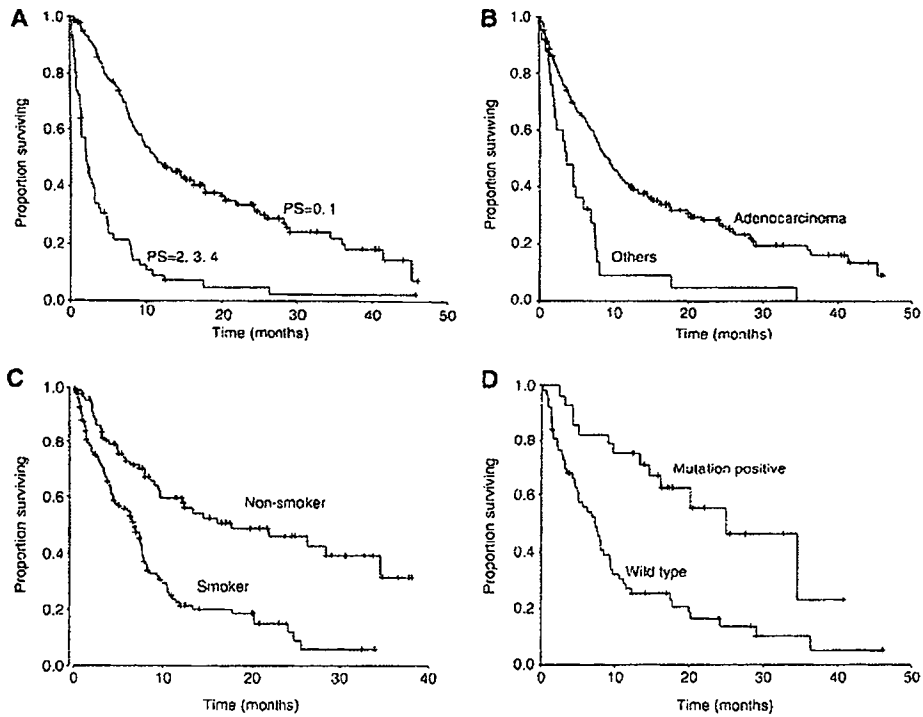
**Figure 1** Kaplan–Meier plots of survival for patients receiving gefitinib treatment classified according to (A) PS, (B) histology, (C) smoking status and (D) EGFR gene mutation status.

**Table 3a** COX Proportional Hazard Model for Survival Analysis in Overall Population (N = 221)

| Variable | HR | 95%CI | P-value |
|---|---|---|---|
| Never-smoker/Ever-smoker | 0.413 | 0.294–0.582 | <0.001 |
| Adeno/Non-adeno | 0.416 | 0.265–0.654 | <0.001 |
| PS 0, 1/2–4 | 0.205 | 0.145–0.291 | <0.001 |

Stepwise method (include <0.05, exclude >0.2). Tested variables: gender, smoking, histology, PS, excluded variable: gender.

**Table 3b** COX proportional hazard model for survival analysis in patients in which EGFR mutation status has been detected (n = 91)

| Variable | HR | 95%CI | P-value |
|---|---|---|---|
| Adeno/Non-adeno | 0.581 | 0.288–1.171 | 0.129 |
| Never-smoker /ever-smoker | 0.607 | 0.351–1.048 | 0.073 |
| Mutation negative/positive | 2.543 | 1.345–4.808 | 0.004 |
| PS 0, 1/2–4 | 0.166 | 0.091–0.303 | <0.001 |

Tested variables: gender, smoking, histology, PS, mutation excluded variable: gender.



| Brinkman index | N | MST | 95% CI |
|---|---|---|---|
| None | 89 | 14.5 | 10.87 – 18.13 |
| 1 ≤ BI < 500 | 25 | 9.5 | 6.41 – 12.59 |
| 500 ≤ BI < 1000 | 59 | 6.9 | 5.64 – 8.16 |
| 1000 ≤ BI | 45 | 4.0 | 3.00 – 5.00 |

