EGFR Mutation and Response of Lung Cancer to Gefitinib

TO THE EDITOR: Kobayashi et al. (Feb. 24 issue)[1] report that a second mutation in the gene encoding the epidermal growth factor receptor (*EGFR*), one resulting in a threonine-to-methionine substitution at amino acid position 790 (T790M), was associated with acquired resistance to gefitinib in their patient and that this mutant gene had been absent from the primary non–small-cell lung cancer. In a reanalysis of the data from the 397 subjects we have previously described,[2,3] we identified two women who had never smoked who had non–small-cell lung cancer and harbored two *EGFR* mutations — T790M and a leucine-to-arginine substitution at amino acid position 858 (L858R) — in resected tumor specimens before treatment with chemotherapy or radiotherapy. Both patients later had recurrent disease and eventually died — outcomes suggesting that tumors with both the L858R and T790M mutations are very aggressive. One patient was treated with gefitinib and had progression.

These findings indicate the existence of cases with inherent double mutations and provide evidence that the T790M mutant genotype is an important factor conferring resistance to gefitinib in non–small-cell lung cancers containing EGFR sensitivity mutations. In addition, detecting T790M may be useful for predicting pretreatment resistance to EGFR tyrosine kinase inhibitors. Our observation, together with data from recent reports,[1,4] may help clarify the role of *EGFR* mutations in the development of EGFR-related non–small-cell lung cancer and help establish effective strategies against specific subtypes of non–small-cell lung cancer.

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THE AUTHORS REPLY: Dr. Toyooka and colleagues describe two patients whose lung tumors harbored a T790M mutation before treatment with chemotherapy or radiotherapy was begun and suggest that this mutation might be a marker of tumor aggressiveness as well as resistance to gefitinib therapy. In the cases we and others[1] have described, the T790M mutation was not found in specimens from untreated patients. Nevertheless, the possibilities do exist that this second mutation might be present in some tumors at a low frequency at the time of diagnosis and that tumor cells harboring the mutation might be enriched over time during treatment with gefitinib or erlotinib. By analogy, imatinib-resistant *BCR-ABL* mutations have, on occasion, been detected in specimens from patients with untreated chronic myeloid leukemia.[2,3] We agree that such interesting findings should motivate further research to improve our understanding of the role of EGFR in non–small-cell lung cancers, to encourage the development of alternative EGFR inhibitors able to overcome such resistance mutations, and to incorporate the knowledge gained into clinical treatment.

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Review Article

Throwing new light on lung cancer pathogenesis: Updates on three recent topics

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Lung cancers have become the leading cause of cancer deaths in Japan, claiming more than 55 000 lives annually. Unfortunately, substantial improvement in terms of cure rates has not been achieved over the last two decades, although during the same period of time in-depth basic knowledge of the molecular mechanisms, which underlies carcinogenesis and progression of this deadly group of neoplasms, has accumulated at an amazing pace. It has consequently become evident that they have many shared but also distinct features, when comparisons are made not only with other common epithelial cancers of adults, such as colon cancer, but also within the various histologic types of lung cancers themselves. This review article provides an up-date on cutting-edge research into the following three different topics, from which important new insights have been obtained. The first concerns genetic instability, especially chromosome instability, and checkpoint failure in lung cancers. Second, we deal with *EGFR* mutations, which shows revealing specificities in various aspects. Finally, advances in the expression profiling analysis of both transcriptomes and proteomes of lung cancers are summarized. (*Cancer Sci* 2005; 96: 63–68)

There are over one million deaths a year worldwide due to lung cancer, making it one of the most prevalent and deadly neoplasms. There is no doubt that smoking of tobacco is the most important causative factor in its development, the disease in fact apparently being rare before the widespread use of tobacco. Japan has been experiencing a steep increase in lung cancer cases, following the footsteps of the USA and other western countries, such as the UK, where increase in tobacco consumption was subsequently followed by an abrupt rise in lung cancer occurrence. Our current problem can be regarded as a ringing alarm bell for other Asian nations, such as China, in which tobacco consumption is now rapidly increasing. Lung cancer currently claims more than 55 000 lives annually in Japan, with a no more than 15% cure rate, meaning that the number of deaths remains unacceptably high. A better understanding of the molecular pathogenesis of this fatal disease is therefore an urgent issue in order to develop better diagnostic approaches and new targeted therapies, as well as effective means for its prevention.

Lung cancers can be classified into two major entities, based on their clinicopathologic characteristics, that is, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter being further divided into three major histologic types, the adenocarcinoma, squamous cell carcinoma and large cell carcinoma. In the development of squamous carcinoma, oncogenic triggering converts normal bronchial epithelium into hyperplastic, metaplastic and dysplastic lesions, leading to the subsequent emergence of carcinoma *in situ* (CIS) and then overt squamous cell carcinomas. Adenocarcinomas are also considered to develop, at least in part, through stepwise morphological changes, with atypical adenomatous hyperplasia (AAH) generally thought to be a premalignant precursor lesion. It has been clearly demonstrated that overt cancers carry multiple genetic and epigenetic alterations occurring during initiation of carcinogenesis and subsequent progression. Since cell-cycle regulation and checkpoint functions are crucial for maintaining genomic integrity, their abrogation is thought to contribute to genomic instability, playing important roles even in the early steps of cancer development.[1] Many of the tumor suppressor genes and oncogenes altered in lung cancer are known to contribute to the regulation of cell cycle progression in either a direct or an indirect manner, and a considerable proportion of the lung cancer-related gene products are components of vital checkpoint mechanisms. It is notable that exposure to carcinogens in tobacco smoke appears to leave fingerprints of the resulting insult to the genome, for example, as distinct mutational spectra of *p53* and *KRAS*.

In this review article on the molecular pathogenesis of lung cancers, we will confine ourselves to concisely summarizing recent advances in three different topics, rather than attempting a comprehensive coverage of all the related subjects (for this purpose, see other review articles by the authors[2–4]). The three topics dealt in this article are: (i) chromosome instability and alterations of cell cycle checkpoints; (ii) the clinicopathologic impact of recent identification of frequent *EGFR* mutations in a specific type of adenocarcinomas; and (iii) accumulating new insights obtained through expression profiling analysis with both genomic and proteomic approaches.

Chromosomal instability and alterations of cell cycle checkpoints

Non-random chromosomal deletion and loss of heterozygosity (LOH) are hallmarks for the involvement of tumor suppressor genes residing in the affected chromosomal regions, while oncogene amplification can be identified by cytogenetic findings, such as homogenously staining lesions and double minutes. In contrast to certain leukemias, lymphomas and sarcomas, specific balanced translocations are not present in lung cancers, whose karyotypes are frequently very complex.[5] Loss of chromosomal arm 3p is among the first non-random genomic aberrations identified in lung cancer. In addition to this common loss of 3p, chromosomal losses are also frequent on 4q, 5q, 8p, 10q, 13q and 17p in SCLC, and 8p, 9p, 13q, 17p in NSCLC. Chromosomal gains are frequent on 3q, 5p and 8q in SCLC, and 1q, 3q, 5p and 8q in NSCLC. Allelic losses and amplifications of multiple small chromosomal regions have also been documented through detailed LOH and comparative genomic hybridization (CGH)

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analyses. It is expected that systematic array CGH analysis will allow further refinement of the affected regions, leading to the identification of candidate genes for the responsible targets.

The frequent presence of complex chromosomal changes suggests significant roles in the development of lung cancer, and it is well known that both numerical and structural aberrations may occur. Failure in cell cycle checkpoint control and defects in the DNA double-strand break repair system are thought to play important roles in the development of chromosome instability (CIN). We have shown that persistent numerical CIN is present in human lung cancer cell lines with a notable association with aneuploid karyotypes, and demonstrated that aneuploidy is not the result of a few past catastrophic processes of missegregations, but rather a reflection of the persistence of an unstable karyotypic state.[6] This feature appears to be common in epithelial cancers in adults, which often show complex aneuploidy. As for the underlying mechanisms, it is notable that mice, deficient in the *Mad2* mitotic checkpoint gene, which is important for correct chromosome segregation, have been shown to specifically develop lung tumors characterized by CIN.[7] In human lung cancers, mitotic checkpoint impairment appears to be present at a considerably high frequency,[8] while mutations in the *MAD1* and *BUB1* mitotic checkpoint genes have been detected in human lung cancer cells, albeit relatively rarely.[9–11] In addition, *p53* inactivation appears to play an indirect role in the acquisition of the CIN phenotype.[6]

Double strand breaks (DSB) can be introduced into genome by intracellular stresses, such as oxidative damage, as well as by environmental factors, such as ionizing irradiation, and this highly detrimental form of DNA damage potentially serves as causes of erroneous rejoining of the broken genome. Cell-cycle checkpoints are built-in safeguards to prevent damaged cells from starting DNA replication (the G1/S checkpoint), from progressing with replication (the intra S checkpoint), or from going into mitosis (the G2 checkpoint).[12] Lung cancers are known to carry frequent G1/S checkpoint abrogation due to *p53* mutations, the most frequent genetic alterations found so far in this common epithelial cancer of adults,[13] and it has been shown that the G2 checkpoint is also frequently impaired in SCLC in a histological type-specific manner.[14] Failure of G2 checkpoint activation in the presence of DNA damage would generate broken chromosomes, the fates of which include: degradation, healing as truncated forms, and incorrect fusion to generate translocated chromosomes. Fusion between two centromere-containing chromosomes can trigger the highly unstable breakage-fusion-bridge cycle. Multiple components of the DNA damage G2 checkpoint mechanism appear to be involved in the development of lung cancers. CHK1 and CHK2 are preferentially activated by their respective upstream kinases, ATR and ATM, leading to phosphorylation of Cdc25C, a phosphatase that normally activates Cdc2, and resulting in its sequestration and inactivation of Cdc25C by 14-3-3 proteins. It is interesting that *14-3-3ε* was recently shown to be involved in a homozygous deletion identified in a SCLC cell line, and that reconstitution of *14-3-3ε* partially restored its G2 checkpoint impairment.[14] Another member of the 14-3-3 family, *14-3-3σ*, which is transactivated by p53 in response to DNA damage, has been shown to contribute to the maintenance of G2 arrest through nuclear exclusion of the Cdc2/cyclin B1.[15] Notably, epigenetic inactivation of *14-3-3σ*, due to aberrant DNA hypermethylation of its promoter region, has been shown to be frequent in SCLC[16,17] as well as in a few other types of human cancers, including, breast, gastric and hepatocellular carcinomas. We have also found that *CHK2* is somatically mutated in lung cancers at a low frequency.[18,19] It remains to be investigated whether there may be an, as yet, unidentified major target gene responsible for the observed checkpoint defects, or whether there might be a large number of affected genes each playing a role in a small proportion of cases.

