factors (7 of 109), both good candidates for molecules involved in the specification of guard cells (fig. S3B). In addition to genes required for guard cell specification, we predicted that transcripts for genes required for guard cell function would appear in this transcriptional cluster. The cell walls of differentiated guard cells are extensively reinforced, and correspondingly, several transcripts (11%, or 12 of 109) were annotated as cell wall modification proteins (fig. S3B).

Sequence-indexed transferred (T-DNA) insertion lines are currently available for ~70% of the transcripts identified in the microarray experiments (18). To validate this genomic approach to identifying new stomatal development genes, we characterized the phenotype of 160 T-DNA insertion lines that represent 82 of 220 differentially expressed genes. Ten loci exhibited stomatal or epidermal phenotypes in at least two independent insertion lines (9). A marked phenotype was observed in lines homozygous for insertions in a putative transcription factor, At3g24140 (FAMA) (Fig. 4, C and D). FAMA exhibited a transcriptional profile similar to that of SDD1 and HIC1 (Fig. 4B). Existing microarray data revealed that FAMA expression is consistent with a role in stomatal pattern: highest in leaves (Affymetrix values 31 ± 13), lower in flowers (212 ± 17), and absent in roots (44 ± 33) (15, 19). The cotyledon epidermis of fama-1 (SALK_100073) (18) had no recognizable guard cells. Instead, chains of small cells that rarely make obvious stomatal pores but that, like guard cells (and unlike pavement cells), often contain mature chloroplasts were intercalated among pavement cells (Fig. 4, E and F). These clusters of incompletely differentiated cells are reminiscent of, but larger than, the clusters of GMCs found in T-DNA insertion lines (18). To validate this genomic approach to identifying new stomatal cell fate regulators, we used T-DNA insertions to manipulate stomatal density. Ten loci exhibited stomatal or epidermal phenotypes in at least two independent insertion lines (9). A marked phenotype was observed in lines homozygous for insertions in a putative transcription factor, At3g24140 (FAMA) (Fig. 4, C and D). FAMA exhibited a transcriptional profile similar to that of SDD1 and HIC1 (Fig. 4B). Existing microarray data revealed that FAMA expression is consistent with a role in stomatal pattern: highest in leaves (Affymetrix values 31 ± 13), lower in flowers (212 ± 17), and absent in roots (44 ± 33) (15, 19). The cotyledon epidermis of fama-1 (SALK_100073) (18) had no recognizable guard cells. Instead, chains of small cells that rarely make obvious stomatal pores but that, like guard cells (and unlike pavement cells), often contain mature chloroplasts were intercalated among pavement cells (Fig. 4, E and F). These clusters of incompletely differentiated cells are reminiscent of, but larger than, the clusters of GMCs found in T-DNA insertion lines (18).

References and Notes


7. Materials and methods are available as supporting material on Science Online.


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Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5676/1494/DC1 Materials and Methods

Figs. 1 to 53

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**EGFR Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy**

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Receptor tyrosine kinase genes were sequenced in non–small cell lung cancer (NSCLC) and matched normal tissue. Somatic mutations of the epidermal growth factor receptor gene **EGFR** were found in 15 of 58 unselected tumors from Japan and 1 of 61 from the United States. Treatment with the EGFR kinase inhibitor gefitinib (Iressa) causes tumor regression in some patients with NSCLC, more frequently in Japan. **EGFR** mutations were found in additional lung cancer samples from U.S. patients who responded to gefitinib therapy and in a lung adenoscarcinoma cell line that was hypersensitive to growth inhibition by gefitinib, but not in gefitinib-insensitive tumors or cell lines. These results suggest that **EGFR** mutations may predict sensitivity to gefitinib.

Protein kinase activation by somatic mutation or chromosomal alteration is a common mechanism of tumorigenesis (1). Inhibition of activated protein kinases through the use of targeted small molecule drugs or antibody-based strategies has emerged as

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As an initial screen, we amplified and sequenced the exons encoding the activation loops of 47 of the 58 human receptor tyrosine kinase genes (16) (table S1) from genomic DNA from a subset of 58 NSCLC samples that included 41 lung adenocarcinomas. Three of the tumors, all lung adenocarcinomas, showed heterozygous missense mutations in EGFR not present in the DNA from normal lung tissue from the same patients (table S2; S0361, S0388, S0389). No mutations were detected in amplicons from other receptor tyrosine kinase genes. All three tumors had the same EGFR mutation, predicted to change leucine-858 to arginine (Fig. 1A; CTG→CGG; L858R).

We next examined exons 2 through 25 of EGFR in the complete collection of 119 NSCLC tumors. Exon sequencing of genomic DNA revealed missense and deletion mutations of EGFR in a total of 16 tumors, all within exons 18 through 21 of the kinase domain. All sequence alterations in this group were heterozygous in the tumor DNA; in each case, paired normal lung tissue from the same patient showed wild-type sequence, confirming that the mutations are somatic in origin. The distribution of nucleotide and protein sequence alterations, and the patient characteristics associated with these abnormalities, are summarized in table S2.