**Figure 2** Survival stratified by smoking exposure (classified by BI).

Several subsequent retrospective studies have reported that female gender, adenocarcinoma histology, bronchioloalveolar subtype, never-smokers and patients with favourable PS are predictive factors of response (Miller et al, 2004; Kim et al, 2005; Lim et al, 2005; Ando et al, 2006). EGFR mutation has been reported as a predictor of efficacy of gefitinib and erlotinib (Lynch et al, 2004; Pao et al, 2004). There have been several reports of clinical factors associated with EGFR mutations, and per the univariate analysis, mutation frequency is high in patients of East Asian ethnicity, females, never-smokers and adenocarcinomas (Kosaka et al, 2004;

Paez et al, 2004; Shigematsu et al, 2005; Tokumo et al, 2005). Moreover, multivariate analysis has shown that adenocarcinoma histology and never-smoker status are independent factors associated with EGFR mutation (Kosaka et al, 2004; Tokumo et al, 2005). Reports to date have shown that approximately 90% of EGFR mutations are centred around the L858R point mutation in exon 21 and deletions centred around codons 746–750 in exon 19 (Kosaka et al, 2004; Shigematsu et al, 2005; Sonobe et al, 2005; Tokumo et al, 2005). As association between these two types of EGFR mutations and the antitumour activity and prolonged survival with gefitinib has been reported (Han et al, 2005; Mitsudomi et al, 2005), we conducted analysis on these two types of mutations only. Our results were compatible with those from

**Figure 3** Survival stratified by smoking status and EGFR gene mutation status.

| | Mutation positive | | Mutation negative | | Log-rank |
|---|---|---|---|---|---|
| Smoking (MST) | N | MST | N | MST | |
| Never smoker | 17 | 34.5 | 21 | 8.0 | 0.0141 |
| Ever smoker | 11 | 24.9 | 42 | 7.3 | 0.0118 |
| Log-rank | 0.8638 | | 0.3412 | | |

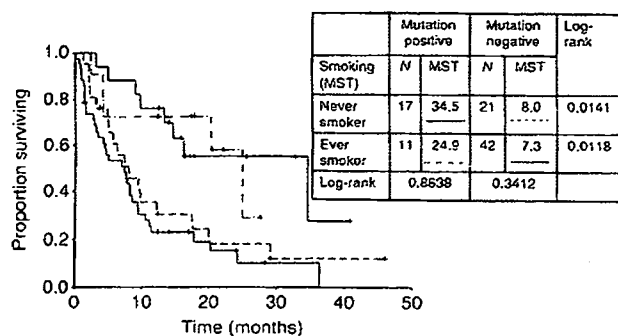prospective Phase II studies conducted in patients with EGFR mutation (Inoue et al, 2006; Okamoto et al, 2006). Epidermal growth factor receptor mutation therefore appears to be a more specific criterion for gefitinib use than patient selection according to clinical characteristics.

The relatively high incidence of ILD (3.5-5.8%) in patients treated with gefitinib has been reported in Japan. It also revealed that male gender, ever-smokers, poor PS and the coincidence of interstitial pneumonia were predictive factors for the development of ILD (Yoshida, 2005; Ando et al, 2006). Although these predictive factors contrast with those for the presence of an EGFR mutation, two of the 28 patients with an EGFR mutation developed ILD in our study.

In our examination of prognostic factors, we analysed the relationship particularly between smoking status (ever-/never-smoker, smoking exposure) and two types of EGFR mutations, as well as the relationship between smoking status and EGFR mutation. Our findings indicated that patients with EGFR mutation had significantly longer MST in both ever- and never-smokers, and there was no significant difference in MST between ever- and never-smokers with the same mutation status. This led to the conclusion that the essential factor associated with survival is EGFR mutation status. Though better MST has been reported in L858R cases in a comparison of survival between exon 19 deletion and L858R missense (Shigematsu et al, 2005), recent reports have shown better survival in patients with exon 19 deletion (Jackman et al, 2006; Riely et al, 2006). Incidentally, we found MST to be better in L858R cases. As reported by Jackman et al (2006), RR was more favourable among the exon 19 deletion cases. Although this was conceivably due to factors including the ILD being experienced in two cases with exon 19 deletion and the impact of post-gefitinib treatment, the relatively small sample did not allow for any clarification in this respect.