In addition, our recent work by Nakagawa *et al.* provided direct evidence that decatenation at the G2 checkpoint,[20] which ensures sufficient chromatid decatenation by topoisomerase II before entering into mitosis,[21] is impaired in a proportion of human lung cancer cell lines. Therefore, the G2 checkpoint may also be important. This is clinically relevant, with considerable interest in the potential association between decatenation impairment and hypersensitivity to catalytic topoisomerase inhibitors, such as ICRF-193,[20] since this could ultimately lead to the development of an attractive strategy for lung cancer treatment, that is, selective killing of targeted cancer cells without causing major toxicity in normal cells. The *CHFR* gene, which has been postulated to play a key role in the prophase checkpoint, is also known to be inactivated by DNA hypermethylation in lung cancers.[22] The findings so far obtained, clearly indicate that multiple cell cycle checkpoints are impaired in lung cancers (Fig. 1), conceivably providing a driving force to acquisition of genetic instability, including CIN, and therefore contributing to the development of lung cancers.

Specific Involvement of *EGFR* and *ras* Mutations in Lung Adenocarcinomas

In the 1980s, various oncogenes were shown to be altered in lung cancers, and the *myc* and *ras* gene families are among the best studied in relation to their pathogenesis.[23] Gene amplification

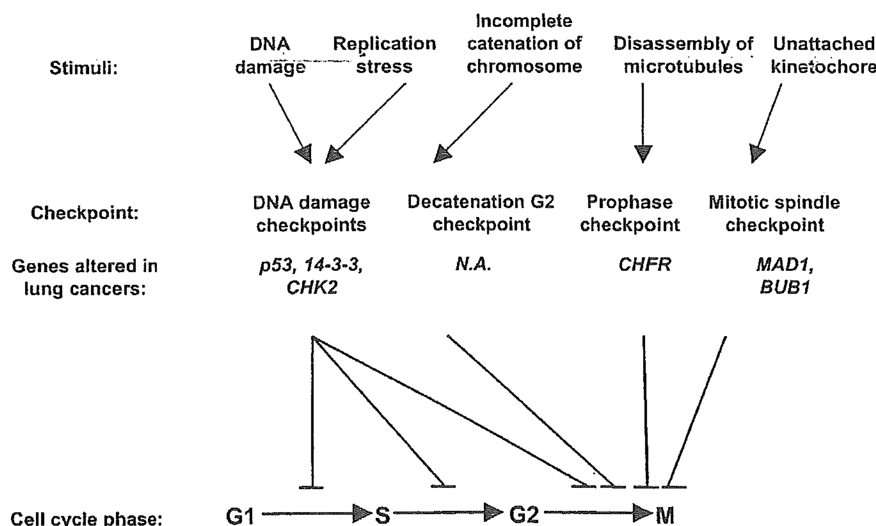

Fig. 1. Cell cycle checkpoints perturbed in human lung cancers. N.A., not available.

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of one of the members of the *myc* family is detectable in about 25% of SCLC cell lines as well as in 5–15% of primary tumor specimens, while overexpression of one of the members is detectable in virtually all SCLC. Therefore, the vast majority of these lesions appear to have both *p53* mutations and MYC overexpression, consistent with the notion that inactivation of the ARF/MDM2/p53 pathway, which is nearly ubiquitous in SCLC, may be required for Myc-induced transformation. By contrast, *K-ras* mutations are detected almost exclusively in adenocarcinomas, usually at codon 12, in association with a poor prognosis of surgically treated cases,[24] whereas *H-ras* or *N-ras* are only very rarely mutated in any type of lung cancer. *K-ras* mutations are predominantly G to T transversions, as is also the case for *p53* mutations, implying their creation through DNA adduct formation from tobacco exposure. In addition, mutations in the *BRAF* gene are present in approximately 3% of adenocarcinomas without *K-ras* mutations,[25,26] consistent with *K-ras* and *BRAF* ultimately signaling through the same pathway.

The discovery of mutations of the two prototype tumor suppressor genes, namely *Rb* and *p53*, in 1989,[27,28] shifted much of the attention of investigators in this field, including ourselves, to the involvement of tumor suppressor inactivation, with development of candidate gene approaches and positional and functional cloning efforts. However, recent reports on *EGFR* mutations by two Boston groups have generated considerable interest because of the highly characteristic and clinically useful nature of this genetic change.[29,30] Therefore, the field of oncogene activation is being revisited in relation to lung carcinogenesis. *EGFR* mutations have been found to be more frequent in cases with an adenocarcinoma histology and female gender, and in Japanese patients (in comparison with American patients), and the presence of *EGFR* mutations was shown to have a potential predictive value for sensitivity to gefitinib (Iressa, ZD1839), a small molecule tyrosine kinase inhibitor that targets EGFR. Interestingly, Paez *et al.* observed a marked predominance of *EGFR* mutations in 26% (15 of 58) of specimens from Japanese patients, compared to only 2% (1 of 61) in a series from American patients.[30] In addition, a group at the Memorial Sloan-Kettering Cancer Center has recently confirmed the utility of detecting *EGFR* mutations as a marker for predicting responsiveness to gefitinib.[31] We have extended their findings by analyzing 277 Japanese NSCLC patients, and found *EGFR* mutations to be present exclusively in adenocarcinomas, with a single exception (an adenosquamous carcinoma case), at a frequency of 59% and 26% in female and male cases, respectively.[32] The vast majority of the mutations proved to be either deletions around codons 746–750 or a missense mutation substituting leucine with arginine at codon 858, generally affecting three functionally important structures (αC helix, activation loop, P-loop) flanking the ATP binding pocket of the tyrosine kinase domain (Fig. 2). In this connection, it is of interest that EGFR activates several downstream substrates in addition to the RAS-MAPK pathway, such as the PI3K/AKT and JAK/STAT pathways. *PIK3CA*, which encodes the p110α catalytic subunit of PI3K, is mutated in lung cancers,[33] though at low frequency, and a recent study by Sordella *et al.* showed that mutated-EGFR targets the PI3K/AKT and JAK/STAT pathways rather than the RAS-MAPK pathway.[34] The findings of a recent immunohistochemical investigation carried out by Cappuzzo *et al.* are consistent with this notion, that is, gefitinib-treated patients with P-Akt-positive tumors had a better response, disease control, and time to progression than those with P-Akt-negative tumors.[35] In addition, we have shown, for the first time, that patients with *EGFR* mutations survive for a longer period than those without *EGFR* mutations when treated with gefitinib for recurrent disease after surgery.[36] Although these findings must be confirmed in a prospective clinical trial, screening for *EGFR* mutations appears to be a promising means to target drugs to specific molecules. This may consequently become a common practice for treating pulmonary adenocarcinoma patients in the near future, making individualized therapy a reality.

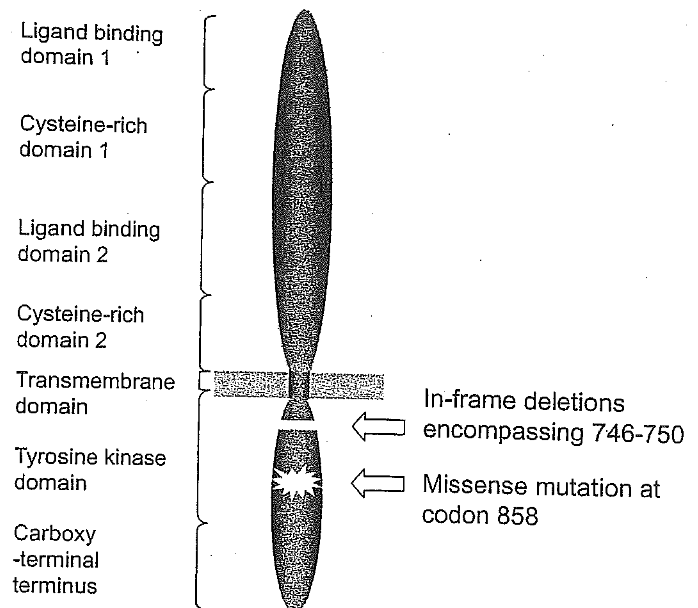

Fig. 2. Schematic diagram of the structure of EGFR as well as of the locations of two major types of mutations.

Notably, our multivariate logistic regression analysis demonstrated a non-smoking status and adenocarcinoma histology, but not female gender, to independently contribute to the occurrence of *EGFR* mutations, suggesting that the apparent difference in frequency observed between female and male may result from differences in lifestyle, including smoking habit, rather than involvement of the sex hormone-related environment, at least in the Japanese population. This is in a striking contrast to other genetic and epigenetic changes previously described in lung cancers. For instance, mutations in the *p53* and *K-ras* genes are known to be more frequent in smokers than in non-smokers.[37,38] Alterations of the *FHIT* gene encompassing a fragile site at 3p14.2, a chromosomal region frequently affected in lung cancers, are also more prevalent in smokers than in non-smokers.[39] The difference in the underlying mechanisms between *EGFR* mutations and the other genetic and epigenetic alterations reported previously, is also consistent with the occurrence of mutations in *EGFR* and *K-ras* in a mutually exclusive manner, as well as with the fact that *K-ras* mutations in adenocarcinomas without *EGFR* mutations are mostly G to T transversions, which may reflect exposure to carcinogens in tobacco smoke.[32] It is also worthy of mention that Yatabe *et al.* have found that even within adenocarcinomas, *EGFR* mutations appear to occur in a distinct subset, that is, terminal respiratory unit-type adenocarcinomas, which is a specific type of lesion conceivably arising from peripheral lung epithelial cells, including alveolar cells and non-ciliated bronchiolar epithelium.[40,41] It should be stressed that *EGFR* mutations appear to be extremely specific to lung cancers, mutations being reported to be present in none of 95 primary tumors and 108 cancer-derived cell lines, of diverse tumor types.[29] At present, it is not clear why the occurrence of *EGFR* mutations shows such specificity in terms of the smoking status and histology. In our opinion, there may be at least two possible explanations, which are not necessarily mutually exclusive. First, as yet unidentified carcinogens other than those in tobacco smoke might be involved in the development of adenocarcinomas in non-smoking individuals through the imposition of *EGFR* mutations in their genome. Cooking oil fumes or HPV 16/18

infection, both of which reportedly have associations with lung cancer occurring in non-smoking women in Taiwan, might be relevant. Alternatively, *EGFR* mutations might provide selective advantage only in peripheral lung epithelial cells. In this regard, it is interesting that EGF stimulates anchorage-independent growth of our HPL1D cell line, the only human peripheral lung epithelial line so far established.[42] These intriguing points await future clarification.

