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Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma

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Abstract: The charge of the Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma Committee was to evaluate the molecular biology, genomic changes, and proteomic findings in patients with bronchioloalveolar carcinoma compared with other types of lung cancer. The literature was reviewed and unpublished information was presented by the committee members at the session. The molecular biology studies have included findings on epidermal growth factor receptor (*EGFR*) mutations, p53 mutations, K-*ras* mutations, and loss of heterozygosity. The genomic changes have mostly focused on the mRNA expression arrays as well as protein studies. The current state of knowledge was reviewed, the missing information was acknowledged, and proposals for future research were identified.

Key Words: Lung neoplasm, Adenocarcinoma, Bronchioloalveolar, Adenocarcinoma, Carcinoma, Non-small cell lung cancer.

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Little information is available about p53 mutations and p53 protein overexpression detected by immunohistochemistry, microsatellite loss of heterozygosity (LOH), and K-*ras* mutations in adenocarcinoma of the bronchioloalveolar subtype, according to the last World Health Organization (WHO) pathological classification proposed in 1999. However, the frequency of these molecular abnormalities seems to increase during the multistep process of carcinogenesis of peripheral adenocarcinoma going from atypical alveolar hyperplasia adenocarcinoma to bronchioloalveolar carcinoma (BAC) and to invasive adenocarcinoma.

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ATYPICAL ADENOMATOUS HYPERPLASIA

There is an increasing body of evidence to support the concept of atypical adenomatous hyperplasia (AAH) as the precursor of at least a subset of adenocarcinomas.[1] AAH is most frequently detected in lungs from patients bearing lung cancers (9–20%), especially adenocarcinomas (up to 40%) compared with squamous cell carcinomas (11%).[2] Several molecular changes frequently present in lung adenocarcinomas are also present in AAH lesions, and there is further evidence that AAH may represent true preneoplastic lesions.[1] The most important findings are the presence in AAHs of K-*ras* (codon 12) mutations (40%),[3] loss of *LKB1* function (20%),[4] allelic losses in chromosomes 3p (20%), 9p ($p16^{INK4a}$, 10%), 9q (50%), 17q, and 17p (*TP53*, 5%),[5,6] and overexpression of cyclin D1 (70%), p53 (ranging from 10 to 60%),[7] and survivin (50%).[8] Despite the evidence that AAH is a precursor lesion for a subset of lung adenocarcinomas, there is general consensus that the pathogenesis of most adenocarcinomas is still unknown. The findings of relatively infrequent tyrosine kinase domain epidermal growth factor receptor (*EGFR*) mutations in AAH lesions (three out of 40 examined)[9,10] and no *EGFR* mutation[11,12] or relatively low frequency in true BACs of the lung[9] support the concept that genetic abnormalities of *EGFR* are not relevant in the pathogenesis of alveolar types of lung neoplasia. In addition, Tang et al.[13] recently reported that *EGFR* mutation is an early event in the pathogenesis of lung cancer, being identified in histologically normal epithelium of small bronchi and bronchioles adjacent to *EGFR* mutant lung adenocarcinomas in nine out of 21 (43%) patients examined, but in none of the patients without mutation in the tumor. These data further support the notion than AAH lesions are not involved in the pathogenesis of *EGFR* mutant lung adenocarcinomas.

BAC, ADENOCARCINOMA WITH BRONCHIOLOALVEOLAR FEATURES, AND ADENOCARCINOMA OF THE LUNG

The frequency of *EGFR* mutations has also been studied in patients with BAC, adenocarcinoma with BAC features, and adenocarcinomas of the lung. Although responses to EGFR tyrosine kinase inhibitors have been reported to be higher[14] and EGFR mutations were preferentially observed in tumors having BAC features,[12,15] we did not find association with the BAC subtype of adenocarcinoma in 97 cases from

the United States[11] using the criteria stated by the 1999 WHO classification of lung tumors.[16,17]

In addition to the WHO system, Noguchi et al.[18,19] have classified adenocarcinomas into different categories that have different frequencies of genetic changes. Koga et al.[20] reported that p53 mutations were present in approximately 0% of 17 pure BAC, 11% of 27 mixed adenocarcinoma with BAC features, and 48% of 101 invasive adenocarcinomas. Similar to the frequency of mutations, the frequency of p53 protein overexpression detected by immunohistochemistry increased from 6% (2/32 tumors) in pure BAC to 28% (27/133) in BAC with foci of active fibroblastic proliferation (Noguchi type C) and to 40% (14/35) in adenocarcinoma.[21] p53 mutation and protein overexpression were also correlated with the size and invasive component of small peripheral adenocarcinomas (≥5 mm: 41%; <5 mm: 20%).[22,23]

The frequency of allelic losses also increased significantly during malignant progression. According to Noguchi's classification,[18,19] frequencies of allelic losses at chromosomal loci 3p, 17p, 18q, and 22q were significantly lower in BAC with or without alveolar collapse (Noguchi types A and B, respectively) than in BAC with active fibroblastic proliferation (Noguchi type C) in a series of 66 small peripheral adenocarcinomas.[24]

The frequency and type of K-*ras* mutation in BAC are related to the cytological features (mucinous versus nonmucinous). This raises the question of whether the mucinous form might represent a biological entity separate from the nonmucinous form. Small series of tumors (all <50) from patients with adenocarcinoma of the lung show that the K-*ras* mutation is present in 73 to 100% of the mucinous types and that the type of the mutation was usually G to A (codon 12), whereas it was seen in 10 to 43% in the nonmucinous types, usually in G to T transversions.[25–27] Mutations at codon 12 of the K-*ras* oncogene were found in 39% of 41 AAH, 42% of 18 adenocarcinomas, and none of five lung neoplasms that were not adenocarcinomas. Of the patients with both an AAH and a synchronous adenocarcinoma, more than half did not have the mutation in both the AAH and the synchronous lung adenocarcinoma, suggesting that peripheral adenocarcinomas arise not always from AAH but sometimes directly from a background of field cancerization.[27]

Adenocarcinomas with BAC features are also characterized by an intense inflammatory reaction especially containing alveolar neutrophils and macrophages. Increased numbers of tumor-infiltrating neutrophils are linked to poorer outcomes in these patients.[28] Tumor environment drives local neutrophil recruitment and activation via C-X-C chemokine release such as interleukin-8 and epithelial cell–derived neutrophil activating protein 78 but also prolongs alveolar neutrophil survival through the production of soluble antiapoptotic factors (granulocyte–macrophage colony–stimulating factor and granulocyte colony–stimulating factor).[29,30] The mechanisms by which neutrophils influence the prognosis of adenocarcinoma with BAC features could be multiple. It has been postulated that the persistence of neutrophil alveolitis would result in persistent release of proinflammatory mediators such as cytokines, proteases, and reactive oxygen and nitrogen species that can damage DNA and activate oncogenes.[31,32] Among these factors released by neutrophils, hepatocyte growth factor seems to be particularly involved in the progression of these types of tumors, especially through its mitogenic and scattering properties, favoring c-Met expressing tumor-cell migration along the alveolar basal membrane.[33] Lastly, neutrophils might be involved in luminal tumor spread by promoting tumor-cell shedding (M. Wislez, AACR 2004), described pathologically as the presence of micropapillary clusters that are also involved in the mechanism of aerogenous progression.[34]

GENOMIC AND PROTEOMIC STUDIES OF BAC

As mentioned before, BAC is thought to arise from AAH and is potentially an intermediate to invasive adenocarcinoma. Extensive analyses of BAC using gene-expression profiling and proteomic-based studies have not yet been performed and are only available for limited numbers of these cancers. These types of studies may have the potential to define similarity or differences in the observed types of adenocarcinoma of the lung. Of particular interest is the potential regulatory pathway involved in the lepidic growth patterns of BAC, which is different from most other adenocarcinomas of the lung. The observation that some adenocarcinomas can exhibit regions of BAC provides complexity and has resulted in multiple pathological-based classifications.[14,16–19] Genomic studies have the potential to define the similarities as well as key differences between BAC, adenocarcinomas with BAC features, and adenocarcinomas of the lung.