Our data also show that the larger the smoking exposure, the shorter the survival.

There have been several reports of an inverse correlation between smoking exposure and EGFR mutation rate (Han et al, 2005; Pham et al, 2006; Sugio et al, 2006; Tam et al, 2006; Toyooka et al, 2006). In line with these studies, our data show that smoking status, unlike EGFR mutation status, is not an independent prognostic factor. Considered in combination with past reports on smoking exposure and EGFR mutation rates, the inverse correlation between smoking exposure and MST shown by our data might conceivably reflect that mutation rates differ according to smoking exposure. They also indicate that smoking status is a very powerful surrogate marker of EGFR mutation status, which is a prognostic factor for prolonged survival with gefitinib treatment. Our multivariate analysis in terms of clinical characteristics indicates that smoking status is a significant predictor. However, the multivariate analysis adding EGFR mutation status eliminates the significant difference with regard to smoking, demonstrating that EGFR mutation status and PS 0-1 are independent prognostic factors. This also suggests that ECOG PS and EGFR mutation status are factors that can be used to predict the intrinsic effect of gefitinib on patients as well as their prognosis, supporting the claim that smoking could be a surrogate marker of EGFR mutation status for prediction of survival benefit. Although RR in never-smokers and cases with EGFR mutation on erlotinib, which is also an EGFR-TKI, has been significantly favourable, there has only been marginal significant interaction between survival and smoking status (Clark et al, 2006). However, no significant differences have been reported in regard to EGFR mutation status, and detection of the EGFR mutation is considered unnecessary in treatment using erlotinib (Tsao et al, 2005; Clark et al, 2006). Our results showing that EGFR mutation and smoking status can function as predictors of survival benefit differ from reports on erlotinib. However, they concur with reports to date on gefitinib, presumably suggesting the necessity to select patients before using gefitinib. Further clinical studies are warranted to examine the survival benefits of gefitinib according to EGFR mutation status, that is, to make the EGFR mutation status an inclusion criterion. Considerations should be made in clinical practise to analyse actively EGFR mutations status where possible. However, it is often very difficult to obtain histological specimens of advanced and recurrent lung cancer for which gefitinib is indicated. In fact, in this study we were only able to obtain analytical results for EGFR mutation status for 91 out of 221 (42%) patients. Another problem is EGFR mutation analysis takes time, about 1-3 weeks, necessitating a wait-time before treatment. Therefore, when a certain clinical environment does not allow for, or complicates the detection of EGFR mutations, smoking exposure/smoking status could be a quick and inexpensive reference as a surrogate marker of EGFR mutation status. In future, it will be necessary to evaluate the survival benefits of gefitinib via a Phase III study in patients with these better predictive factors.

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## ORIGINAL ARTICLE

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# Preliminary experience with a modified premedication protocol that included intravenous diphenhydramine and calcium bromide for the prophylaxis of paclitaxel-related hypersensitivity reactions

**Abstract**

*Background.* Paclitaxel often causes severe hypersensitivity reactions (HSRs) rapidly after infusion, even in patients given prophylactic therapy. The purpose of this study was to analyze the incidence of paclitaxel-related HSRs in patients with non-small cell lung cancer (NSCLC) retrospectively, and to assess the feasibility of a modified premedication protocol.

*Methods.* One hundred and seven patients who were pretreated with either a conventional premedication regimen (two doses of dexamethasone) or a short premedication regimen (single dose of dexamethasone with oral diphenhydramine and intravenous ranitidine), prior to paclitaxel infusion were retrospectively analyzed. A modified premedication regimen, consisting of 12.5 ml of Rescalmin (intravenous diphenhydramine 50 mg and calcium bromide 437.5 mg), intravenous ranitidine 100 mg, and intravenous dexamethasone 20 mg, was given 30 min prior to paclitaxel, with oral dexamethasone 8 mg given on the night before the paclitaxel. Patients received paclitaxel intravenously at 175 mg/m² over 3 h, followed by carboplatin, AUC 5, over 1 h on day 1 every 3 weeks.