Genomic and Proteomic Profiling of Lung Cancers for Better Understanding and Future Applications

The recent rapid progress in microarray technology has made it possible to analyze gene expression profiles on a genome-wide basis in order to better understand molecular pathogenesis of human cancers, as well as to search for molecular markers for classification and prediction of outcome (Fig. 3). While lung cancers are known to be very heterogeneous in various aspects, recent expression profiling studies have clearly shown the presence of several distinct subclasses.[43–48] SCLC express a set of genes which are related to neuroendocrine differentiation, including *ASH1*, a key transcription factor that is indispensable for the development of neuroendocrine cells of the lung. Squamous cell carcinomas show marked elevation of a group of keratin isoforms related to squamous differentiation, as well as of *p63*, a *p53* homolog that is believed to play a role in squamous cell differentiation. Although these results are not unexpected, considering the characteristics evident even on routine pathology examination, it is certainly interesting that expression-profiling analysis has proven sufficiently powerful to detect the presence of distinct expression profiles, even within a subgroup of tumors with adenocarcinoma histology. Garber *et al.* identified three subgroups in adenocarcinomas through unsupervised hierarchical clustering analysis,[43] while Bhattacharjee *et al.* made a subclassification into four subgroups.[44] We have also reported the identification of four distinct subsets of adenocarcinomas based on unsupervised hierarchical clustering analysis.[48] Similarly, Virtanen *et al.* observed adenocarcinomas to form three distinct clusters.[46] Although the expression signatures identified in these studies do not show complete correspondence with each other, there is a high degree of consistence in the fact that all these studies have identified a subset of adenocarcinomas with high-level expression of genes related to differentiation of normal peripheral lung epithelial cells, such as *TTF-1* and *SP-C*. Notable features of this subclass are a significantly higher proportion in female non-/light smokers and the tumors are well-differentiated, suggesting that they may arise from cells committed to becoming peripheral lung epithelial cells, that is, terminal respiratory unit-type adenocarcinomas.[41] With these lesions, smoking presumably has no or only a weak influence. In fact, we note that these features correspond to those of adenocarcinoma cases with a high frequency of *EGFR* mutations and hence higher sensitivity to the gefitinib treatment (see above). Indeed, we observed *EGFR* mutations in 50% of the cases belonging to this cluster (unpublished observation). Therefore, it will be interesting to examine adenocarcinoma cases for expression profiles and the presence of *EGFR* mutations in a comprehensive manner in order to verify this intriguing possibility.

In addition, the advantages of expression profiling analysis have been shown through the identification of previously undefined subgroups in other types of lung cancers. The existence of two clinically relevant subsets of squamous cell carcinomas has been suggested, with distinct gene expression signatures and markedly different postoperative survival,[1,48] while two prognostically significant subtypes of high-grade neuroendocrine tumors, which appear to be independent of the currently employed pathologic subclassification, have been identified.[49] Based on the hypothesis that genes specifically expressed in various types of NSCLC may be associated with developmentally regulated genes and pathways, Borczuk *et al.* conducted an interesting exercise.[50] They found that the gene set specific for adenocarcinoma histology corresponded to those expressed in the late stage (terminal sac and alveolar stages) of murine lung development, whereas the large-cell carcinoma set was associated with genes expressed in the earlier pseudoglandular and canalicular stages. It is also interesting to note that a metastatic signature, obtained by comparing expression profiles of adenocarcinoma metastases of multiple tumor types to unmatched primary adenocarcinomas, was shown to be associated with a poor prognosis,[51] as to be expected from the fact that deaths from lung adenocarcinomas are in most cases attributable to metastasis.

Expression profiling analysis, aimed at individualized patient outcome prediction, is another important and much needed application, since survival or death is a matter of all or nothing, and currently available information regarding what percentage of those at a certain disease stage are likely to survive after a

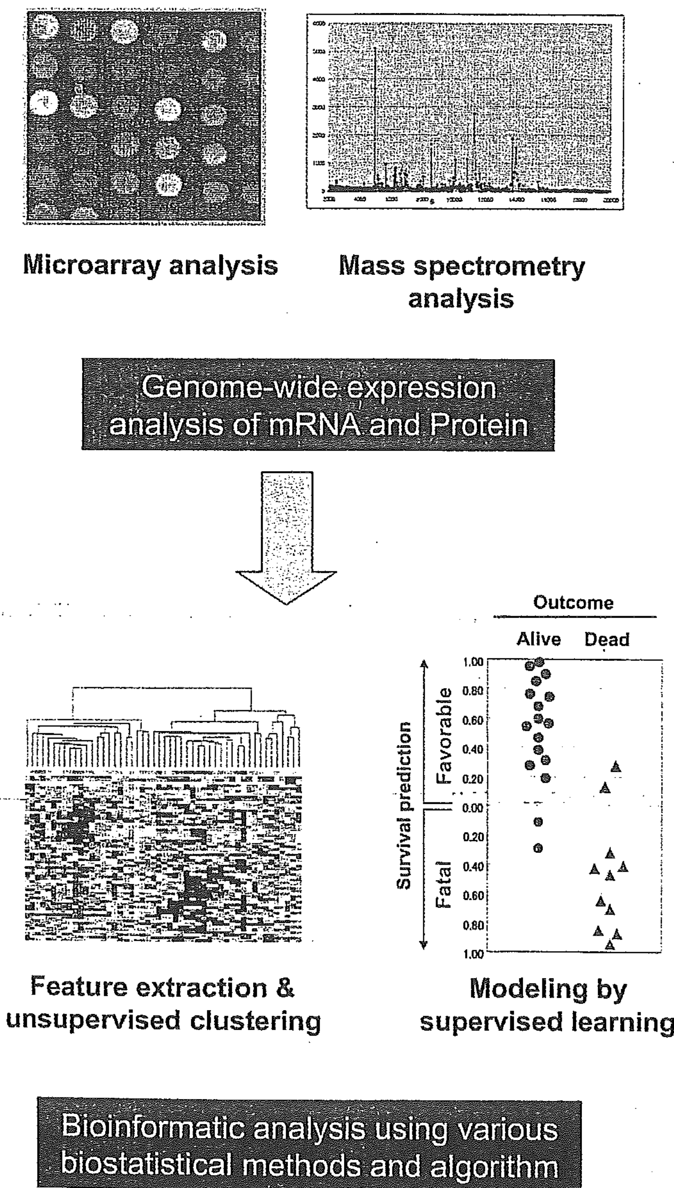

Fig. 3. Expression profiling analyzes of both transcriptome and proteome of lung cancers.

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certain period of time is insufficient in many respects. In this regard, there are two good examples of obvious benefit. Beer *et al.* identified prognosis-related genes using the *t*-test, and constructed a prognosis prediction classifier of adenocarcinomas by defining a risk index as a linear combination of gene-expression values for selected genes weighted by their estimated regression coefficient in the preceding COX regression analysis.[45] We have also succeeded in constructing individualized outcome classifiers of NSCLC, using gene expression profiling data- and a weighted-voting algorithm-based approach with genes selected by the signal-to-noise metric.[48] Reasoning from potential differences in outcome-related expression signatures in two major histologic types of NSCLC, histologic type-specific outcome classifiers were further constructed, showing an accuracy of more than 90% for the prediction of 5-year survival or death. A study by Endoh *et al.*, in which they selected 44 candidate genes from those previously identified through exploratory expression profiling analysis, which was validated by real-time reverse transcriptase-polymerase chain reaction using a completely separate large cohort, points to a sensible direction for the next step aimed at translation into the clinical setting.[52] It can be envisaged that the establishment of new disease classifications and highly accurate, individualized outcome classifiers for identifying those who are at high risk of future failure, and therefore most eligible for intensive adjuvant therapy with the intention of eradicating undetectable micrometastases, sources of future recurrence, would be a realistic immediate goal. In addition, expression profiling employing sophisticated bioinformatic analyzes should soon transform current strategies for clinical evaluation of drug combinations for cancer treatment, making it possible to provide more sound treatment decisions than in current practice.

In addition to analyzing global changes in the transcriptome, recent advances in biomedical technology have enabled new proteomic approaches to be developed. Yanagisawa *et al.* used matrix-assisted laser desorption/ionization mass spectrometry to identify more than 1 600 protein peaks from 79 cases of lung tumors.[53] Biostatistical selection of differentially expressed peaks allowed discrimination between normal and malignant tissue, subclassification of primary tumors, identification of patients with nodal involvement, and classification according to their prognosis based on 15 distinct peaks on mass spectrometry. Protein profiling of serum or plasma using high throughput mass spectroscopy has also been reported for lung cancers, distinguishing cases and controls based on key protein patterns with 50–70% detection rates and a 10% false positive rate.[54] Although anonymous peaks or protein profile spectra might be useful for classification, identification and functional investigation of these proteins are essential for understanding the underlying molecular biology. In this regard, we should mention that candidate molecular markers have been identified,[53,55] and far more examples will be found with recently developed sophisticated technologies for proteomic analysis, such as multidimensional liquid chromatography combined with tandem mass spectrometry. This should make it possible to further extend appropriate means of generating and exploiting new biological insights and clinical applications for the benefit of patients.

Conclusions

Accumulating evidence clearly indicates that perturbation of the integrity of the genome leads to the genesis and progression of lung cancers. It is becoming evident that the sequential accumulation of various genetic and epigenetic alterations confers various capabilities on lung cancer cells, including escape from growth inhibitory signals as well as from excess shortening of telomeres, resistance to apoptosis, sensitivity to stimuli for proliferation and angiogenesis, and invasive and metastatic characteristics. In the coming decades, taking advantage of the ample information on the human genome sequence as well as emerging new technologies including sophisticated informatics, we should be able to acquire a complete picture of lung cancer biology and revolutionize the prevention, diagnosis and treatment strategies for this presently fatal disease.

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ORIGINAL REPORT

Mutations of the Epidermal Growth Factor Receptor Gene Predict Prolonged Survival After Gefitinib Treatment in Patients With Non–Small-Cell Lung Cancer With Postoperative Recurrence

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
To evaluate the relationship between mutations of the epidermal growth factor receptor (*EGFR*) gene and the effectiveness of gefitinib treatment in patients with recurrent lung cancer after pulmonary resection.

Patients and Methods
We sequenced exons 18-21 of the *EGFR* gene using total RNA extracted from 59 patients with lung cancer who were treated with gefitinib for recurrent lung cancer. Gefitinib effectiveness was evaluated by both imaging studies and change in serum carcinoembryonic antigen (CEA) levels.

Results
EGFR mutations were found in 33 patients (56%). Of these mutations, 17 were deletions around codons 746-750 and 15 were point mutations (12 at codon 858, three at other codons), and one was an insertion. *EGFR* mutations were significantly more prevalent in females, adenocarcinoma, and never-smokers. Gefitinib treatment resulted in tumor shrinkage and/or CEA decrease to less than half of the baseline level in 26 patients, tumor growth and/or CEA elevation in 24 patients, and gefitinib effect was not assessable in nine patients. Female, never-smoking patients with adenocarcinoma tended to respond better to gefitinib treatment. Gefitinib was effective in 24 of 29 patients with *EGFR* mutations, compared with two of 21 patients without mutations ($P < .0001$). Of note, del746-750 might be superior to L858R mutations for prediction of gefitinib response. Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment ($P = .0053$).

Conclusion
EGFR mutations were a good predictor of clinical benefit of gefitinib in this setting.