Substitution mutations G719S and L858R were detected in two and three tumors, respectively. These mutations are located in the GXGXXG motif of the nucleotide triphosphate binding domain or P-loop and adjacent to the highly conserved DFG motif in the activation loop (17), respectively. The mutated residues are nearly invariant in all protein kinases, and the analogous residues (G463 and L596) in the B-Raf protein serine-threonine kinase are somatically mutated in colorectal, ovarian, and lung carcinomas (5, 18) (Fig. 1, A and B).

We also detected multiple deletion mutations clustered in the region spanning codons 746 to 759 within the kinase domain of EGFR. Ten tumors carried one of two overlapping 15-nucleotide deletions eliminating EGFR codons 746 to 759, starting at nucleotide 2232 or 2236 (Del-1) (Fig. 1C and table S2). EGFR DNA from another tumor displayed a heterozygous 24-nucleotide gap leading to the deletion of codons 752 to 759 (Del-2) (Fig. 1C). Representative chromatograms are shown in fig. S1.

The positions of the substitution mutations and the Del-1 deletion in the threedimensional structure of the active form of the EGFR kinase domain (19) are shown in Fig. 2. Note that the sequence alterations cluster around the active site of the kinase and that the substitution mutations lie in the activation loop and glycine-rich P-loop, structural elements known to be important for autoregulation in many protein kinases (17).

The EGFR mutations show a striking correlation with patient characteristics. Mutations were more frequent in adenocarcinomas (15/70 or 21%) than in other NSCLCs (1/49 or 2%), more frequent in women (9/45 or 20%) than in men (7/74 or 9%), and more frequent in the patients from Japan (15/58 or 26%, and 14/41 adenocar-

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Fig. 1. Sequence alignment of selected regions within the EGFR and B-Raf kinase domains. Depiction of each type of EGFR mutation in human NSCLC. EGFR (gb:X005588) mutations in NSCLC tumors are highlighted in yellow. B-Raf (gb:M95712) mutations in multiple tumor types (5) are highlighted in blue. Asterisks denote residues conserved between EGFR and B-Raf. (A) L858R mutations in activation loop. (B) G719S mutant in P-loop. (C) Deletion mutants in EGFR exon 19.

Fig. 2. Positions of missense mutations G719S and L858R and the Del-1 deletion in the three-dimensional structure of the EGFR kinase domain. The activation loop is shown in yellow, the P-loop in blue, and the C-lobe and N-lobe are as indicated. The residues targeted by mutation or deletion are highlighted in red. The Del-1 mutation targets the residues ELREA in codons 746 to 750.
cinomas or 32%) than in those from the United States (1/61 or 2%, and 1/29 adenocarcinomas or 3%). The highest fraction of EGFR mutations was observed in Japanese women with adenocarcinoma (8/14 or 57%). Notably, the patient characteristics that correlate with the presence of EGFR mutations are those that correlate with clinical response to gefitinib treatment.

To investigate whether EGFR mutations might be a determinant of gefitinib sensitivity, pretreatment NSCLC samples were obtained from 5 patients who responded and 4 patients who progressed during treatment with gefitinib out of more than 125 patients treated at the Dana-Farber Cancer Institute either on an expanded access program or after regulatory approval of gefitinib (13). Four of the patients had partial radiographic responses (≥50% tumor regression in a computed tomography scan after 2 months of treatment), whereas the fifth patient experienced dramatic symptomatic improvement in less than 2 months. All of the patients were from the United States and were Caucasian.

While sequencing of the kinase domain (exons 18 through 24) revealed no mutations in tumors from the four patients who progressed on gefitinib, all five tumors from gefitinib-responsive patients harbored EGFR kinase domain mutations. The chi-square test revealed the difference in EGFR mutation frequency between gefitinib responders (5/5) and nonresponders (0/4) to be statistically significant with P = 0.0027, whereas the difference between the gefitinib responders and unselected U.S. NSCLC patients (5/5 versus 1/61) was also significant with P < 10−12 (20). The EGFR L858R mutation, previously observed in the unselected tumors, was identified in one gefitinib-sensitive lung adenocarcinoma (Fig. 1A and table S3, IR3T). Three gefitinib-sensitive tumors contained heterozygous in-frame deletions (Fig. 1C and table S3, Del-3 in two cases and Del-4 in one), and one contained a homozygous in-frame deletion (Fig. 1C and table S3, Del-5). Each of these deletions was found within codons 746 to 753 of EGFR, where deletions were also found in unselected tumors. Each of these three deletions is also associated with an amino acid substitution (table S3). In all four samples where matched normal tissue was available, these mutations were confirmed as somatic.

To determine whether mutations in EGFR confer gefitinib sensitivity in vitro, the mutation status and response to gefitinib were determined in four lung adenocarcinoma and bronchioloalveolar carcinoma cell lines. The H3255 cell line was originally derived from a malignant pleural effusion from a Caucasian female nonsmoker with lung adenocarcinoma (21). This cell line was 50 times as sensitive to gefitinib as the other lines, with an IC50 of 40 nM for cell survival in a 72-hour assay (Fig. 3A).