Recent studies examining individual genes have hinted at differences between BAC and adenocarcinomas. The tumor suppressor in the lung cancer-1 gene encodes an adhesion molecule and is frequently associated with LOH at that locus in non–small-cell lung cancer. Both normal lung cells and BAC retain expression of tumor suppressor in lung cancer-1, whereas 63% of adenocarcinomas demonstrated decreased expression detected by immunohistochemistry.[35] BACs have very low p53 DNA mutation frequencies compared with adenocarcinomas of the lung.[20] LOH at the 3p FHIT loci was observed in 43% of BAC, and 12th codon K-*ras* mutations are detected in the mucinous form of BAC.[36] A comparative LOH study between 14 BAC and 20 stage I lung adenocarcinomas using nine chromosomal regions revealed that the most frequently affected chromosomal regions in BAC were 8q and 17p.[37] In adenocarcinomas of the lung, LOH at 1p, 3p, 7q, and 18q was more frequent than in BAC, and fractional allele loss was greater in adenocarcinomas of the lung than BAC.

Using immunocytochemistry to examine protein expression, detection of the thyroid transcription factor-1 (TTF-1), cytokeratin 7, and cytokeratin 20 were measured in both mucinous and nonmucinous BAC.[38] TTF-1 was detected in 17% of mucinous and 94% of nonmucinous BAC, cytokeratin 7 was detected in 100% of mucinous and 23% of nonmucinous BAC, and cytokeratin 20 was detected in 60% of mucinous and 0% of nonmucinous BAC.[38] In a study that examined MUC protein expression in AAH, BAC, and adenocarcinomas with BAC features, MUC1 decreased from

AAH to BAC and from BAC to adenocarcinoma, whereas MUC2, MUC5AC, MUC6, and depolarized MUC6 increased.[39] Alterations in p53 and the increased expression of MUC1, MUC5AC, and MUC6 were noted.

ADDITIONAL GENOMIC AND PROTEOMIC STUDIES

A comparison of normal lung tissue and BAC using oligonucleotide arrays was reported by Goodwin et al.[40] and identified 12 up-regulated and six down-regulated genes in the BAC tumors. Although this analysis provides some information, a comparison of BAC and adenocarcinomas was not included, which may be most relevant in defining critical genes involved in the development of these cancers. We used oligonucleotide arrays to examine gene expression in 14 BAC and 73 adenocarcinomas.[41] The most highly expressed genes that were significantly different between the BAC tumors and adenocarcinomas and higher in BAC included the surfactant pulmonary-associated proteins A1, A2, C and D, MUC1, TTF-1 and TTF-3, villin 2, and prostaglandin D2 synthetase. Interestingly, higher mRNA expression for both fos and jun B were detected in BAC, which may reflect an elevated AP-1 activity and upstream signaling events in these tumors. The higher level of expression of surfactant genes is consistent with the well-differentiated phenotypic characteristics of BAC. TTF-1 was the most differentially expressed gene between BAC and adenocarcinomas, consistent with the high TTF-1 protein expression reported in BAC.[38] Because of the small numbers of tumors for our analyses, it was not possible to divide the BAC tumors into separate categories such as mucinous, nonmucinous, and mixed histology. Although we found MUC1 mRNA present in both BAC and adenocarcinomas of the lung, the significantly increased expression in BAC is consistent with the higher MUC1 protein levels that have been reported in these tumors.[39]

Analysis of survival-related genes revealed prostaglandin D2 synthetase and neutrophil elastase 2 to be more highly expressed in BAC than the other adenocarcinomas. In contrast, much lower levels of vascular endothelial growth factor were detected in the BAC, possibly reflecting a lesser level of angiogenesis and hypoxia in these tumors relative to the adenocarcinomas. Adenocarcinomas also expressed increased levels of metallothionein 2A and thioredoxin reductase mRNA. We speculate that these genes may correspond to smoking-related alterations because these genes may change in response to reactive oxygen species originating from tobacco smoking or in response to inflammatory cells. Alternately, the expression of thioredoxin reductase and metallothionein 2 may reflect the higher rates of cell proliferation in the lung adenocarcinomas relative to BAC.

Few, if any, large-scale proteomic analyses of BAC have been reported. We examined the same BAC and lung adenocarcinomas for mRNA using oligonucleotide arrays and also at the protein level with two-dimensional gel electrophoresis and mass spectrometry.[42] A total of 682 protein spots were quantified, and 75 proteins were found to differ significantly ($p < 0.05$) between BAC and lung adenocarcinomas. Thirty-eight protein spots were successfully identified using mass spectometry. Of interest were the relatively higher expression of the ras-related protein RAB-14, glutathione-s-transferase-pi, cytokeratin 7, and three isoforms of the selenium-binding protein 1 in BAC compared with adenocarcinomas of the lung. Adenocarcinomas expressed higher levels of phosphoglycerate kinase 1, pyruvate kinase M1/M2, and stathmin (OP-18) compared with BACs. Increased phosphoglycerate kinase 1 is consistent with higher hypoxia-induced glycolysis in the adenocarcinomas of the lung relative to BAC.[42]

Future studies that include sufficient numbers of the various histological subtypes of BAC are needed to provide insight into the similarities and differences among these tumors and as compared with lung adenocarcinomas. The NCI Director's Challenge: Validation Study of Lung Adenocarcinomas will examine gene expression using Affymetrix 133A oligonucleotide arrays among approximately 500 tumors. Thus, a relatively large number of BACs will be included in this study, allowing potential gene pathways to be defined that may be relevant to our understanding of the growth- and cell-signaling systems in BAC. These analyses will also incorporate detailed pathologic assessment of each tumor so that the subtypes of each BAC can be compared. It is expected that these data, made available to the research community, will then stimulate further research into potential new markers for early diagnosis and possible therapeutic intervention strategies that may be effective for BAC.

FUTURE DIRECTIONS

The Committee responsible for Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma outlined studies that will provide further insights into BAC. The most important part of the meeting was partial agreement and understanding about the interpretation of the pathological classification. The participants in the meeting agreed on a common set of descriptors for the pathological interpretation of BAC that will be used more consistently in the future.

TABLE 1. Different Biological Properties in Atypical Adenomatous Hyperplasia, Pure Bronchioloalveolar Cancer, Adenocarcinoma with Bronchioloalveolar Cancer Features, and Adenocarcinoma

	Atypical Adenomatous Hyperplasia	Bronchioloalveolar Carcinoma	Adenocarcinoma with Bronchioloalveolar Carcinoma Features	Adenocarcinoma of the Lung
EGFR mutation	↓ <5%	10%		↑ 40%
TP53 mutations	Not reported	↓ 0%	↓ 10%	↑ 50%
p53 by immunohistochemistry	Not reported	↓ 5%	↑ 30%	↑ 50%

Upcoming technological improvements will provide additional insights into the biology of BAC. These will include the increasing ability to detect genetic changes in BAC and adenocarcinomas including, but not be limited to, *EGFR*, HER-2/neu, B-raf, K-*ras*, and *TP53*. In addition, there is the ability to detect genetic loss in the whole genome using studies with single-polynucleotide polymorphisms or array chromosomal genomic hybridization. There is increasing ability to use small and smaller amounts of DNA and DNA from paraffin-embedded tissues. Future studies will provide information on the degree of genetic changes seen in early lesions (<1cm) that are being detected more often as computerized tomographic scanning of the chest is becoming more widely used. These findings can be compared with the more advanced lesions. The genetic changes can also provide insights into the clonality of the BACs to determine whether the multiple lesions in the lungs arise from single or multiple clones. Table 1