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INTRODUCTION

Lung cancer has long been the leading cause of cancer death in North America. In 1998, it became the leading cause of cancer death in Japan, and now claims more than 55,000 lives annually.[1] Lung cancer is divided into two morphologic types: small-cell lung cancer and non–small-cell lung cancer (NSCLC). NSCLCs are further subdivided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Adenocarcinoma is the predominant histologic subtype, and is increasing among patients with lung cancer who are candidates for surgical treatment in Japan. In our institution, adenocarcinoma accounted for 76% of 407 patients who were operated on from 2001 through 2003. Adenocarcinomas are characterized by a high degree of morphologic heterogeneity. Analyses of various cancer-associated genes, including *K-ras*,[2] *p53*,[3,4] cyclin D1,[5] $p27^{Kip1}$,[6] and cyclooxygenase-2,[7]

suggests a different molecular pathway for carcinogenesis in lung adenocarcinomas at least partly accounts for this heterogeneity. In addition, the NSCLC frequently overexpresses receptors of the ErbB family, including the epidermal growth factor receptor (EGFR) encoded by ErbB1 (HER-1).[8,9]

EGFR is a 170 kd receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues upon binding of several specific ligands including epidermal growth factor and transforming growth factor alpha.[8] These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes.[8] Downstream pathways include ras-raf-MEK-ERK, phosphatidylinositol-3 kinase-Akt, and PAK-JNKK-JNK.[8]

Gefitinib is an orally bioavailable small molecule that specifically inhibits EGFR tyrosine phosphorylation.[10] Clinical trials revealed that there is significant variability in response to gefitinib. Good clinical responses have been observed most frequently in women, in nonsmokers, in patients with adenocarcinomas, and in Japanese patients.[11,12] However, it was not possible to predict gefitinib sensitivity by levels of EGFR overexpression as determined by immunohistochemistry[13] or immunoblotting.[14] The factors that determine gefitinib sensitivity have long been an enigma. Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib[15-17] or erlotinib, another EGFR TK inhibitor. Furthermore, the incidence of *EGFR* mutations is significantly higher in female, never-smoking, Japanese patients with adenocarcinoma.[15] These features coincide with those of good responders to gefitinib.

In this study, we studied patients who had recurrent disease after pulmonary resection for NSCLC and who were subsequently treated with gefitinib. We searched for mutations of the *EGFR* gene in tumor specimens taken at the time of surgery and we correlated *EGFR* mutations with gefitinib effectiveness, including tumor response and patient survival.

PATIENTS AND METHODS

Patients

Seventy-five patients were treated with gefitinib for their recurrent diseases after they had undergone surgery between 1999 and 2003. We studied 59 patients whose tumors were available for RNA extraction, which was a sole determinant of inclusion into the present study. There were 32 men and 27 women with ages ranging from 48 to 79 years. Fifty patients had adenocarcinomas, five had squamous cell carcinomas, three had large-cell carcinomas, and one had adenosquamous carcinoma. Eight patients had stage IA disease; seven stage IB; three stage IIA; five stage IIB; 24 stage IIIA; eight stage IIIB; and three stage IV at the time of surgery. Lobectomy had been performed in 57, and pneumonectomy and partial resection in one patient each. Four patients received postoperative adjuvant chemotherapy (two with oral uracil/tegafur and two with gemcitabine monotherapy). Forty patients had had chemotherapy before gefitinib treatment (23 patients, platinum doublet; 16 patients, monotherapy with vinorelbine or gemcitabine, one patient, oral uracil/tegafur). Gefitinib treatment with a daily dose of 250 mg was initiated between July 2002 and May 2004, with the median interval between operation and gefitinib treatment being 778 days (range, 107 to 1,931 days). Fifty patients had distant metastatic tumors, eight patients had pleural dissemination and malignant effusion, and one patient had hilar lymph-node metastasis at initiation of gefitinib treatment.

Molecular Analysis of Lung Cancer Specimens

After we obtained appropriate approval from the institution and written informed consent for comprehensive use of molecular and pathologic analysis from the patients, tumor samples were collected during surgery, rapidly frozen in liquid nitrogen and stored at −80°C. A surgical pathologist (Y.Y.) grossly dissected the frozen tumor specimens to enrich the tumor cell population as much as possible. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18-21) of the seven exons (exons 18-24) that code for TK domain of the *EGFR* gene (which includes all the mutations reported so far[15-17]) was amplified with primers F1 (5′-AGCTTGTGGAGCCTCTTACACC-3′) and R1 (5′-TAAAATTGATTCCAATGCCATCC-3′) in a one-step reverse transcription polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (Qiagen). The cDNA sequence of the *EGFR* gene was obtained from GenBank (accession number NM 005228). The RT-PCR conditions were: one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 60 seconds, followed by one cycle of 72°C for 10 minutes.

RT-PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST (basic local alignment search tool) and chromatograms by manual review. High-quality sequence variations found in both directions were scored as candidate mutations.

Definition of Effectiveness of Gefitinib

Because this study was a retrospective analysis of the daily clinical practice of oncology, the evaluation of tumor response could not be performed strictly according to predefined criteria, such as Response Evaluation Criteria in Solid Tumors (RECIST).[18] RECIST are not necessarily applicable or complete in such a context and the evaluation may instead be based on a subjective medical judgment that results from clinical and laboratory data.[18] Therefore, gefitinib treatment was judged as effective when the tumors showed at least a 30% decrease in tumor diameter in imaging studies. However, because of the nature of the study, confirmation of tumor response no less than 4 weeks apart, as in RECIST,[18] was not necessarily required.

As patients with recurrent lung cancer often do not have measurable disease, we also included change in serum carcinoembryonic antigen (CEA) level (cut off, 5 ng/mL) as an evaluation

criterion to avoid underestimating gefitinib effectiveness. CEA has been reported as a useful clinical therapeutic marker.[19] When the elevated CEA level decreased to a level less than half of the baseline level, gefitinib treatment was judged as effective. On the other hand, gefitinib treatment was judged as ineffective when the tumors showed any growth or a new lesion appeared in the imaging studies, or when the serum CEA level increased. Any patient who did not fit either of these criteria was classified as not assessable. All these evaluations were done before the *EGFR* gene analysis, without knowledge of mutational status of the *EGFR* gene.

Statistical Analysis

For comparisons of proportions, the χ^2 test or Fisher's exact test was used. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed by the log-rank test. The two-sided significance level was set at $P < .05$. To identify which independent factors had a joint significant influence on gefitinib effectiveness, the logistic regression modeling technique was used, and for multivariate analysis of the overall survival, the Cox proportional hazards modeling technique was applied. All analyses were performed using StatView version 5 (SAS institute Inc, Cary, NC) software on a Macintosh computer.

RESULTS

EGFR *Mutations*

Mutations of the *EGFR* gene were detected in 33 (56%) of 59 patients. Seventeen were deletions, 15 were point mutations, and one was an insertion. Details of these mutations are shown in Figure 1. As previously reported,[15-17] *EGFR* mutations were significantly associated with adenocarcinoma histology, female sex, and never-smoking status (Table 1). However, the mutations were not associated with the age or stage of the patients. Furthermore, median time from the original surgery to

I. Deletions **17**

```
719    740         750         760          860
        *           *           *            *
G • • • KIPVAIKELREATSPKANKEILD • • • FGLAKLLG

G • • • KIPVAIK-----TSPKANKEILD • • • FGLAKLLG          12
G • • • KIPVAIK---RPTSPKANKEILD • • • FGLAKLLG           1
G • • • KIPVAIK------APKANKEILD • • • FGLAKLLG           1
G • • • KIPVAIKE---PTSPKANKEILD • • • FGLAKLLG           1
G • • • KIPVAIKE------SKANKEILD • • • FGLAKLLG           2
```

II. Point mutations **15**

```
719    740         750         760          860
        *           *           *            *
G • • • KIPVAIKELREATSPKANKEILD • • • FGLAKLLG

Codon 719                                          2
C • • • KIPVAIKELREATSPKANKEILD • • • FGLAKLLG +E709H    1
A • • • KIPVAIKELREATSPKANKEILD • • • FGLAKLLG           1
Codon 858                                         12
G • • • KIPVAIKELREATSPKANKEILD • • • FGRAKLLG          10
G • • • KIPVAIKELREATSPKANKEILD • • • FGRAKLLG +A871G    1
G • • • KIPVAIKELREATSPKANKEILD • • • FGRAKLLG +E709G    1
Codon 861
G • • • KIPVAIKELREATSPKANKEILD • • • FGRAKQLG
```

III. Simple insertions **1**

```
        740         750         760         770
         *           *           *           *
G • • • KIPVAIKELREATSPKANKEILDEAYVMASVDNP
          ↑
          KIPVAI                                         1
```

Fig 1. Analysis of 33 epidermal growth factor receptor (EGFR) mutations in tyrosine kinase domain of the *EGFR* gene found in unselected patients with lung cancer.

Table 1. Incidence of EGFR Mutations and Clinical and Pathologic Features

Variable	EGFR Mutation No. of Patients	%	Wild-Type	*P*
All cases	33	56	26	
Sex				
Male	14	44	18	.0402
Female	19	70	8	
Age, years				
≤ 64	22	55	18	.8342
> 64	11	58	8	
Histologic type				
Adenocarcinoma	32	64	18	.0033
Nonadenocarcinoma	1	11	8	
Squamous cell carcinoma	0	0	5	
Large-cell carcinoma	0	0	3	
Adenosquamous carcinoma	1	100	0	
Smoking status				
Never smoker	20	71	8	.0227
Former or current smoker	13	42	18	
Stage				
I-II	12	50	12	.4472
III-IV	21	60	14	

Abbreviation: EGFR, epidermal growth factor receptor.

recurrence was almost identical in patients with *EGFR* mutations (362 days) and in those without *EGFR* mutations (363 days; $P = .8265$).

Clinical Improvement After Gefitinib Treatment

Forty-one of 59 patients had measurable disease at recurrence with imaging studies. Of these, 20 showed appreciable tumor shrinkage after gefitinib treatment, whereas 17 tumors increased in size, and there was no change in tumor size in four patients. All of these 20 tumors (pulmonary metastases in 11, pleural disseminated nodules in two, hepatic metastases in two, mediastinal lymph node swelling in two, brain metastases in two, and chest wall tumor in one) showed at least a 30% decrease in diameter. Figure 2 shows representative imaging studies. A computed tomography scan of the chest in patient L703 (73-year-old woman, adenocarcinoma) showed masses in the right-lower lobe and marked improvement 8 weeks after gefitinib initiation. A computed tomography scan of the liver in patient L1492 (52-year-old woman, adenocarcinoma) showed masses in the right lobe of the liver and dramatic improvement 10 days after gefitinib initiation. A large chest-wall mass in the left back of patient L1362 (62-year-old man, adenosquamous carcinoma) before gefitinib treatment almost disappeared 13 weeks after gefitinib initiation. A left-lung tumor in patient L1171 (70-year-old woman, adenocarcinoma) was smaller 6 weeks after gefitinib initiation.