Treatment with 100 nM gefitinib completely inhibited EGFR autophosphorylation in H3255 (Fig. 3B). Such treatment also inhibited the phosphorylation of known downstream targets of EGFR such as the extracellular signal-regulated kinase 1/2 (ERK1/2) and the v-akt murine thymoma viral oncogene homolog (AKT kinase) (Fig. 3B), a correlation that has been noted by others (22). In contrast, the other three cell lines showed comparable levels of inhibition of target protein phosphorylation only when gefitinib was present at concentrations roughly 100 times as high (Fig. 3B).

The sequence analysis of EGFR cDNA in these four cell lines showed the L858R mutations in H3255 (table S3), whereas the other three cell lines did not contain EGFR mutations. We also confirmed the presence of the L858R mutation in the primary tumor from which H3255 was derived (table S3, IRG), although no matched normal tissue was available. The results suggest that L858R mutant EGFR is particularly sensitive to inhibition by gefitinib compared with the wild-type enzyme and that this likely accounts for the extraordinary drug sensitivity of the H3255 cell line.

The identification of EGFR mutations in a subset of human lung carcinomas and the association between EGFR mutation and gefitinib sensitivity extend the emerging paradigm whereby genetic alterations in specific kinases, and not simply kinase expression, render tumors sensitive to selective inhibitors as is the case for imatinib treatment of c-kit mutant gastrointestinal stromal tumors (23). Thus, although randomized trials of cytotoxic therapy with or without gefitinib revealed no survival benefit for the gefitinib-treated NSCLC patients (24, 25), our current data suggest that gefitinib may be particularly effective for treating lung cancers with somatic EGFR mutations and that prospective clinical trials of EGFR inhibition in patients with EGFR mutations might reveal increased patient survival. Identification of EGFR mutations in other malignancies, perhaps including glioblastomas in which EGFR alterations are already known (26), may identify other patients who could similarly benefit from treatment with EGFR inhibitors.

Important questions remain to be answered, including whether these alterations result in activated and transforming alleles of EGFR, whether receptors harboring such mutations will show differential sensitivity to any of the multiple EGFR small molecule inhibitors and what the implications of these findings are for the development of other selective inhibitors.
Regulation of an ATG7–beclin 1 Program of Autophagic Cell Death by Caspase-8

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Caspases play a central role in apoptosis, a well-studied pathway of programmed cell death. Other programs of death potentially involving necrosis and autophagy may exist, but their relation to apoptosis and mechanisms of regulation remains unclear. We define a new molecular pathway in which activation of the receptor-interacting protein (a serine-threonine kinase) and Jun amino-terminal kinase induced cell death with the morphology of autophagy. Autophagic death required the genes ATG7 and beclin 1 and was induced by caspase-8 inhibition. Clinical therapies involving caspase inhibitors may arrest apoptosis but also have the unanticipated effect of promoting autophagic cell death.

Apoptosis is a well-studied pathway of programmed cell death conserved from Caenorhabditis elegans to humans (1). Caspases, a family of cysteinyl aspartate-specific proteases, produce the morphological changes associated with apoptotic death (2, 3). Nonapoptotic forms of cell elimination include those with features of necrosis and autophagy (4–7). Necrosis can result when cell metabolism and integrity are compromised by a nonphysiological insult. Recently, evidence has emerged that death receptors and receptor-interacting protein (RIP) can induce caspase-independent cell death that appears necrotic (6, 7). Autophagy promotes a cell survival response to nutritional starvation involving membrane-bound vacuoles that target organelles and proteins to the lysosome for degradation (8, 9). Two pathways functioning in autophagy contain ubiquitin-like genes that are highly conserved from yeast to humans (ATG genes). Certain examples of cell death have autophagic features, but a role for ATG genes in cell death has not been established (10).

In mouse L929 fibroblastic cells, tumor necrosis factor, oxidants, ceramide, and radiation can induce caspase-independent death (11). However, benzyloxy-carbonyl-valyl-alanyl–aspartic acid (O-methyl)–fluoro-methylketone (zVAD), a caspase inhibitor with broad specificity, also directly induced the death of L929 cells. Death began at 12 hours after zVAD treatment and was complete after 40 hours (Fig. 1A and B). The dead cells appeared to be round and detached, and they had a convoluted plasma membrane permeable to vital dyes; this differed from apoptosis, in which nuclei are condensed and membrane integrity is preserved. Transmission electron microscopy (TEM) revealed intact mitochondria and endoplasmic reticulum, condensed osmophilic cytoplasm, and numerous large cytoplasmic inclusions that were membrane-bound vacuoles characteristic of autophagy (Fig. 1C). A time course revealed that vacuolated cells accumulated before cell death (Fig. 1D). Similar results were obtained in human U937 monocytoid cells (Fig. 1E and fig. S2). The zVAD treatment also induced cell death in mouse RAW 264.7 macrophage cells and primary mouse peritoneal macrophages (figs. S3 and S4).

The association of autophagic vacuoles with cell death has been observed in developing animals, but it has not been clear whether the process serves to rescue or condemn the cell (12). Drosophila cells mani-
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