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Common arm analysis: One approach to develop the basis for global standardization in clinical trials of non-small cell lung cancer

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Summary The global development of new anticancer treatments is desirable. However, whether results of clinical trials performed in one population can be fully extrapolated to another population remains in question. We retrospectively compared "common arms" of platinum-based doublet phase III trials among Japanese, European, and American patients with non-small cell lung cancer to develop the basis for global standardization in clinical trials. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors including socioeconomic factors, medical service background, and patient selection process for clinical trials may be consistent between geographically different oncology groups. The doses of docetaxel, gemcitabine, and vinorelbine were lower in Japanese studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations in these countries. Response rates seemed to be distributed randomly from one study to another, whereas patient survival might be better in Japanese studies. In conclusion, geographical differences in the dose of anticancer agents, response, survival and toxicity of lung cancer chemotherapy were actually observed. However, extrapolation of clinical data obtained in one country to another population and global clinical trials were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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1. Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and the incidence lung caner is increasing globally at a rate of 0.5% per year [1]. Lung cancer currently claims more than 55 000 lives annually in Japan, and this figure is projected to double during the next three decades due to the aging of the Japanese population [2]. Non-small cell lung cancer (NSCLC) comprises 80% of all lung cancers, and more than half of the patients with this disease are found to have developed distant metastases or pleural effusion at the time of the initial diagnosis. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely livelong [3].

The development of new anticancer agents and chemotherapeutic regimens are among the urgent tasks for medical oncologists who are involved in the treatment of lung cancer. Since it is time- and money-consuming work, the development of new agents and regimens is desirable on a global scale. Under the present situation in Japan, in that we are considerably behind with the development of new anticancer agents, it is worth evaluating the possibility that the results of clinical trials held outside Japan could be used for approval of these agents by the Japanese authorities. However, whether the results of clinical trials performed in one population can be fully extrapolated to another population remains in question due to the potential differences in trial designs, study-specific criteria, patient demographics, and population-related pharmacogenomics. According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline E5, Ethnic Factors in the Acceptability of Foreign Clinical Data, the impact of genetic and physiologic (intrinsic) factors and cultural and environmental (extrinsic) factors upon the efficacy and safety of anticancer agents at a particular dosage and dose regimen must be assessed for the application of new agent approval [4].

One approach to develop the basis for global standardization in clinical trials of anti-NSCLC agents is a planned comparative analysis of a "common arm" with similar eligibility, staging, response and toxicity criteria of prospectively designed and conducted separate phase III trials for the treatment of advanced NSCLC, although this approach may have potential limitation in comparability [5]. In this review we retrospectively compared the outcome of phase III trials conducted in Japan, Europe, and USA for chemotherapy doublet regimens using a platinum and a third-generation cytotoxic agent, including paclitaxel, docetaxel, gemcitabine, and vinorelbine.

2. Methods

Combinations of paclitaxel and carboplatin, docetaxel and cisplatin, gemcitabine and cisplatin, and vinorelbine and cisplatin were evaluated in patients with advanced NSCLC as the post-marketing sponsored phase III trials in Japan [6,7]. Phase III trials evaluating these regimens conducted outside Japan were identified by Medline searches. The selection criteria of phase III trials for this analysis were (1) first-line treatment for stage IIIB or IV NSCLC; (2) not intended for a special cohort of patients such as the elderly or those with poor performance status; (3) each arm included more than 120 patients; (4) tumor response was evaluated according to the World Health Organization (WHO) criteria, modified WHO criteria such as Eastern Cooperative Oncology Group (ECOG) criteria and Southwest Oncology Group (SWOG) criteria, or response evaluation criteria in solid tumors (RECIST) criteria; (5) toxicity was evaluated according to the WHO criteria or the National Cancer Institute-Common Toxicity Criteria (NCI-CTC). The dose and schedule of anticancer agents, patient demographics, treatment delivery, tumor response, patient survival, and toxicity were compared between common arms in separate phase III trials. To assess the influence of demographic variables on tumor response and survival, multiple linear regression analysis was performed as previously described [8].

3. Results

3.1. Taxane and platinum

The schedule was identical between the studies in both paclitaxel and carboplatin, and docetaxel and cisplatin combinations (Tables 1 and 2). The dose of paclitaxel ranged from 175 to 225 mg/m^2 without ethnic tendency. The dose of docetaxel was set to be 20% lower in a Japanese study [7] than that of USA studies [9,10]. This difference was mainly attributable to differences in the criteria of the maximum tolerated dose in phase I studies of docetaxel between Japan and the USA. Patient demographics were very similar among these studies. Response rates (RRs) in the combination of paclitaxel and carboplatin varied widely from 17% to 46%, and median survival time (MST) from 7.8 to 12.3 months. The RR and MST in Japanese and Greek studies appeared to be better than those in ECOG study, but did not differ from those in other American studies. A multiple linear regression analysis failed to show correlation between demographic variables and the RR or MST. In the docetaxel and cisplatin combination, the RR and survival in the Japanese study appeared to be better that those in the ECOG study [9], but similar to those in the other USA study [10].

Among paclitaxel and carboplatin studies, the incidence of grade 3-4 neutropenia and febrile neutropenia was higher in the Japanese study than in the other studies. The toxicity profile of the docetaxel and cisplatin combination was identical among all studies.

3.2. Gemcitabine and cisplatin

The dose of gemcitabine per one course was smaller in the Japanese study than in other studies outside Japan (Table 3). The RR in ECOG study was lower than that in European studies, while the MST of 14.8 months and 1-year survival rate of 60% in the Japanese study seemed higher than those in the other studies [6]. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.

Table 1 The combination of carboplatin and paclitaxel

Characteristics	Japan [6]	Greece [13]	Greece [14]	EU [18]	ECOG [19]	SWOG [19]	SWOG [5]	USA [20]	USA [12]
Chemotherapy dose									
CBDCA (AUC)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)
PTX (mg/m^2)	200 (day 1)	175 (day 1)	200 (day 1)	200 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)
Demographics (% not specified)									
No. of patients	145	185	252	309	290	206	182	190	345
Age (median) (range)	63 (33–74)	65 (30–83)	63 (31–81)	58 (27–76)	63 (30–85)	62 (26–80)	63 (28–80)	62 (28–80)	63 (31–85)
Female	32	14	13	17	38	30	37	34	39
PS 0–1	100	80	86	83	95	100	100	NA	91
Stage IV	81	49	62	68	86	88	87	77	78
Non-squamous	79	63	69	63	NA	NA	82	NA	81
Treatment delivery and efficacy (% not specified)									
Cycles (median)	3	NA	NA	4	4	NA	4	NA	6
Response rate (95% CI)	32 (25–40)	46 (39–53)	28 (22–34)	23 (20–30)	17 (13–21)	25 (19–31)	34 (27–41)	23 (17–29)	29 (24–34)
MST (month) (95% CI (month))	12.3 (NA)	11.0 (10–12)	10.4 (8.8–12)	8.2 (7.4–9.6)	8.1 (7.0–9.5)	8.6 (7.2–10.7)	9.0 (NA)	7.8 (NA)	9.9 (NA)
1-year survival (%)	51	43	42	32	34	38	37	32	42
Grade 3-4 toxicity (%)									
Neutropenia	88	14	15	51	63	57	NA	65	6
Febrile neutropenia	16	9	0	4	4	2	3	NA	NA
Thrombocytopenia	11	2	2	2	10	10	8	8	NA
Neuropathy	5	26	8	9	10	13	16	5	1