CEA was above the upper normal limit (5 ng/mL) at baseline in 32 patients. Serum CEA level decreased to < 10%, < 50%, and to > 50% of the baseline level in three, 12, and five patients, respectively, whereas CEA level increased in 12 patients. When we combined the results of imaging studies with CEA and judged according to our criteria, gefitinib treatment was effective in 26 (52%), not effective in 24 (48%), and not assessable in nine patients (Table 2). There was a good correlation between these two examinations. The imaging studies and change in CEA levels did not conflict in any patients. In 17 patients with measurable diseases and whose baseline CEA level was elevated, the CEA level decreased in all 11 patients showing tumor shrinkage and increased in all five patients showing tumor growth, except for one patient whose tumors showed no change in size ($P < .001$, Fisher's exact test), supporting the validity of our criteria.

We searched for a relation between gefitinib effectiveness and various clinical and pathologic features (Table 2). Never-smokers and patients with adenocarcinoma had a significantly higher incidence of gefitinib effect. However, we could not detect significant difference in gefitinib sensitivity by sex or presence of prior chemotherapy, probably because of the small sample size, although there was a trend that female and chemotherapy-naïve patients were more responsive.

Relationship Between Clinical Response to Gefitinib Treatment and EGFR Mutations

The incidence of *EGFR* mutations in terms of response to gefitinib treatment as judged by imaging studies and CEA levels is shown in Table 3. Of 20 patients who showed tumor shrinkage, 19 (95%) had mutations of the *EGFR* gene. On the other hand, two (12%) of 17 patients whose tumors grew after gefitinib treatment harbored *EGFR* mutations ($P < .001$, Fisher's exact test). In Figure 2, patient L703, L1492, and L1362 had *EGFR* mutations (delE746-A750, L858R, and E746-S752insA, respectively). Of three, 12, and five patients whose CEA level decreased to less than 10%, less than 50%, and to more than 50% of the baseline level after gefitinib treatment, three (100%), 10 (83%), and four (80%) had *EGFR* mutations, respectively. On the other hand, of 12 patients whose CEA level increased, three (25%) had *EGFR* mutations ($P = .004$, Fisher's exact test).

When we used our criteria combining the results of imaging studies with CEA, gefitinib was effective in 24 (83%) of 29 patients with *EGFR* mutations, whereas it was effective only in two (10%) of 21 patients without *EGFR* mutations ($P < .0001$; Table 2). There were three patients with *EGFR* mutations (two with L858R and one with G719A) whose CEA level increased after gefitinib treatment but did not have measurable diseases. There were also two patients with *EGFR* mutations, one with L858R+E709H and one with I744-K745 ins KIPVAI whose tumor progressed.

Logistic regression analysis (Table 4) showed that *EGFR* mutation was the only significant factor contributing to gefitinib sensitivity.

On the other hand, patient L1171, who showed a decrease in size of multiple pulmonary metastatic nodules

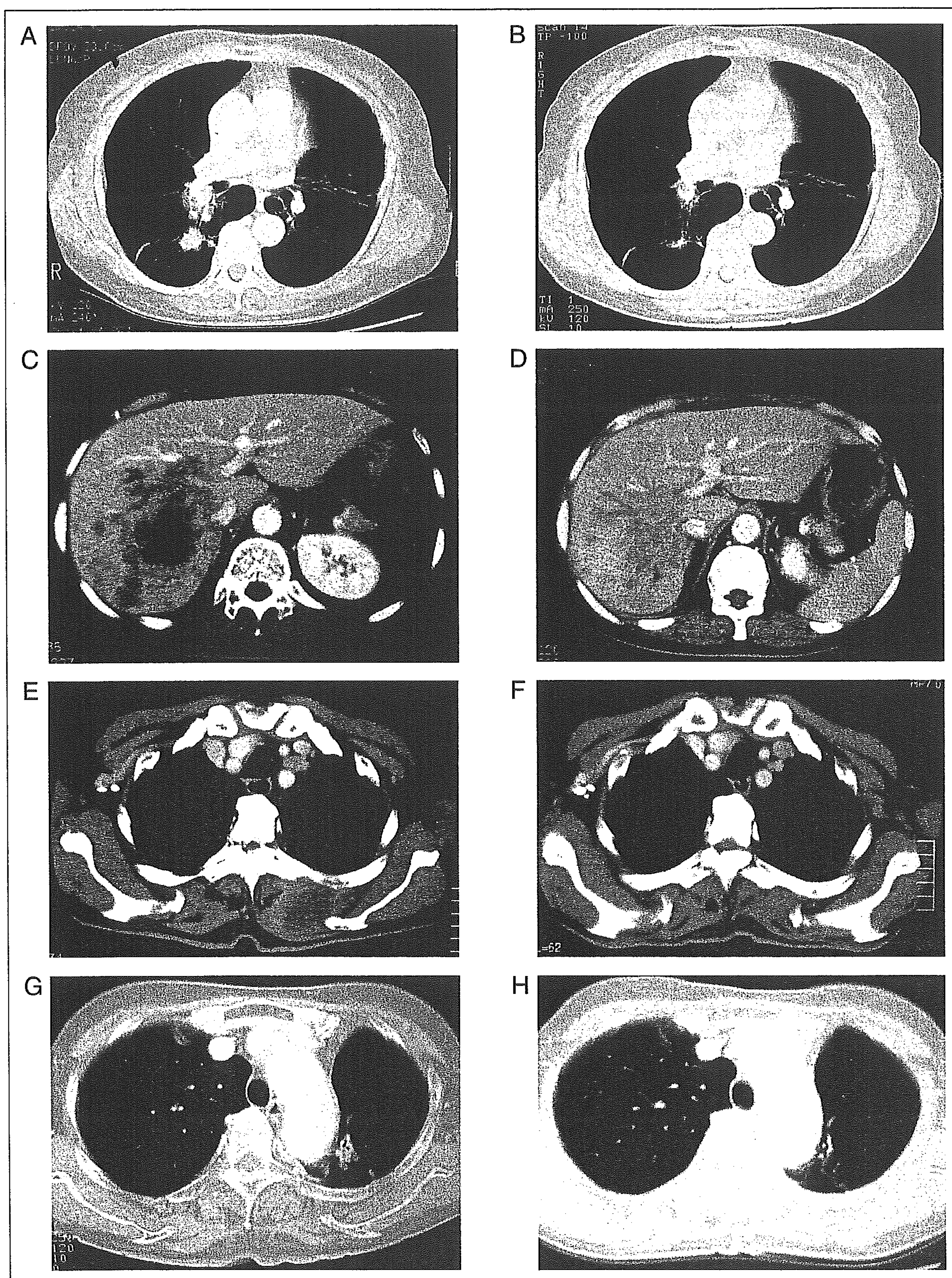

Fig 2. Examples of the response to gefitinib in representative four patients with recurrent non–small-cell lung cancer. Computed tomography (CT) scans before gefitinib treatment (A, C, E, G) and after the gefitinib was initiated (B, D, F, H) are shown. CT scans of patient L703 (A, B), patient L1492 (C, D), patient L1362 (E, F), and patient L1171 (G, H).

Table 2. Relation Between Gefitinib Effectiveness and Various Clinical and Pathologic Features

Variable	Effective: No. of Patients	Effective: %*	Not Effective	Not Assessable	P†
All patients	26	52	24	9	
Sex					
Male	11	41	16	5	.0842
Female	15	65	8	4	
Smoking status					
Never-smoker	17	68	8	3	.0235
Former or current smoker	9	36	16	6	
Histologic type					
Adenocarcinoma	25	58	18	7	.0313
Nonadenocarcinoma	1	14	6	2	
Prior chemotherapy					
Present	17	47	19	4	.2782
Absent	9	64	5	5	
EGFR mutation					
Mutation	24	83	5	4	< .0001
Deletion	16	100	0	1	.0108‡
Insertion	0	0	1	0	
Point mutation	8	67	4	3	
Wild-type	2	10	19	5	

Abbreviation: EGFR, epidermal growth factor receptor.
*Percentages were calculated excluding patients who were not assessable.
†*P* values were calculated excluding patients who were not assessable.
‡*P* value for Fisher's exact test comparing deletion mutants with the other mutants.

(Figs 2G and H) and a decrease in CEA level from 16.8 to 4.3 ng/mL, did not have *EGFR* mutations. In this patient, we extended our search for mutations to exons 22 and 23 of the *EGFR* gene, and still found none. Another patient without *EGFR* mutation in whom gefitinib was effective was a 59-year-old man who showed a decrease in serum CEA level from 10.6 to 1.5 ng/mL after 2 weeks of gefitinib treatment; this low level of CEA was maintained at least for 7 months.

When we further analyzed gefitinib response by classes of *EGFR* mutation, we found that there was a difference of response between patients with deletion mutations and those with the other types of mutations. Gefitinib was effective in all 16 patients with deletions, and effective in eight of 13 with other types of mutation ($P = .0108$).

Effect of *EGFR* Mutation on Patient Survival After Gefitinib Treatment

Patients with *EGFR* mutations survived for a significantly longer time calculated from the day of gefitinib initiation than those without *EGFR* mutations ($P = .0053$, log-rank test; Fig 3). Likewise, 26 gefitinib responders survived for a longer time than 24 nonresponders ($P = .0320$, log-rank test; not shown). Multivariate analysis revealed that *EGFR* mutation was the only factor that significantly and independently affected overall survival (Table 5). *EGFR* mutation class did not affect overall survival (not shown).

DISCUSSION

Recurrence after complete resection of NSCLC often presents as a form of distant metastases.[20] In clinical practice, chemotherapy is given to these patients except for a small number in whom re-resection of the tumor is indicated. Many studies have shown that chemotherapy prolongs survival and improves quality of life in unresectable stage IV tumors.[21] However, patients with unresectable tumors and patients with recurrent diseases may not be the same. There have been no large-scale randomized clinical trials addressing whether chemotherapy improves survival of patients with recurrence. Yoshino et al[22] found that chemotherapy for recurrence only tended to prolong survival in 118 of 468 consecutive patients who had recurrence after pulmonary resections. After introduction of gefitinib to clinical practice in 2002 in Japan, some patients with recurrent disease showed dramatic responses to gefitinib treatment, but many others did not respond. It has been unclear which patients respond to gefitinib and also whether gefitinib treatment prolongs survival in these patients.