*Results.* In the conventional premedication group, 21 patients had HSRs (32.3%); in 1 of these patients the HSR was considered to be severe (1.5%). In the short premedication group, 19 patients had HSRs (45.2%); in 6 of these patients the HSRs were considered to be severe (14.3%).

The incidence of severe HSRs was significantly higher in the short premedication group than in the conventional premedication group (P = 0.027). In the modified premedication protocol study, HSR events were recorded in 14 patients (63.6%); 14 showed flushing, 2 had skin rash, and 1 had tachycardia. No severe HSRs were seen.

*Conclusions.* The incidence of HSRs in the short premedication group tended to be higher than that in the conventional premedication group. The modified premedication protocol was found to be feasible for preventing paclitaxel-related HSR, but case accumulation is needed.

**Key words** Paclitaxel · Premedication · Hypersensitivity reactions · Prophylaxis · Diphenhydramine

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## Introduction

Paclitaxel is a highly active drug used for the treatment of lung, ovarian, breast, head and neck, bladder, and other epithelial cancers. In early phase I trials, a high frequency of severe hypersensitivity reactions (HSRs) was observed when paclitaxel was administered.[1–3] HSRs usually occur just after the start of paclitaxel administration. The reaction likely occurs due to the release of histamine and other vasoactive compounds from mast cells in response to the polyoxyethylated castor oil vehicle (Cremophor EI, Sigma Chemical Co., St. Louis, MO).[3] Severe HSRs, characterized by chest pain, dyspnea, bronchospasm, urticaria, and/or hypotension, were initially reported in 10.6% of patients who were not premedicated prior to paclitaxel infusion.[3] After the initial report of HSR events to the National Cancer Institute, it was recommended that all patients receiving paclitaxel be given conventional premedication, which contained two doses of oral dexamethasone, intravenous diphenhydramine, and intravenous cimetidine or ranitidine.[3] Consequently, the incidence of severe HSRs has decreased to 1%–3%.[6–8] It has become common practice to pretreat patients with various regimens prior to paclitaxel administration.

Currently, the standard recommended prophylactic therapy regimen is a single dose of intravenous dexamethasone, intravenous diphenhydramine, and intravenous cimetidine or ranitidine,[8-11] which is called a short premedication regimen. However, it has been reported that short premedication may not be the optimum prophylactic therapy for paclitaxel-related HSRs.[12,13] In Japan, oral diphenhydramine has usually been used as a prophylactic H1 antagonist, because pure intravenous diphenhydramine has not been not available. We hypothesized that the blood concentration of diphenhydramine when the oral form is used may be more influenced by the patient's condition (for example, by the presence of gastrointestinal disease, or advanced age) than when the intravenous form is used.

In this study, we retrospectively analyzed the incidence of paclitaxel-related HSRs in patients with non-small cell lung cancer (NSCLC). Also, we carried out prophylactic treatment with a modified premedication protocol, using Rescalmin (diphenhydramine with calcium bromide; Nissin, Yamagata, Japan) – a product which is usually used intravenously for allergic rhinitis – to assess its feasibility for preventing paclitaxel-related HSRs.

## Patients and methods

### Definition of paclitaxel-related hypersensitivity reactions

Reactions were scored as "severe" if, during paclitaxel infusion, the patient experienced one or more of the following grade 3–4 toxicities: angioderma, chest and/or back pain, dyspnea and/or wheezing, hypotension requiring vasopressor agent support, or cardiac arrest. If patients with a grade 2 HSR, such as chest or back pain, strongly desired to stop the infusion, we classified the HSR as severe in such patients. Reactions were scored as mild if one of the following grade 1–2 toxicities was recorded: flushing, mild hypotension, skin rash, or palpitation.

### Retrospective historical cohort analysis of paclitaxel-related hypersensitivity reactions

We retrospectively analyzed the incidence of paclitaxel-related HSRs in patients with NSCLC. A pharmacy database at Osaka Prefectural Medical Center for Respiratory and Allergic Diseases identified all patients who had received paclitaxel with either conventional or short premedication from April 1999 to March 2002 (Table 1). All the patients had received both H1 and H2 antagonists (50 mg oral diphenhydramine and 100 mg intravenous ranitidine) 30 min prior to the paclitaxel infusion. In addition, the conventional premedication group had received two 20 mg doses of intravenous dexamethasone, at 12 and 6 h prior to the paclitaxel. The short premedication group had received a single 20-mg dose of intravenous dexamethasone 30 min prior to the paclitaxel. Paclitaxel was administered at a dose of 175–200 mg/m² by infusion over 3 h.