Table 2 The combination of cisplatin and docetaxel

Characteristics	Japan [7]	ECOG [9]	USA [10]
Chemotherapy dose			
CDDP (mg/m^2)	80 (day 1)	75 (day 1)	75 (day 1)
DTX (mg/m^2)	60 (day 1)	75 (day 1)	75 (day 1)
Demographics (% not specified)			
No. of patients	151	289	408
Age (median) (range)	63 (30–74)	63 (34–84)	61 (30–81)
Female	36	37	28
PS 0–1	96	94	96
Stage IV	100	86	67
Non-squamous	89	NA	68
Treatment delivery and efficacy (% not specified)			
Cycles (median)	3	4	5
Response rate (95% CI)	37 (29–45)	17 (12–21)	32 (27–36)
MST (month) (95% CI (month))	11.3 (NA)	7.4 (6.6–8.8)	11.3 (10.1–12.4)
1-year survival	48	31	46
Grade 3-4 toxicity (%)			
Neutropenia	74	69	75
Febrile neutropenia	2	11	5
Thrombocytopenia	1	3	3
Neuropathy	0	5	4

The toxicity was similar among many studies except for the gemcitabine and cisplatin arm of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT) study [11], where the incidence of grade 3-4 neutropenia and thrombocytopenia was reported to be about one tenth of that in other studies (Table 3).

3.3. Vinorelbine and cisplatin

The dose of vinorelbine per one course was also smaller in the Japanese study than in other studies outside Japan (Table 4). The RR in the Greek study was higher than that in an American study. There was no difference in survival for this combination among all studies. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.

Grade 3-4 neutropenia was less common in the Greek study than in other studies, but the frequency of febrile neutropenia in that study was intermediate among studies.

4. Discussion

This study showed that geographical differences in the outcome of lung cancer chemotherapy may be present. However, extrapolation of clinical data in a country to another population and global clinical trials were considered possible with adequate considerations as discussed below.

The dose of third-generation cytotoxic agents was smaller in Japanese studies than in European and American studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations of these countries. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors may be comparable and consistent between geographically different oncology groups. These factors include socioeconomic factors, medical service background, and patient selection process for clinical trials.

RRs in phase III studies including third-generation cytotoxic agents seemed to be distributed randomly from one study to another, whereas patient survival might have been better in Japanese studies. The Japanese phase III trials were performed in academic institutes, including university-affiliated hospitals, cancer center hospitals, and central city general hospitals. Thus, the distribution of patients selected for Japanese phase III trials may be skewed, in that they were in good general condition, although established prognostic factors in patients with NSCLC were almost identical among Japanese and non-Japanese studies. In addition, better survival among Japanese patients may be attributable to true ethnic differences. One possibility is the relatively high frequency of non-squamous histology in Japanese studies, but the reason is largely unknown.

The severity and frequency of common toxicity were comparable in all these phase III studies with a few exceptions. The incidence of grade 3-4 neutropenia was only 5–6% in the carboplatin and paclitaxel arm of the INTACT2 study [12] and in the cisplatin and gemcitabine arm of the INTACT1 study [11], both of which were sponsored by one pharmaceutical company. Similarly, the incidence of neutropenia was lower in Greek studies [13–15] than in other studies. These differences in the incidence of toxicity may be associated with the frequency of monitoring, including patient hospital visits and blood cell count and chemistry evaluation.

Table 3 The combination of cisplatin and gemcitabine

Characteristics	Japan [6]	Italy [21]	Spain [22]	EORTC [23]	EU [11]	ECOG [9]	EU + USA [24]
Chemotherapy dose							
CDDP (mg/m^2)	80 (day 1)	100 (day 2)	100 (day 1)	80 (day 1)	80 (day 1)	100 (day 1)	100 (day 1)
GEM (mg/m^2)	1000 (day 1, 8)	1000 (day 1, 8, 15)	1250 (day 1, 8)	1250 (day 1, 8)	1250 (day 1, 8)	1000 (day 1, 8, 15)	1000 (day 1, 8, 15)
Demographics (% not specified)							
No. of patients	146	155	182	160	363	288	260
Age (median) (range)	61 (34–74)	62 (28–76)	59 (33–75)	57 (28–75)	61 (33–81)	64 (32–87)	62 (36–88)
Female	33	37	12	29	28	37	30
PS 0–1	100	93	85	89	90	95	80
Stage IV	81	79	77	79	69	86	67
Non-squamous	81	68	55	74	71	NA	70
Treatment delivery and efficacy (% not specified)							
Cycles (median)	3	NA	4	5	6	3	4
Response rate (95% CI)	30 (23–38)	38 (30–46)	42 (35–50)	37 (29–45)	47 (42–53)	22 (17–27)	30 (25–36)
MST (month) (95% CI (month))	14.8 (NA)	8.6 (NA)	9.3 (8.1–10.5)	8.9 (7.8–10.5)	10.9 (NA)	8.1 (7.2–9.4)	9.1 (8.3–10.6)
1-year survival	60	33	38	33	44	36	39
Grade 3-4 toxicity (%)							
Neutropenia	63	40	32	43	5	63	57
Febrile neutropenia	2	1	4	3	NA	4	5
Thrombocytopenia	35	64	19	36	6	50	50

Table 4 The combination of cisplatin and vinorelbine

Characteristics	Japan [6]	Greece [15]	France [25]	EU [26]	SWOG [19]	USA [10]
Chemotherapy dose						
CDDP (mg/m^2)	80 (day 1)	80 (day 8)	100 (day 1)	120 (day 1)	100 (day 1)	100 (day 1)
VNR (mg/m^2)	25 (day 1, 8)	30 (day 1, 8)	30 (day 1, 8, 15, 22)	30 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)
Demographics (% not specified)						
No. of patients	145	204	156	206	202	404
Age (median) (range)	61 (28–74)	64 (46–75)	57 (39–74)	59 (NA)	61 (32–83)	61 (35–80)
Female	30	25	21	12	33	25
PS 0–1	100	90	92	80	100	96
Stage IV	83	64	86	59	89	67
Non-squamous	81	54	76	44	NA	65
Treatment delivery and efficacy (% not specified)						
Cycles (median)	3	4	4	3	NA	4
Response rate (95% CI)	33 (25–41)	39 (33–46)	36 (28–43)	28 (22–34)	28 (22–34)	25 (20–29)
MST (month) (95% CI (month))	11.4 (NA)	9.7 (8.3–11.2)	9.6 (8.1–12.2)	9.3 (NA)	8.1 (6.7–9.6)	10.1 (9.2–11.3)
1-year survival	48	41	42	37	36	41
Grade 3-4 toxicity (%)						
Neutropenia	88	37	83	79	76	79
Febrile neutropenia	18	11	22	4	1	5
Thrombocytopenia	1	6	3	3	4	4

Anticancer agents are considered to be sensitive to ethnic factors, because of a steep pharmacodynamic curve for both efficacy and safety, a narrow therapeutic dose range, non-linear pharmacokinetics, their metabolic enzymes with the potential for drug-drug interaction, and these enzymes with the potential for ethnically variable activity caused by genetic polymorphism. Thus, bridging studies using pharmacologic endpoints are extremely important to apply efficacy, safety, and dose data from one place to another [16]. These pharmacologic studies can be incorporated into phase I trials and, when it is necessary, phase II trials. Furthermore, the current study suggests that, once the pharmacological property and recommended dose of a new cytotoxic agent are established in one country, the outcome of randomized controlled trials developed in other countries can be extrapolated to the population.