Recent studies have showed striking correlation between gefitinib sensitivity and *EGFR* mutations both in vitro and in clinical studies.[15-17] Because this study was a retrospective analysis of response to gefitinib prescribed as routine care, judgment of gefitinib effectiveness tended to be less strict than that in a prospective clinical trial. Yet, changes in serum CEA level never conflicted with imaging studies. We were able to confirm a relation between *EGFR*

Table 3. Response to Gefitinib Treatment in 59 Patients With Recurrent Disease

CEA Level	Imaging Results: Shrinkage	No Change	Not Measurable	Growth	Total
Decreased					
<10% of the baseline	**3 (3)**				3 (3)
<50% of the baseline	**6 (5)**	**1 (1)**	**5 (4)**		12 (10)
>50% of the baseline	**2 (2)**		*3 (2)*		5 (4)
Not assessable	**9 (9)**	*3 (1)*	*3 (1)*	12 (2)	27 (13)
Elevated			7 (3)	5 (0)	12 (3)
Total	20 (19)	4 (2)	18 (10)	17 (2)	59 (33)

NOTE. Numbers in bold indicate that gefitinib treatment resulted in clinical improvement in these patients; numbers with underlines indicate the treatment resulted in progression of the disease; numbers in parentheses show number of patients with EGFR mutations in each category; and italicized numbers indicate that gefitinib treatment could not be assessed.
Abbreviations: EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen.

Table 4. Logistic Regression Analysis of Various Factors That Predict EGFR Effectiveness

Variable	Odds Ratio	95% CI	*P*
Sex			
Male/female	1.139	0.130 to 9.953	.9063
Smoking status			
Never/former/current	1.496	0.165 to 13.535	.7202
Histologic type			
Adenocarcinoma/ nonadenocarcinoma	1.727	0.091 to 33.33	.7159
Prior chemotherapy			
Yes/no	0.427	0.060 to 3.027	.3948
EGFR mutation			
Mutant/wild-type	40.000	6.024 to 2750	< .0001

Abbreviation: EGFR, epidermal growth factor receptor.

Table 5. Cox Proportional Hazards Model for Survival Analysis

Variable	Hazard Ratio	95% CI	*P*
Sex			
Female/male	0.359	0.068 to 1.900	.2280
Smoking status			
Never/former/current	0.511	0.092 to 2.854	.4445
Histologic type			
Adenocarcinoma/ nonadenocarcinoma	0.335	0.095 to 1.184	.0894
Prior chemotherapy			
Yes/no	0.653	0.222 to 1.923	.4397
Stage			
I-II/III-IV	0.848	0.322 to 2.232	.7380
Age, years			
> 64/≤ 64	0.964	0.342 to 2.717	.9457
EGFR mutation			
Mutant/wild-type	0.342	0.117 to 0.998	.0496

Abbreviation: EGFR, epidermal growth factor receptor.

mutations and gefitinib sensitivity in a slightly different clinical setting. We correlated *EGFR* mutations found in specimens taken at the time of surgery with response to gefitinib, often after several courses of cytotoxic chemotherapy for recurrent disease. Multivariate analysis revealed that *EGFR* mutation was the only independent predictor for gefitinib response among several allegedly contributing factors. As in previous studies, *EGFR* mutation was not a perfect predictor of gefitinib effectiveness.[15-17] Two patients without *EGFR* mutations showed response to gefitinib. It is not clear at this time whether *EGFR* mutations are present in other parts of the gene or whether mechanisms other than *EGFR* mutations govern sensitivity in these patients.

We found a significant difference in gefitinib sensitivity according to classes of *EGFR* mutations. All 16 patients with deletion mutants responded to gefitinib, compared with eight of 12 patients with other mutations (P = .0108). It is not clear whether this difference is based on differences in biologic activity of these mutant proteins.

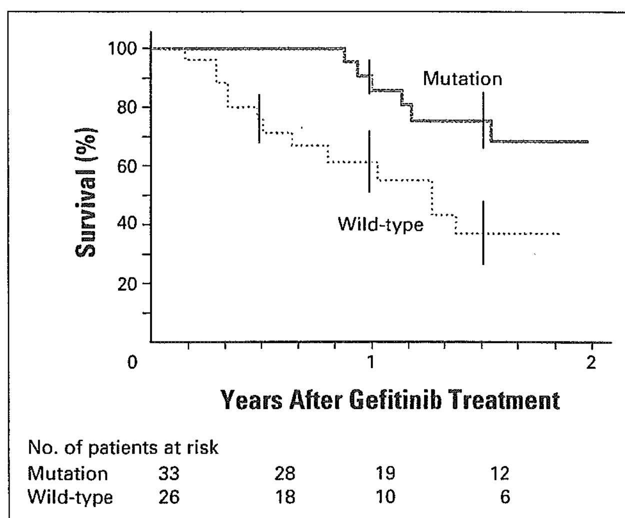

Fig 3. Effect of epidermal growth factor receptor mutations on survival, calculated from the day of initiating gefitinib treatment in patients who had recurrent disease after surgery (P = .0053, log-rank test).

Gefitinib sensitivity was essentially the same in COS cells transfected with L858R and in cells transfected with del L747-P753insS.[16] A more recent study showed that the tyrosine residue at codon 845 is highly phosphorylated in L858R mutants, but not in deletion mutants after epidermal growth factor binding.[23] This might explain the difference in gefitinib response between tumors with L858R and those with deletions.

Although our criteria for tumor response are soft, these are merely a surrogate marker for the effect on survival. We were able to show, for the first time, that *EGFR* mutation was the only significant and independent predictor for a prolonged survival after gefitinib treatment. In a previous study, we showed that *EGFR* mutation itself is not a predictor for better postoperative survival in 236 unselected patients with adenocarcinoma,[24] and in the present study, median disease-free interval was almost identical in patients with or without *EGFR* mutations. A recent placebo-controlled clinical trial showed that treatment with erlotinib, another oral EGFR TK inhibitor, significantly prolongs survival after first and second chemotherapy for NSCLC,[25] although *EGFR* mutation frequency is reported to be around 10% in Western countries.[15-17] This result is interpreted to mean that a subset of patients without mutations have also benefited from erlotinib therapy. The present study suggests that if patients were selected by presence of *EGFR* mutations, it would be possible to concentrate patients with benefits from gefitinib treatment, avoiding unnecessary adverse reactions such as fatal interstitial lung disease, which is relatively common in Japanese patients.[26] Furthermore, our results provide a basis for postoperative adjuvant gefitinib treatment in NSCLC patients with *EGFR* mutations, as adjuvant treatment is considered the earliest treatment of metastatic disease. These possibilities should be tested in future clinical trials.

It is common for patients to show progressive disease soon after presenting an initial striking response to

gefitinib. However, we could not detect any evidence that differences in classes of *EGFR* mutations are associated with duration of response (data not shown).

In conclusion, tumors with *EGFR* mutations showed good, but not perfect, correlation with clinical response in patients with postoperative recurrence of NSCLC. Furthermore, patients with *EGFR* mutations survived for a significantly longer period than those without *EGFR* mutations. Future clinical trials using gefitinib should examine *EGFR* mutations for effective selection of patients who are most likely to benefit from this molecular-targeted drug.

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Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Honoraria: Tetsuya Mitsudomi, AstraZeneca Japan, Bristol-Myers Squibb Japan, TAIHO Pharmaceutical. For a detailed description of this category, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and Disclosures of Potential Conflicts of Interest found in Information for Contributors in the front of each issue.

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Reduced expression of *Dicer* associated with poor prognosis in lung cancer patients

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Emerging evidence suggests that microRNA, which are well-conserved, abundant and small regulatory RNA, may be involved in the pathogenesis of human cancers. We recently reported that expression of *let-7* was frequently reduced in lung cancers, and that reduced *let-7* expression was significantly associated with shorter patient survival. Two members of the double-stranded RNA-specific endonuclease family, *Dicer* and *Drosha*, convert precursor forms of microRNA into their mature forms using a stepwise process. In the present study, we examined expression levels of these genes in 67 non-small cell lung cancer cases, and found for the first time that *Dicer* expression levels were reduced in a fraction of lung cancers with a significant prognostic impact on the survival of surgically treated cases. It should be noted that multivariate COX regression analysis showed that the prognostic impact of *Dicer* ($P = 0.001$) appears to be independent of disease stage ($P = 0.001$), while logistic regression analysis demonstrated that the higher incidence of reduced *Dicer* expression in poorly differentiated tumors remained significant even after correction for other parameters ($P = 0.02$). Given the fundamental and multiple biological roles of *Dicer* in various cellular processes, our results suggest the involvement of reduced *Dicer* expression in the development of lung cancers, thus warranting further investigations of the underlying mechanisms, which can be expected to enhance understanding of the molecular pathogenesis of this fatal cancer. (*Cancer Sci* 2005; 96: 111–115)

Introduction

Lung cancer is the leading cause of cancer-related death in Japan, as it is in many other economically developed countries.[1,2] The mutation, amplification and epigenetic changes of various genes, which may eliminate the normal function of gene products, have been identified in lung cancers, suggesting that they may be involved in pathogenesis.[3] In addition, emerging evidence suggests that microRNA, which constitute a well-conserved and abundant class of approximately 22-nucleotide regulatory RNA, could also be involved. We previously reported that the expression of *let-7* was frequently reduced in lung cancers, both *in vitro* and *in vivo*, and that reduced *let-7* expression was significantly associated with shorter patient survival.[4] Furthermore, we were able to demonstrate that over-expression of *let-7* resulted in significant inhibition of *in vitro* growth of lung cancer cells. In addition to our findings in lung cancers, a number of studies have dealt with microRNA alterations in other types of human cancers. These alterations include down-regulation of *miR15* and *miR16* in chronic lymphocytic leukemia as well as of *miR-143* and *miR-145* in human colon cancers.[5,6] The biological functions of microRNA are not yet fully understood, but it has been suggested that they play a role in the coordination of cell proliferation and cell death during development, in addition to their involvement in stress resistance.[7–9] This evidence appears to lend support to the notion that microRNA alterations could be involved in the genesis and/or progression of various human cancers.

A double-stranded RNA (dsRNA)-specific endonuclease converts precursor forms of microRNA into mature forms through a stepwise process, which includes the generation of ~70nt pre-microRNA with a characteristic hairpin structure from the longer nascent transcripts (pri-microRNA), and further processing into its mature form.[7–9] In humans, *Dicer* and *Drosha* are thought to collaborate in this stepwise processing of microRNA, with *Drosha* executing the initial step of microRNA processing in the nucleus,[10] and the resultant premicroRNA being exported to the cytoplasm where they are cleaved by *Dicer* to generate the final products of ~22nt.[11–16]

In this study, we posed a question as to whether expressions of *Dicer* and *Drosha*, which are essential for the processing of microRNA, are altered in lung cancers, and whether changes in the expression have any effect on clinicopathological features. To this end, we examined 67 non-small cell lung cancer (NSCLC) cases, which had undergone potentially curative surgical resection, by means of real-time RT-PCR. We report here for the first time that the reduced expression of *Dicer* in a significant fraction of lung cancers was associated with shorter postoperative survival.