Treatment evaluations consisted of a complete medical history and physical examination, which included a blood

Table 1. Premedication details and characteristics in patients receiving paclitaxel

| | Total | Conventional | Short |
|---|---|---|---|
| Total no. of patients | 107 | 65 | 42 |
| Sex | | | |
| Male | 77 | 48 | 29 |
| Female | 30 | 17 | 13 |
| Median age, years | 61 | 61 | 62.5 |
| (range) | (32–75) | (32–75) | (34–74) |
| Performance status | | | |
| 0–1 | 83 | 50 | 33 |
| 2–3 | 24 | 15 | 9 |
| Histological type | | | |
| Adenocarcinoma | 84 | 52 | 32 |
| Squamous cell carcinoma | 19 | 10 | 9 |
| Large cell carcinoma | 4 | 3 | 1 |
| Prior chemotherapy | | | |
| Yes | 25 | 13 | 12 |
| No | 82 | 52 | 30 |
| Allergic history | | | |
| Yes | 5 | 2 | 3 |
| No | 102 | 63 | 39 |

cell count, urinalysis, ECG, chest X-ray, bone scan, and computed tomography. HSRs were graded according to the National Cancer Institute common toxicity criteria (NCI-CTC version 2.0; January 30, 1998) for adverse reactions to chemotherapy. Statistical significance was calculated with the Yate's corrected $\chi^2$ statistic. A difference with a $P$ value of less than 0.05 was considered to be significant. Statistical analysis software (StatMate III, ATMS, Tokyo, Japan) was used for the analysis.

### Modified premedication protocol for prophylaxis of paclitaxel-related hypersensitivity reactions

We conducted a prospective trial to assess the feasibility of using a modified premedication protocol for the prophylaxis of paclitaxel-related HSRs. To be eligible, patients had to have histologically or pathologically documented NSCLC. Measurable disease was not necessary. Patients were required to have, at study entry, an Eastern Cooperative Oncology Group (ECOG) performance score of 0 to 2, and were required to have an absolute neutrophil count of 2000/μl or more, a platelet count of 100 000/μl or more, a WBC count of 3500/μl or more, and a hemoglobin level of 9.5 g/dl or more. The total bilirubin level was required to be less than 1.5 times the upper normal limit. The serum creatinine level was required to be less than the upper normal limit. Patients were required to have recovered from toxicities of prior chemotherapy, and may not have had either radiation therapy or investigational drug therapy within 4 weeks of initiating paclitaxel and carboplatin. This protocol was reviewed and approved by the institutional review board, and all patients gave written informed consent before participation.

All patients received 12.5 ml of Rescalmin (50 mg intravenous diphenhydramine with 437.5 mg calcium bromide; Nissin), 100 mg intravenous ranitidine, and 20 mg intravenous dexamethasone, 30 min prior to the paclitaxel infusion.

after having oral dexamethasone 8mg the night before the paclitaxel. Paclitaxel was administered intravenously at 175mg/m² over 3h, followed by carboplatin, AUC 5, over 1 h on day 1 every 3 weeks. The calculated dose of paclitaxel was diluted in 500ml of 5% dextrose in water. Polyolefin containers and polyethylene-lined tubing were used for drug administration because of concern that the vehicle in which paclitaxel was prepared. Cremophor EL, might leach plasticizer from polyvinylchloride-containing intravenous sets.

During the infusion, patients' vital signs (heart rate, respiratory rate, and blood pressure) were determined every 15 min for the first hour, and every 30 min for the next 2h. Continuous cardiac monitoring was required until 6h after the completion of the paclitaxel infusion.