We defined ethnic populations in the current study according to the country where the study was performed. However, patients enrolled into multicenter European and North American studies may include patients with a diverse ethnicity. It would be greatly interesting to see RR, MST and toxicity in subgroups of patients with different ethnicity in those trials, although there has been no such data published.

Randomization of patients in a trial guarantees the comparability between treatment arms within the trial, but not between treatment arms in different trials. Thus, it is impossible to compare the outcome of different trials exactly. Nevertheless, we frequently refer to the outcome of trials performed outside Japan and they furnish us with much information. To compensate this limitation, we tried to compare patient characteristics between trials, but other factors including the frequency of monitoring may also affect the outcome greatly. The number of combination regimens evaluated in this study is insufficient, but no large scale Japanese trials of other combination regimens have been available so far.

This study failed to demonstrate whether this approach to clinical trial analysis was really helpful. For future clinical trials, consistency in monitoring, as well as the use of the common toxicity and response criteria, is important to keep comparability between trials. A meta-analysis using individual patient data may be more useful than a subgroup analysis within a trial to compare the outcomes between ethnic subgroups with adequate statistical power.

A phase II study of gefitinib in patients with advanced NSCLC who had previously received one or two chemotherapy regimens was conducted in cooperation with 43 hospitals across Europe, Australia, South Africa, and Japan. The population was prospectively stratified into Japanese and non-Japanese patients to investigate whether there were any differences between the two patient populations with respect to efficacy [17]. This study clearly showed that a global study of NSCLC using the same protocol was completed, and this global strategy was an effective method to speed up the development of a new anticancer agent in Japan. In addition, the stratification by the county or ethnicity is important in a global study of an investigational new drug to investigate geographical differences in efficacy and toxicity.

In conclusion, the dose of anticancer agents, RR, survival and toxicity of lung cancer chemotherapy showed some differences among Japanese, European, and USA studies. However, extrapolation of clinical data in a country to another population and global clinical trials including many countries were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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Establishment of a human non-small cell lung cancer cell line resistant to gefitinib

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The epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitor gefitinib (Iressa®, ZD1839) has shown promising activity preclinically and clinically. Because comparative investigations of drug-resistant sublines with their parental cells are useful approaches to identifying the mechanism of gefitinib resistance and select factors that determine sensitivity to gefitinib, we established a human non-small cell lung carcinoma subline (PC-9/ZD) that is resistant to gefitinib. PC-9/ZD cells are ~180-fold more resistant to gefitinib than their parental PC-9 cells and PC-9/ZD cells do not exhibit cross-resistance to conventional anticancer agents or other tyrosine kinase inhibitors, except AG-1478, a specific inhibitor of EGFR. PC-9/ZD cells also display significant resistance to gefitinib in a tumor-bearing animal model. To elucidate the mechanism of resistance, we characterized PC-9/ZD cells. The basal level of EGFR in PC-9 and PC-9/ZD cells was comparable. A deletion mutation was identified within the kinase domain of *EGFR* in both PC-9 and PC-9/ZD, but no difference in the sequence of *EGFR* cDNA was detected in either cell line. Increased EGFR/HER2 (and EGFR/HER3) heterodimer formations were demonstrated in PC-9/ZD cells by chemical cross-linking and immunoprecipitation analysis in cells unexposed to gefitinib. Exposure to gefitinib increased heterodimer formation in PC-9 cells, but not in PC-9/ZD cells. Gefitinib inhibits EGFR autophosphorylation in a dose-dependent manner in PC-9 cells but not in PC-9/ZD cells. A marked difference in inhibition of site-specific phosphorylation of EGFR was observed at Tyr1068 compared to other tyrosine residues (Tyr845, 992 and 1045). To elucidate the downstream signaling in the PC9/ZD cellular machinery, complex formation between EGFR and its adaptor proteins GRB2, SOS, and Shc was examined. A marked reduction in the GRB2-EGFR complex and absence of SOS-EGFR were observed in PC-9/ZD cells, even though the protein levels of GRB2 and SOS in PC-9 and PC-9/ZD cells were comparable. Expression of phosphorylated AKT was increased in PC-9 cells and inhibited by 0.02 μM gefitinib. But the inhibition was not significant in PC-9/ZD cells. These results suggest that alterations of adaptor-protein-mediated signal transduction from EGFR to AKT is a possible mechanism of the resistance to gefitinib in PC-9/ZD cells. These phenotypes including EGFR–SOS complex and heterodimer formation of HER family members are potential biomarkers for predicting resistance to gefitinib.
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Key words: resistance; gefitinib; EGFR; Grb2; SOS; non-small cell lung cancer

Chemotherapy has played a central role in the treatment of patients with inoperable NSCLC for over 30 years, although its efficacy seems to be of very limited value.[1,2] Human solid tumors, including lung cancer, glioblastoma, breast cancer, prostate cancer, gastric cancer, ovarian cancer, cervical cancer and head and neck cancer, express epidermal growth factor receptor (EGFR) frequently, and elevated EGFR levels are related to disease progression, survival, stage and response to therapy.[2–10] The therapies directed at blocking EGFR function are attractive.

Interest in target-based therapy has been growing ever since the clinical efficacy of STI-571 was first demonstrated,[11–13] and small molecules and monoclonal antibodies that block activation of the EGFR and HER2 have been developed over the past few decades. The leading small-molecule EGFR tyrosine-kinase inhibitor, gefitinib (Iressa®, ZD1839), has shown excellent antitumor activity in a series of Phase I and II studies,[14,15] and Phase II international multicenter trials (Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and 2) yield an overall RR of 11.8–18.4% and overall disease control rate of 42.2–54.4% (gefitinib 250 mg/day) in patients with advanced non-small cell lung cancer (NSCLC) who had undergo at least 2 previous treatments with chemotherapy. INTACT 1 and 2 ('Iressa' NSCLC Trials Assessing Combination Therapy) have demonstrated that gefitinib does not provide improvement in survival when added to standard first line platinum-based chemotherapy *vs.* chemotherapy alone in advanced NSCLC.[16,17] Two small retrospective studies reported recently that activating mutation of *EGFR* correlate with sensitivity and clinical response to gefitinib and erlotinib.[18–20] Although information of *EGFR* mutation may enable to identify the subgroup of patients with NSCLC who will respond to gefitinib and erlotinib, it would be expected that acquired resistance would develop in such patients after treatment. The problem of acquired resistance to gefitinib might be growing, but there has been no preclinical research about the mechanism of developing resistance to gefitinib. We established resistant subline using PC-9 that is highly sensitive to gefitinib.

Establishment of drug-resistant sublines and comparative investigations with their parental cells to identify their molecular, biological and biochemical properties are useful approaches to elucidating the mechanism of the drug's action. Our study describes the establishment of a gefitinib-resistant cell line and its characterization at the cellular and subcellular levels. The PC-9/ZD cell line is the first human NSCLC cell line resistant to gefitinib ever reported. PC-9 is a lung adenocarcinoma cell line that is highly sensitive to gefitinib at its IC_{50}-value of 0.039 μM, but the PC-9/ZD subline, which has a level of EGFR expression comparable to that of PC-9 cells, is specifically resistant to gefitinib. Thus, PC-9 and PC-9/ZD cells will provide useful information about the mechanism of developing resistance to gefitinib and molecules as surrogate markers for predicting chemosensitivity to gefitinib.

Material and methods

Drugs and cells

Gefitinib(*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy] quinazolin-4-amine) was supplied by AstraZeneca Pharmaceuticals (Cheshire, UK). AG-1478, AG-825, K252a, staurosporin, genistein, RG-14620 and Lavendustin A were purchased from Funakoshi Co. Ltd (Tokyo, Japan).