Materials and methods

Patients and tumor sample preparations. NSCLC samples were obtained from 67 patients who underwent potentially curative resection at the Aichi Cancer Center Hospital (Nagoya, Japan) between January 1996 and January 1998. Approval from the institutional review board and the patients' written informed consent were obtained. Stages were determined after pathologic evaluation of resected specimens according to the International System for Staging Lung Cancer, revised in 1997. The cohort consisted of 41 males and 26 females, with age at diagnosis ranging from 32 to 84 years (median age, 62 years). Thirty-seven patients had stage I disease, 13 patients stage II and 17 patients stage III. There were 15 patients with poorly differentiated, 43 with moderately and nine patients with well-differentiated tumors. Thirty-eight patients were smokers, and the remaining 29 had never smoked. A surgical pathologist (Y.Y.) performed a gross examination of the tissue specimens immediately after surgical removal, and pieces of tumor tissue were carefully selected for maximum tumor content. Half of each piece was snap frozen in liquid nitrogen, followed by storage at −80°C until use, and the other half was fixed with prechilled acetone and embedded in paraffin for confirmation of tumor contents. Total RNA was isolated by means of the standard acid

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guanidinium isothiocyanate/cesium chloride procedure using ultracentrifugation.

Relative quantification by real-time RT-PCR analysis. First-strand cDNA were synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen, Carlsbad, CA, USA) and random hexamer primers (Roche Applied Science, Alameda, CA, USA). Real-time quantitative PCR amplification of a cDNA template corresponding to 20 ng total RNA was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7900-HT (Applied Biosystems). PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 55 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. Standard curves were plotted by using serially diluted cDNA of the BEAS2B lung epithelial cell line, and the expression level of the samples was normalized with that of *18S rRNA* and expressed as a ratio of the normalized expression of the gene of interest in a mixture of the RNA of 38 normal lung tissues. Expression levels in this mixture of normal lung RNA were adopted as 1. The primer pairs used for *Dicer* were 5′-GTACGACTACCACAAGTACTTC-3′ and 5′-ATAGTACACCTGCCAGACTGT-3′, for *Drosha*, 5′-GTGCTGTCCATGCACCAGATT-3′ and 5′-TGCATAACTCAACTGTGCAGG-3′; and for *18S rRNA*, 5′-AATCAGGGTTCGATTCCGGA-3′ and 5′-CCAAGATCCAACTACGAGCT-3′.

DNA methylation analysis of *Dicer*. Extraction of genomic DNA from tissues was performed according to standard procedures. Genomic DNA were treated with the bisulfite conversion method as described in a previous study.[17] After conversion, the promoter region of *Dicer* was amplified by PCR and every CpG site within the region was examined with direct sequencing for the presence of DNA methylation. The primer sequence was designed on the basis of the converted sense strand sequences without CpG sites 5′-TTTATTTGGGTTTGTAGTAGT-3′ and 5′-AACCCTATCCAATCACAAACT-3′. The PCR mixture contained 1 unit of Platinum Taq DNA polymerase (Invitrogen) together with 1 × PCR buffer, 2.5 m*M* of $MgCl_2$, 25 pmol of each primer, and 0.2 m*M* of dNTP. PCR conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 56°C for 30 s, at 72°C for 45 s, and at 72°C for 5 min. The PCR products were gel extracted (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA, USA) and sequenced directly with the aid of an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Statistical analysis. The following biostatistical analyses were performed with the STATA statistical package release 7.0 (STATA, College Station, TX, USA). The χ^2 goodness-of-fit test was used to analyze whether the distribution of expression levels at $\log_2$ of *Dicer* or *Drosha* could be fitted to the normal distribution. Student's *t*-test was employed to determine the best cut-off value for separating two characteristic groups in terms of gene expression levels. The association between expression levels of *Dicer* and *Drosha* was analyzed by computing the Pearson correlation coefficient, and associations between various clinicopathologic characteristics and the expression levels of *Dicer* and *Drosha* were examined by means of Fisher's exact test. The Kaplan-Meier estimates of overall survival time were compared by using the log-rank test. Cox regression analysis of factors potentially related to survival was used to identify which independent factors might jointly have a significant effect on survival. All tests were two-tailed, and the significance level was set at $P < 0.05$.

Results

Reduced expression of *Dicer* in NSCLC. We used real-time RT-PCR analysis to examine 67 NSCLC cases, which had undergone potentially curative resection, for *Dicer* and *Drosha* expression. We found that there was a significant correlation between *Dicer* and *Drosha* expression in NSCLC, with a Pearson correlation coefficient of 0.79 ($P < 0.001$; Fig. 1a).

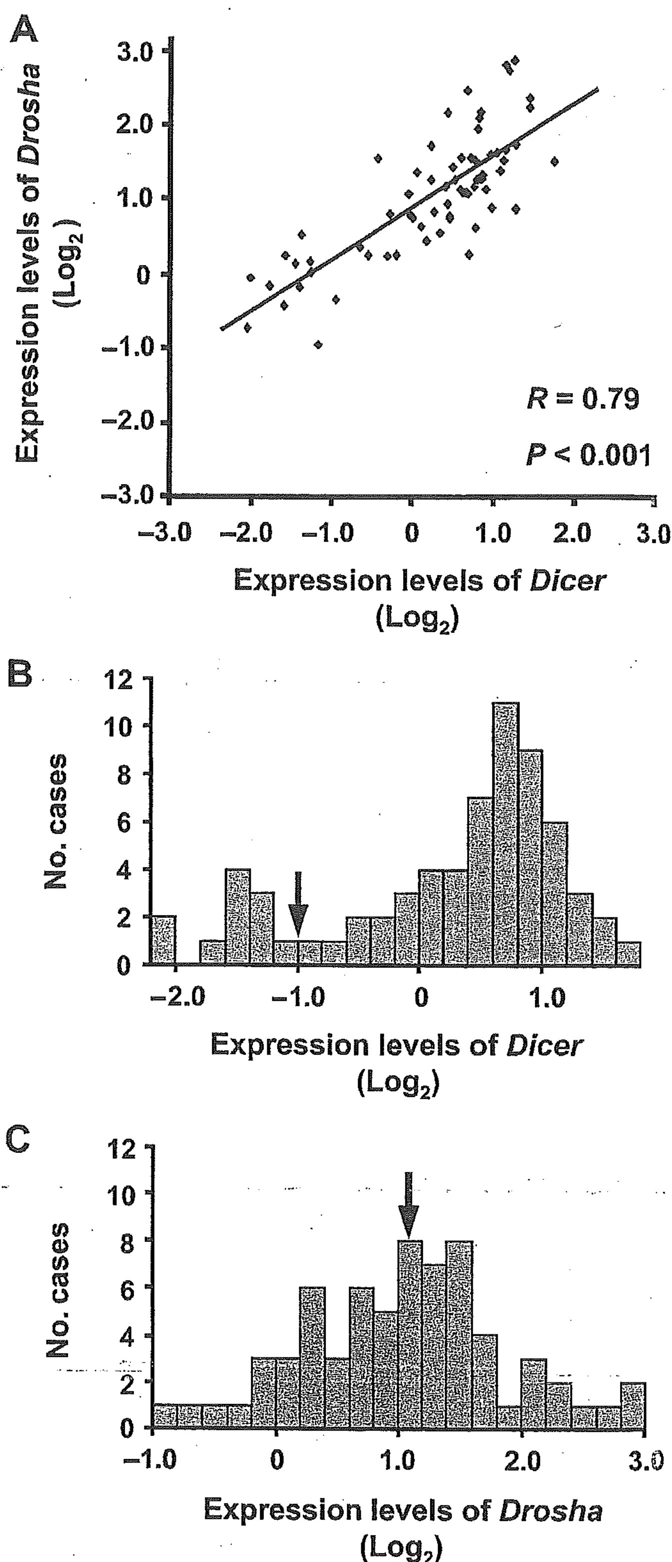

Fig. 1. Histograms of distributions of non-small cell lung cancers (NSCLC) according to their expression of double-stranded RNA-specific endonucleases mRNA. (A) Scattered plot analysis of expression levels of *Dicer* and *Drosha*. (B) Histogram of *Dicer* expression level at $\log_2$ value in NSCLC. With the threshold set at –1.0 of $\log_2$ value, patients were divided into two groups: low, with *Dicer* $\log_2$ value expression <–1.0; and high, with *Dicer* $\log_2$ value expression = –1.0. (C) Histogram of *Drosha* $\log_2$ value expression level in NSCLC. With the threshold set at 1.1 of $\log_2$ value, patients were divided into two groups: low, with *Drosha* $\log_2$ value expression <1.1; and high, with *Dicer* $\log_2$ value expression = 1.1. X-axis, $\log_2$ value; Y-axis, number of cases.

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able 1. Relationship between expression levels of *Dicer* and *Drosha* nd various clinicopathologic characteristics

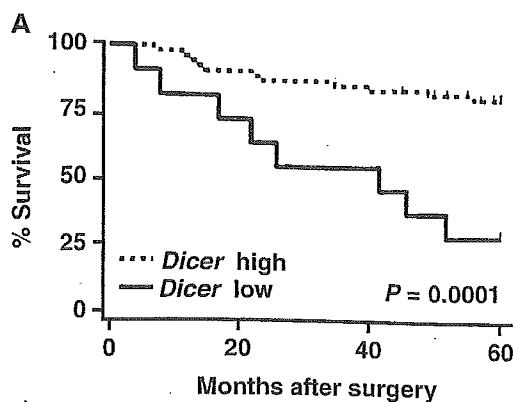
haracteristics	No. cases	*Dicer*			*Drosha*		
		High	Low	*P**	High	Low	*P**
,ge (years)							
≤62	34	29	5	0.75	22	12	0.03
>62	33	27	6		12	21	
ex							
Male	41	34	7	1.00	21	20	1.00
Female	26	22	4		13	13	
listology							
Squamous	11	10	1	0.68	5	6	0.75
Non-squamous	56	46	10		29	27	
moking history							
Smoker	38	31	7	0.75	19	19	1.00
Non-smoker	29	25	4		15	14	
isease stage							
I	37	31	6	1.00	20	17	0.63
II-III	30	25	5		14	16	
ifferentiation							
Poor	15	9	6	0.01	5	10	0.15
Well or moderate	52	47	5		29	23	

Two-sided Fisher's exact test.