Treatment cycles were repeated every 3 weeks, provided toxic effects were not prohibitive and there was no evidence of tumor progression. Doses were to be reduced in the event of treatment-related febrile neutropenia, grade 4 neutropenia, or grade 3 nonhematological toxicity. Paclitaxel was discontinued if there was more than grade 2 neurologic toxicity, cardiac arrhythmias, heart block, or a significant HSR. Minor reactions were to be managed by stopping the infusion, if judged medically necessary, and by administering symptomatic medications such as additional antihistamines, corticosteroids, or bronchodilators.

## Results

### Retrospective historical cohort analysis

One hundred and seven patients were identified in the database. Up to November 2000, 65 patients had received the conventional prophylactic regimen, and from December 2000, 42 patients had received the short prophylactic regimen. Table 2 shows the incidence of HSRs in the two prophylactic regimens. In the conventional premedication group, 21 patients had HSRs (32.3%); in 1 of these patients, the HSR was considered to be severe (1.5%). In the short premedication group, 19 patients had HSRs (45.2%); in 6 of these patients, the HSRs were considered to be severe (14.3%). In this historical cohort analysis, the overall incidence of HSRs in the short premedication group was not significantly different from that in the conventional premedication group ($\chi^2$; $P = 0.177$), but the incidence of severe HSRs was found to be significantly higher in the short pre-

medication group ($\chi^2$; $P = 0.027$). Table 3 shows a summary of the severe HSRs in the 7 patients. In the 6 patients in the short premedication group, the hypersensitivity events occurred soon after the paclitaxel was initiated in the second course, and the reactions included chest or back pain, and dyspnea with or without bronchospasm. In the 1 patient in the conventional premedication group, grade 3 dyspnea with bronchospasm occurred during the first course. Paclitaxel infusion was discontinued immediately in all the patients with severe HSRs, and they received corticosteroid treatment. None of the patients were in a critical state, and there were no treatment-related deaths.

### Modified premedication regimen experience

#### Patients

From January 2004 to May 2004, 22 patients were enrolled in this study (Table 4). The patients were predominantly male (20 of 22 patients), and the median age was 65 years (range, 38 to 74 years). Nineteen (86.4%) of the 22 patients had an ECOG performance status of 0 or 1, 8 (36.4%) had metastatic lesions (stage IV), 17 (77.3%) had adenocarcinoma, and 7 (31.9%) had had prior chemotherapy.

#### Adverse events

Toxicity data were available for all 22 patients who had received at least one dose of paclitaxel. Overall, the therapy was generally well tolerated and manageable. The patients' nonhematological toxicities are listed in Table 5. In this study, HSRs were recorded in 14 patients (63.6%); 14 showed flushing (grade 1), 2 had skin rash (1 of grade 1 and 1 of grade 2), and 1 had tachycardia (grade 1). No severe

**Table 2.** Comparison of incidence of hypersensitivity reactions (HSRs) with the two prophylactic regimens

|  | Conventional | Short | P value |
|---|---|---|---|
| Overall HSR |  |  | 0.177 |
| (+) | 21 (32.3%) | 19 (45.2%) |  |
| (−) | 44 (67.7%) | 23 (54.8%) |  |
| Severe HSR |  |  | 0.027 |
| (+) | 1 (1.5%) | 6 (14.3%) |  |
| (−) | 64 (98.5%) | 36 (85.7%) |  |

**Table 3.** Summary of severe hypersensitivity reactions

| Patient no. | Age (years) | Sex | Premedication | Symptoms | Onset[a] | Course | NCI-CTC |
|---|---|---|---|---|---|---|---|
| 1 | 71 | M | Short | Back pain | Soon | 2 | 2 |
| 2 | 70 | F | Short | Angioderma, dyspnea without bronchospasm | 5 min | 2 | 3 |
| 3 | 64 | F | Short | Chest pain, back pain, flushing | Soon | 2 | 2 |
| 4 | 52 | F | Short | Chest pain, back pain, flushing | Soon | 2 | 2 |
| 5 | 71 | M | Short | Dyspnea with bronchospasm | 10 min | 2 | 3 |
| 6 | 34 | M | Short | Dyspnea with bronchospasm | Soon | 2 | 3 |
| 7 | 69 | M | Conventional | Dyspnea with bronchospasm | 10 min | 1 | 3 |

[a] In relation to paclitaxel infusion