NSCLC cell line PC-9 (derived from a patient with adenocarcinoma untreated previously) was provided by Prof. Hayata of Tokyo Medical University (Tokyo, Japan).[21] PC-9 and PC-9/ZD cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (GIBCO-BRL, Grand Island, NY), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively: GIBCO-BRL) in a humidified atmosphere of 5%

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CO_2 at 37°C. Gefitinib-resistant PC-9/ZD cells were selected from a subculture that had acquired resistance to gefitinib using the following procedure. Cultured PC-9 cells were exposed to 2.5 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 24 hr and then washed and cultured in medium containing 0.2 μM gefitinib for 7 days. After exposure to gefitinib, they were washed and cultured in drug-free medium for 14 days. When variable cells had increased, they were seeded in medium containing 0.3–0.5 μM of gefitinib on 96-well cultured plates for subcloning. After 21–28 days, the colonies were harvested and a single clone was obtained. The subcloned cells exhibited an 182-fold increase in resistance to the growth-inhibitory effect of gefitinib as determined by MTT assay, and the resistant phenotype has been stable for at least 6 months under drug-free conditions.

In vitro *growth-inhibition assay*

The growth-inhibitory effects of cisplatin, carboplatin, adriamycin, irinotecan, gemcitabine, vindesine, paclitaxel, genistein, K252a, staurosporin, AG-825, AG-1478, Tyrophostin 51, RG-14620, Lavendustin A and gefitinib in PC-9 and PC-9/ZD cells were examined by using a 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay.[22] A 180 μl volume of an exponentially growing cell suspension (6×10^3 cells/ml) was seeded into a 96-well microtiter plate, and 20 μl of various concentrations of each drug was added. After incubation for 72 hr at 37°C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 hr at 37°C. After centrifuging the plates at 200*g* for 5 min, the medium was aspirated from each well and 180 μl of DMSO was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm with a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340, Bio-Metallics, Princeton, NJ). Each experiment was carried out in 6 replicate wells for each drug concentration and carried out independently 3 or 4 times. The IC_{50}-value was defined as the concentration needed for a 50% reduction in the absorbance calculated based on the survival curves. Percent survival was calculated as: (mean absorbance of 6 replicate wells containing drugs − mean absorbance of six replicate background wells)/(mean absorbance of 6 replicate drug-free wells − mean absorbance of 6 replicate background wells) × 100.

In vivo *growth-inhibition assays*

Experiments were carried out in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (2nd ed.). Female BALB/c nude mice, 6-weeks-old, were purchased from Japan Charles River Co. Ltd (Atsugi, Japan). All mice were maintained in our laboratory under specific-pathogen-free conditions. *In vivo* experiments were scheduled to evaluate the effect of oral administration of gefitinib on pre-existing tumors. Ten days before administration, 5×10^6 PC-9 or PC-9/ZD cells were injected subcutaneously (s.c.) into the back of the mice, and gefitinib (12.5, 25 or 50 mg/kg, p.o.) was administered to the mice on Days 1–21. Tumor diameter was measured with calipers on Days 1, 4, 8, 11, 14, 19 and 22 to evaluate the effect of treatment, and tumor volume was determined by using the following equation: tumor volume = $ab^2/2$ (mm^3) (where *a* is the longest diameter of the tumor and *b* is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated, and Day "1" denotes the first day of treatment. All mice were sacrificed on Day 22, after measuring their tumors. We considered absence of a tumor mass on Day 22 to indicate a cure. Differences in tumor sizes between the treatment groups and control group at Day 22 were analyzed by the unpaired *t*-test. A *p*-value of <0.05 was considered statistically significant.

cDNA expression array

The gene expression profile of PC-9/ZD was assessed with an Atlas Nylon cDNA Expression Array (BD Bioscience Clontech, Palo Alto, CA). Total RNA was extracted by a single-step guanidinium thiocyanate procedure (ISOGEN, Nippon Gene, Tokyo, Japan). An Atlas Pure Total RNA Labeling System was used to isolate RNA and label probes. The materials provided with the kit were used, and the manufacturer's instructions were followed for all steps. Briefly, streptavidin-coated magnetic beads and biotinylated oligo(dT) were used to isolate poly A RNA from 50 μg of total RNA and the RNA obtained was converted into ^{32}P-labeled first-strand cDNA with MMLV reverse transcriptase. The ^{32}P-labeled cDNA fraction was purified on NucleoSpin columns and was added to the membrane on which fragments of 777 genes were spotted. Hybridization was allowed to proceed overnight at 68°C. After washing, the radiolabeled spots were visualized and quantified by BAS-2000II and Array Gauge 1.1 (Fuji Film Co., Ltd., Tokyo, Japan). The data were adjusted for the total density level of each membrane.

Quantitative real-time RT-PCR analysis

Total RNAs extracted from PC-9 cells and PC-9/ZD cells (1×10^6 cells each) were incubated with DNase I (Invitrogen, Carlsbad, CA) for 30 min. First-strand cDNA synthesis was carried out on 1 μg of RNA in 10 μl of a reaction mixture with 50 pmol of Random hexamers and 50 U of M-MLV RTase. Oligonucleotide primers for human *EGFR* were obtained from Takara (HA003051, Takara Bio Co., Tokyo, Japan). For PCR calibration, we generated a calibrator dilution series for *EGFR* cDNA in pUSEamp vector (Upstate, Charlottesville, VA) ranging from 10^8–10^2 copies/1 μl. A total of 2 μl of reverse transcriptase products was used for PCR amplification using Smart Cycler system (Takara) according to manufacturer's instructions. Absolute copy numbers were calculated back to the initial cell numbers, which were set into the RNA extraction. As a result we obtained copies/cell:ratio representing the average *EGFR* RNA amount per cell.

Immunoprecipitation and immunoblotting

The cultured cells were washed twice with ice-cold PBS, and lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na orthovanadate, and 10 mg/ml each of leupeptin, aprotinin, pepstatin A and phenylmethylsulphonyl fluoride). The lysate was cleared by centrifugation at 15,000 r.p.m. for 10 min, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce, Rockford, IL). The membrane was probed with antibody against EGFR (1005; Santa Cruz, Santa Cruz, CA), HER2/neu (c-18; Santa Cruz), HER3 (c-17; Santa Cruz), HER4 (c-18; Santa Cruz), PI3K (4; BD), Grb2 (81; BD), SOS1/2 (D-21; Santa Cruz), Shc (30; BD, San Jose, CA), PTEN (9552; Cell Signaling, Beverly, MA), AKT (9272; Cell Signaling), phospho-EGFR specific for Tyr 845, Tyr 992, Tyr 1045, and Tyr 1068 (2231, 2235, 2237, 2234; Cell Signaling), phospho-AKT (Ser473) (9271; Cell Signaling), phospho-Erk (9106; Cell Signaling), and phospho-Tyr (PY-20; BD) as the first antibody, and then with by horseradish-peroxidase-conjugated secondary antibody. The bands were visualized by enhanced chemiluminescence (ECL Western Blotting Detection Kit, Amersham, Piscataway, NJ). For Immunoprecipitation, 5×10^6 cells were washed, lysed in EBC buffer, and centrifuged, and the supernatants obtained (1,500 μg) were incubated at 4°C with the anti-EGFR (1005), -HER2 (c-18), and -HER3 (c-17) Ab overnight. The immunocomplexes were absorbed onto protein A/G-Sepharose beads, washed 5 times with lysate buffer, denatured, and subjected to electrophoresis on a 7.5% polyacrylamide gel.