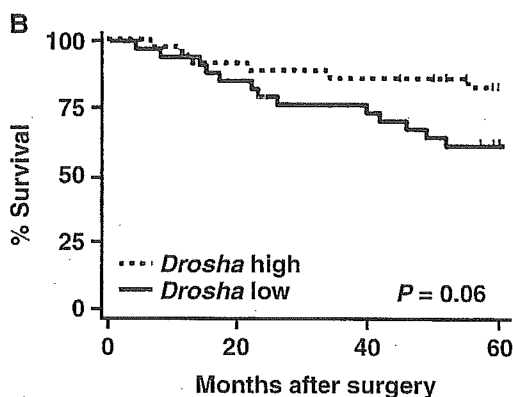
Iowever, close inspection of the distributions of their expression isclosed a clear difference. A histogram of the expression of)*icer* showed a frequency distribution with two prominent eaks at $\log_2$ values from −1.6 to −1.4 and from 0.6 to 0.8 Fig. 1B), which was in marked contrast to that of *Drosha* Fig. 1C). We used the χ^2 goodness-of-fit test to determine vhether the observed frequency distributions of expression of)*icer* and *Drosha* could be fitted to the normal distribution. In he case of *Dicer*, it was clear that the data were not normally listributed ($P < 0.001$). The Student's *t*-test was therefore used o identify the cut-off value with the highest potential for liscriminating two distinct groups in terms of *Dicer* expression. 'atients could be divided most clearly and consistently into wo groups with low and high expression of *Dicer* when the listribution threshold was set at −1.0 of the $\log_2$ ratio of *Dicer* xpression. In contrast to the findings for *Dicer*, the hypothesis hat the distribution of *Drosha* follows a normal distribution attern could not be rejected ($P = 0.97$). Accordingly, the nedian expression level (i.e. 1.1 of the $\log_2$ value) was chosen s the threshold value to be used for further analysis.

Relationships between expression of *Dicer* and *Drosha* and various linicopathologic characteristics. Our next investigation was conerned with whether expression levels of either *Dicer* or *Drosha* howed any relationship with the clinicopathologic characeristics of lung cancers, and found that there was a statistically ignificant association between *Dicer* expression levels and lifferentiation grade (Table 1). Cases with low *Dicer* expression howed significantly greater prevalence of poorly differentiated umors than those with high *Dicer* expression ($P = 0.01$), which vas also observed in the multivariate logistic regression analysis vith adjustment for all the variables analyzed in the univariate nalysis ($P = 0.02$).

Association between low *Dicer* expression and shortened ostoperative survival. The next question to be examined was vhether expression levels of *Dicer* and *Drosha* were associated vith patient survival after surgery. The Kaplan-Meier survival urves demonstrated that the probability of survival was ignificantly lower for the group of patients with low levels of)*icer* expression ($P = 0.0001$ by log-rank test; Fig. 2A), while ow expression of *Drosha* tended to be associated with a worse rognosis ($P = 0.06$ by log-rank test; Fig. 2B). Prognostic values f various factors were studied by univariate Cox regression



Numbers at risk				
Dicer high	56	51	47	37
Dicer low	11	8	6	3



Numbers at risk				
Drosha high	34	31	29	21
Drosha low	33	28	24	17

Fig. 2. Analysis of overall survival of patients with high or low expression of double-stranded RNA-specific endonucleases. (A) Kaplan-Meier survival curves for lung cancer patients, who were classified as showing either high or low *Dicer* expression. *Dicer* status was found to be strongly associated (log-rank, $P = 0.0001$) with patient survival. (B) Kaplan-Meier survival curve for lung cancer patients, who were classified as showing either high or low *Drosha* expression. *Drosha* status did not show a significant (log-rank, $P = 0.06$) relationship with patient survival. X-axis, length of survival after surgery; Y-axis, percentage of survivors.

analysis (Table 2). It was shown that, in addition to disease stage ($P = 0.003$), low *Dicer* expression was a significant predictive factor for poor prognosis ($P < 0.001$), whereas the *Drosha* expression level did not show a significant association with survival ($P = 0.07$).

The interrelationship of possible prognostic factors and survival was further analyzed by means of the Cox proportional hazards modeling using age, sex, histology, smoking history, disease stage and differentiation as well as expression levels of *Dicer* and *Drosha* as variables. As a result, reduced expression of *Dicer*, in addition to disease stage ($P = 0.001$), was identified as a significant and independent prognostic factor ($P = 0.001$) for surgically treated NSCLC patients after potentially curative resection. The hazard ratio for earlier death was 17.6 [95% confidence interval: 3.49–89.1] for low versus high expression levels of *Dicer*. These findings provided a strong indication that the expression levels of *Dicer* appeared to have a significant impact on the postoperative survival of NSCLC patients.

Table 2. Univariate and multivariate Cox regression analyses of the relationship between expression levels of *Dicer* and various clinical characteristics

Univariate analysis

Variables	HR [95% CI]	Unfavorable/Favorable	*P*
Age (years)	2.02 [0.80–5.14]	>62/≤62	0.14
Sex	2.79 [0.92–8.41]	Male/Female	0.07
Histology	1.37 [0.45–4.14]	Squamous/Non-squamous	0.58
Smoking history	2.52 [0.91–7.01]	Smoker/Non-smoker	0.08
Disease stage	4.61 [1.66–12.85]	II-III/I	0.003
Differentiation	2.55 [1.00–6.49]	Poor/Well or moderate	0.05
Dicer	5.18 [2.07–12.97]	Low/High	<0.001
Drosha	2.45 [0.93–6.45]	Low/High	0.07

Multivariate analysis

Variables	HR [95% CI]	Unfavorable/Favorable	*P*
Age (years)	1.86 [0.64–5.43]	>62/≤62	0.26
Sex	1.08 [0.25–4.64]	Male/Female	0.92
Histology	1.14 [0.29–4.51]	Squamous/Non-squamous	0.85
Smoking history	2.89 [0.75–11.1]	Smoker/Non-smoker	0.12
Disease stage	11.3 [2.87–44.3]	II-III/I	0.001
Differentiation	0.48 [0.12–1.86]	Poor/Well or moderate	0.29
Dicer	17.6 [3.49–89.1]	Low/High	0.001
Drosha	0.91 [0.25–3.36]	Low/High	0.88

HR, hazard ratio; CI, confidence interval.

Lack of DNA methylation of the *Dicer* promoter region. Because DNA methylation of the promoter region is thought to be significantly involved in transcriptional regulation,[18] we used the bisulfite conversion technique to study DNA methylation of the *Dicer* promoter region in 15 NSCLC (10 with low *Dicer* expression and five with high *Dicer* expression), as well as in three normal lung tissues. No methylation of the *Dicer* promoter region was found in any of the cases regardless of the level of *Dicer* expression, thus suggesting the involvement of other underlying mechanisms in the reduction of *Dicer* expression.

Discussion

In the study presented here, we have shown that the reduced expression of *Dicer* in a significant fraction of lung cancers is associated with shorter postoperative survival. To the best of our knowledge, ours is the first report of alterations of *Dicer* in human cancers. It should be noted that among the variables used in the multivariate COX regression analysis (i.e. age, sex, histology, smoking history, disease stage and differentiation as well as expression levels of *Dicer* and *Drosha*), *Dicer* appears to have a significant prognostic impact ($P = 0.001$) independent of disease stage ($P = 0.001$). Because logistic regression analysis demonstrated that the higher incidence of reduced *Dicer* expression in poorly differentiated tumors remained significant even after correction for other parameters ($P = 0.02$), one can speculate that prognostic impact of poor differentiation may well be represented by the presence of reduced expression of *Dicer*. Although our finding needs to be confirmed, for example on the cutoff value of *Dicer* expression level, by a further validation study using an independent and larger cohort, reduced expression of *Dicer* appears to be clinically useful for the prognosis of lung cancer patients. As for the underlying mechanisms involved in reduced *Dicer* expression in lung cancers, our study suggests that the involvement of hypermethylation of CpG sites in the promoter region is unlikely, so that other possibilities such as altered chromatin conformation and haploinsufficiency need to be pursued.[18] Corresponding to this, the frequent occurrence of loss of heterozygosity (LOH) on the long arm of chromosome 14, where *Dicer* resides, has been reported in lung cancers,[19,20] while a number of studies have also indicated that this chromosomal region is often affected in various other human cancers.[21–26] It is interesting that LOH on 14q appears to be related to tumor progression of colon cancer, with a higher incidence of this anomaly in metastatic sites than in primary tumors.[27]

Accumulating evidence supports the notion that the prognostic impact of reduced *Dicer* expression observed in our study might have a functional role in the development of lung cancers rather than being a mere surrogate marker. In correspondence with this, we recently reported that expression levels of *let-7* microRNA were frequently reduced in lung cancers, both *in vitro* and *in vivo*, and that lung cancer patients with reduced *let-7* expression had a significantly worse prognosis after potentially curative resection independent of disease stage.[4] We note that significant associations between reduced expression of *Dicer* and those of *let-7a-1* ($R = 0.66$, $P < 0.001$) and *let-7f-1* ($R = 0.65$, $P < 0.001$) were observed in this study. Since *Dicer* is required in the processing and generation of a fully mature form of microRNA,[11–16] it is not inconceivable that reduced *Dicer* expression may constitute an alternate post-transcriptional mechanism, which can also reduce expression levels of *let-7* and probably other microRNA in cancer cells.

In addition, other factors may underlie the potential biological effects of reduced *Dicer* expression in lung cancer cells. In fact, accumulating evidence suggests that the RNAi machinery may be functionally linked to the regulation of chromosome dynamics and genomic integrity. Furthermore, eukaryotic heterochromatin is characterized by a high density of repeats as well as by modified histones, and influences both gene expression and chromosome segregation. It was also found that deletion of *Dicer* in the fission yeast *Schizosaccharomyces pombe* resulted in the aberrant accumulation of complementary transcripts from centromeric heterochromatic repeats, loss of histone H3 lysine-9 methylation, and impairment of centromere function, resulting in defects in proper chromosome segregation.[28–32] The presence of marked aneuploidy is one of the key features of lung cancers,[33] while we previously reported the presence of a persistent increase in the rate of chromosomal losses and gains (i.e. chromosome instability, or CIN),[34] as well as of frequent impairment of mitotic checkpoints in lung cancer cell lines.[35] Therefore, the results of the present study raise the possibility that reduced *Dicer* expression in lung cancers may render cancer cells susceptible to chromosomal missegregation, in part because of the dysfunction of centromeres in the absence of a surveillance mechanism, which is the impairment of mitotic checkpoints.

It has also been suggested that the RNAi machinery might be involved in X inactivation and imprinting through sequence-specific histone modification and consequential DNA methylation and epigenetic silencing.[28] Therefore, it is possible that reduced expression of *Dicer* may affect such transcriptional regulation resulting from the altered activity of the RNAi machinery. In this connection, it should be noted that we previously found that loss of genomic imprinting is a frequent event in human lung cancers.[36,37] It would therefore be of considerable interest to study the involvement of a reduction in *Dicer* expression in relation to altered genomic imprinting in lung cancers.

In conclusion, we have been able to demonstrate for the first time that *Dicer* expression levels are reduced in some lung cancers with a significant prognostic impact on the survival of surgically treated cases. Given the fundamental and multiple biological roles of *Dicer* in various cellular processes, our results suggest the involvement of reduced *Dicer* expression in the development of lung cancers, and clearly warrant further investigations of the underlying mechanisms by which this alteration affects patient prognosis for a better understanding of the molecular pathogenesis of this fatal cancer. In addition, future studies to investigat

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whether altered *Dicer* expression is present in other types of human cancers should be both interesting and important.

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