Analysis of the genes of the HER families by direct sequencing

Total RNAs were extracted from PC-9 and PC-9/ZD cells with ISOGEN (Nippon Gene) according to manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by using 400 U of SuperScript II (Invitrogen, Carlsbad, CA). After reverse transcription with oligo (dT) primer (Invitrogen) or random primer (Invitrogen), the first-strand cDNA was amplified by PCR by using specific primers for *EGFR*, *HER2* and *HER3*. The

reaction mixture (50 µl) contained 1.25 U AmpliTaq DNA polymerase (Applied Biosystem, Foster City, CA), and amplification was carried out by 30 cycles of denaturation (95°C, 30 sec), annealing (55–59°C, 30 sec), and extension (72°C, 30 sec) with a GeneAmp PCR System 9600 (Applied Biosystem). After amplification, 5 µl of the RT-PCR products was subjected to electrophoretic analysis on a 2% agarose gel with ethidium bromide. DNA sequencing of the PCR products was carried out by the dideoxy chain termination method using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

Chemical cross-linking

Chemical cross-linking in intact cells was carried out as described previously.[23] In brief, after 6 hr exposure to 0.2 µM gefitinib, cells were washed with PBS and incubated for 25 min at 4°C in PBS containing 1.5 mM of the nonpermeable cross-linker bis (sulfosuccinimidyl) substrate (Pierce, Rockford, IL). The reaction was terminated by adding 250 mM glycine for 5 min while rocking. Cells were washed in EBC buffer and 20 µg of protein was resolved by 5–10% gradient SDS-PAGE, and then immunoblot analyzed for EGFR, HER2, HER3 and P-Tyr.

Results

Sensitivity of PC-9/ZD cells to cytotoxic agents and tyrosine kinase inhibitors

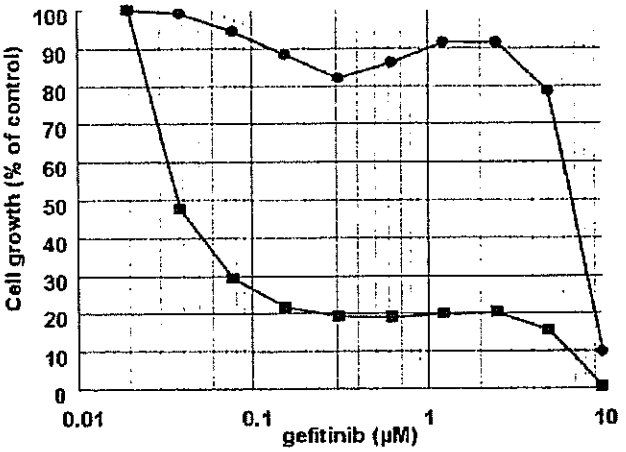
No significant difference between PC-9 and PC-9/ZD cells was observed in *in vitro* cell growth (doubling time of 20.3 hr and 21.4 hr, respectively) and microscopic morphology. Figure 1 shows the growth-inhibitory effect of gefitinib on the parent PC-9 cell line and its resistant subline, PC-9/ZD. The IC_{50}-value of gefitinib in PC-9 cells was 0.039 µM, as compared to 7.1 µM in PC-9/ZD cells (182-fold resistance). PC-9/ZD cells exhibited no cross-resistance to other conventional anticancer agents, including cisplatin, carboplatin, adriamycin, vindesine, paclitaxel and irinotecan. We also examined the growth-inhibitory effect of the EGFR tyrosine kinase inhibitors AG-1478, RG-14620 and Lavendustin A and other tyrosine kinase inhibitors in PC-9 and PC-9/ZD cells. PC-9/ZD cells show cross-resistance to AG1478, but not to all of the tyrosine kinase inhibitors (Tables I, II). It is likely that PC-9/ZD would also be resistant to EGFR-targeted quinazoline derivatives including gefitinib and erlotinib.[20]

PC-9/ZD cells show significant resistance to gefitinib in an in vivo *model*

To ascertain whether the resistance of PC-9/ZD occurs *in vivo*, we investigated the growth-inhibitory effect of gefitinib on PC-9 cells and PC-9/ZD cells in a xenotransplanted model. There was no significant difference in the size of the of PC-9 and PC-9/ZD cell tumor masses in nude mice before the start of gefitinib injection. Figure 2 shows the growth-inhibition curve of PC-9 (Fig. 2*a*) and PC-9/ZD (Fig. 2*b*) cells *in vivo* during the observation period. The PC-9 tumor masses decreased markedly in volume at all doses of gefitinib. In the 50 mg/kg/day p.o. group, the PC-9 masses were eradicated in all mice and did not regrow within the observation period. Growth of the PC-9/ZD masses, on the other hand, was inhibited by gefitinib administration in a dose-dependent manner, but significant tumor reduction was observed only in the 25 and 50 mg/kg/day groups, and the PC-9/ZD masses were not eradicated even in 50 mg/kg/day group. These results clearly demonstrate the significant *in vivo* resistance of PC-9/ZD cells to gefitinib.

Expression of HER family members and related molecules in PC-9 and PC-9/ZD cells

We examined the gene expression and protein levels of HER family members and related molecules by cDNA expression array (followed by confirmation using RT-PCR, data not shown) and immunoblotting. The ratios of the protein expression levels of PC-9 cells to PC-9/ZD cells almost paralleled the expression levels of their genes (Fig. 3*a*). The basal level of EGFR was comparable or slightly higher in PC-9/ZD cells (Fig. 3*a,b*), whereas the HER3 and AKT levels were lower in resistant cells.



	PC-9	PC-9/ZD
IC_{50} value (µM)	0.039± 0.002	7.1± 0.06
Doubling time (hr)	20.3	21.0

FIGURE 1 – Growth-inhibitory effect of gefitinib on PC-9 and PC-9/ZD cells determined by MTT assay. The cells were exposed to the concentrations of gefitinib indicated for 72 hr. The growth-inhibition curves of PC-9 (■) and PC-9/ZD (●) are shown. Doubling time was determined by MTT assay.

TABLE I – CHEMOSENSITIVITY TO OTHER ANTICANCER DRUGS

Drug	IC_{50} values (µM)[1]		RR[2] 1.6
	PC-9	PC-9/ZD	
Cisplatin	1.9 ± 0.7	3.1 ± 1.5	2.0
Carboplatin	25 ± 21	49 ± 23	1.3
Adriamycin	0.16 ± 0.13	0.20 ± 0.15	2.2
Irinotecan	15 ± 10	32 ± 11	1.5
Etoposide	4.5 ± 1.5	6.6 ± 1.3	1.5
Gemcitabine	18 ± 1.5	27 ± 1.5	0.7
Vindesine	0.0046 ± 0.0004	0.0032 ± 0.0009	1.2
Paclitaxel	0.0041 ± 0.0011	0.0048 ± 0.0004	1.6

[1]As assessed by MTT assay in PC-9 and PC-9/ZD cells. Values are the mean ± SD of >3 independent experiments.–[2]Relative resistance value (IC_{50} of resistant cells/IC_{50} of parental cells).

We carried out quantitative RT-PCR to measure the copy numbers of *EGFR*. Estimated transcript levels of *EGFR* were 786.3 and 712.1 copies/cell for PC-9 cells and PC-9/ZD cells, respectively (Fig. 3*d*). Relative ratio of *EGFR* expression levels in PC-9 cells and PC-9/ZD cells is 1.104. Microarray analysis using CodeLink Bioarray (Amersham Bio, Piscataway, NJ) confirmed equivalent gene expression of *EGFR* with ratio of 1.002 between PC-9 and PC-9/ZD cells (data not shown).

Expression of PI3K, Grb2, SOS, and Shc, the adaptor proteins of EGFR, and PTEN was almost the same in PC-9 and PC-9/ZD cells, and no change in the protein levels was observed after exposure to gefitinib (data not shown). The relative densitometric units of each protein are shown in Figure 3*c*. These results suggest that the difference in protein levels of EGFR, HER2, and related proteins can not explain the high resistance of PC-9/ZD cells to gefitinib.

Sequence of HER family member in PC-9/ZD cells

Several reports suggest that the resistance to receptor tyrosine kinase inhibitor STI-571 is partially due to mutations in the

TABLE II – CHEMOSENSITIVITY TO PROTEIN KINASE INHIBITORS[1]

Inhibitor	Target	IC_{50} values (μM)		RR[2]
		PC-9	PC-9/ZD	
AG-1478	EGFR	0.052 ± 0.02	6.0 ± 0.8	117
RG-14620	EGFR	13 ± 1.0	13 ± 2.5	1.0
Lavendustin A	EGFR	20 ± 4.6	27 ± 2.6	1.3
Genistein	TK	18 ± 1.5	27 ± 1.5	1.5
K252a	PKC	0.47 ± 0.17	0.63 ± 0.04	1.3
Staurosporin	PKC	0.0036 ± 0.0019	0.004 ± 0.0014	1.1
AG-825	HER2	>50	>50	

[1]Assessed by MTT assay in PC-9 and PC-9/ZD cells. Values are the mean ± SD of >3 independent experiments.–[2]Relative resistance value (IC_{50} of resistant cells/IC_{50} of parental cells).

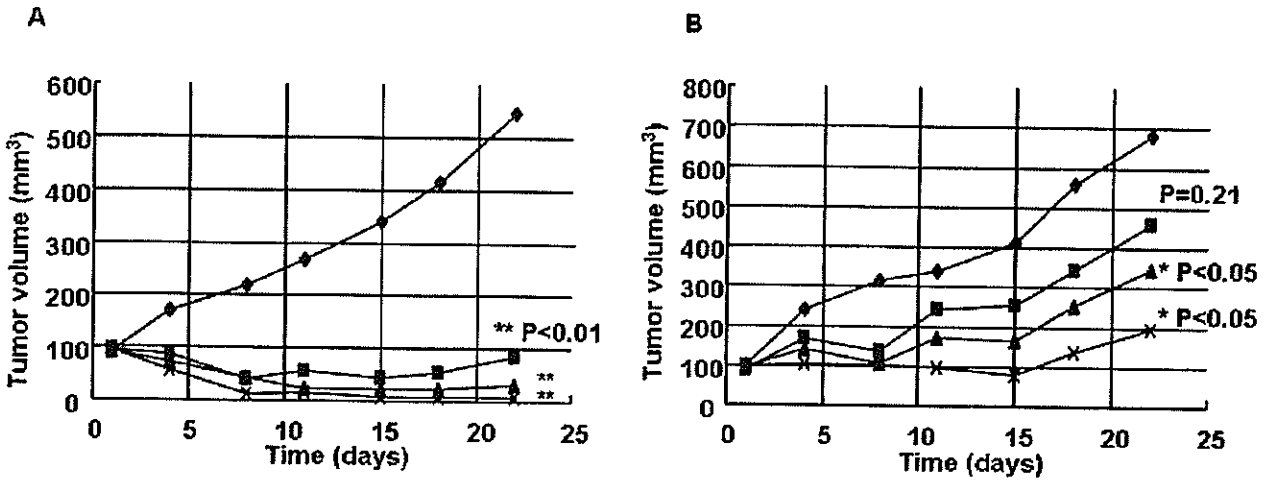


FIGURE 2 – Growth-inhibitory effect of gefitinib on PC-9 and PC-9/ZD cells xenotransplanted into nude mice. Ten days before gefitinib administration, 5×10^6 PC-9 (*a*) or PC-9/ZD (*b*) cells were injected s.c. into the back of mice. The mice were divided into 4 groups (◆, control group; ■, 12.5 mg/Kg group; ▲, 25 mg/Kg group; ×, 50 mg/Kg group). Gefitinib was administered p.o. to the tumor-inoculated mice on Days 1–21. Each group consisted of 6 mice. The statistical analysis was carried out by using the unpaired *t*-test.

ATP-binding site of the Bcr-Abl, the target of the drug.[24–27] We analyzed the sequences of the cDNAs of *EGFR*, *HER2*, and *HER3*, but found no differences in their sequences between PC-9 and PC-9/ZD cells. We did detect a deleted position of *EGFR* in both cell lines that results in deletion of 5 amino acids (Glu722, Leu723, Arg724, Glu725, and Ala726) (Fig. 4). Our findings indicate that the deletion does not directly contribute to the cellular resistance.

Inhibitory effect of gefitinib on autophosphorylation of EGFR in PC-9/ZD cells

Phosphorylation of EGFR is necessary for EGFR-mediated intracellular signaling. Although the EGFR phosphorylation levels of tumors were thought to be correlated with sensitivity to gefitinib, the basal level of phosphorylated EGFR in PC-9 and PC-9/ZD cells is almost the same. Gefitinib inhibited EGFR autophosphorylation in a dose-dependent manner and completely inhibited its phosphorylation at 0.2–2 μM in PC-9 cells (Fig. 5*a*), but its inhibitory effect on autophosphorylation of EGFR in PC-9/ZD cells was less than in PC-9 cells (Fig. 5*a*). Because each phosphorylation site of EGFR has a different role in the activation of downstream signaling molecules, we examined the inhibitory effect of gefitinib on site-specific phosphorylation of EGFR. Phosphorylation of several different EGFR tyrosine residues (Tyr845, Tyr992 and Tyr1068) was dose-dependently inhibited by gefitinib in PC-9 cells, whereas no clear inhibitory effects of gefitinib on phosphorylation at Tyr 845 and Tyr1068 residues in PC-9/ZD cells was observed (Fig. 5*b,c,e*). The most marked difference of in inhibition between the cells was observed at Tyr1068 (Fig. 5*e*). Tyr1045 showed resistance to inhibition of autophosphorylation by gefitinib in both PC-9 and PC-9/ZD cells (Fig. 5*d*).

Complex formation of EGFR and its adaptor proteins

Tyr1068 of EGFR is the tyrosine that is most resistant to inhibition of autophosphorylation by gefitinib in PC-9/ZD cells. Because the Tyr 1068 is a direct binding site for the GRB2/SH2 domain, and its phosphorylation is related to the complex formation of EGFR-adaptor proteins and their signaling, we examined complex formation between EGFR and the adaptor proteins GRB2, SOS, Shc, and PI3K by immunoprecipitation. The level of expression of these proteins in PC-9 and PC-9/ZD cells were similar (Fig. 3*a*). A smaller amount of EGFR-GRB2 complex was observed in PC-9/ZD cells and no EGFR-SOS complex was detected at all (Fig. 6). The amount of HER2- or HER3-GRB2 complex in PC-9 and PC-9/ZD cells was similar, and no decreases in complex formation were observed after exposure to gefitinib. A decreased amount of HER2-SOS complex and inability to detect HER3-SOS complex were also observed in PC-9/ZD cells. HER2-PI3K complex increased in PC-9/ZD. There are no significant differences in complex formation between SHC and EGFR, HER2, or HER3 between PC-9 and PC-9/ZD cells. These results suggest that GRB2-SOS-mediated signaling may be inactivated in PC-9/ZD cells.

Heterodimerization of HER family member in PC-9/ZD cells

Dimerization of members of the HER family is essential for activation of their catalytic activity and their signaling. We examined the effect of gefitinib on the dimerization of HER family members by immunoblotting, immunoprecipitation and chemical cross-linking analysis (Figs. 3*a*, 5*a*, 7*a*). The expression levels of EGFR and HER2 were similar and the HER3 level was lower in PC-9/ZD cells by immunoblotting (Fig. 3*a*). A chemical cross-

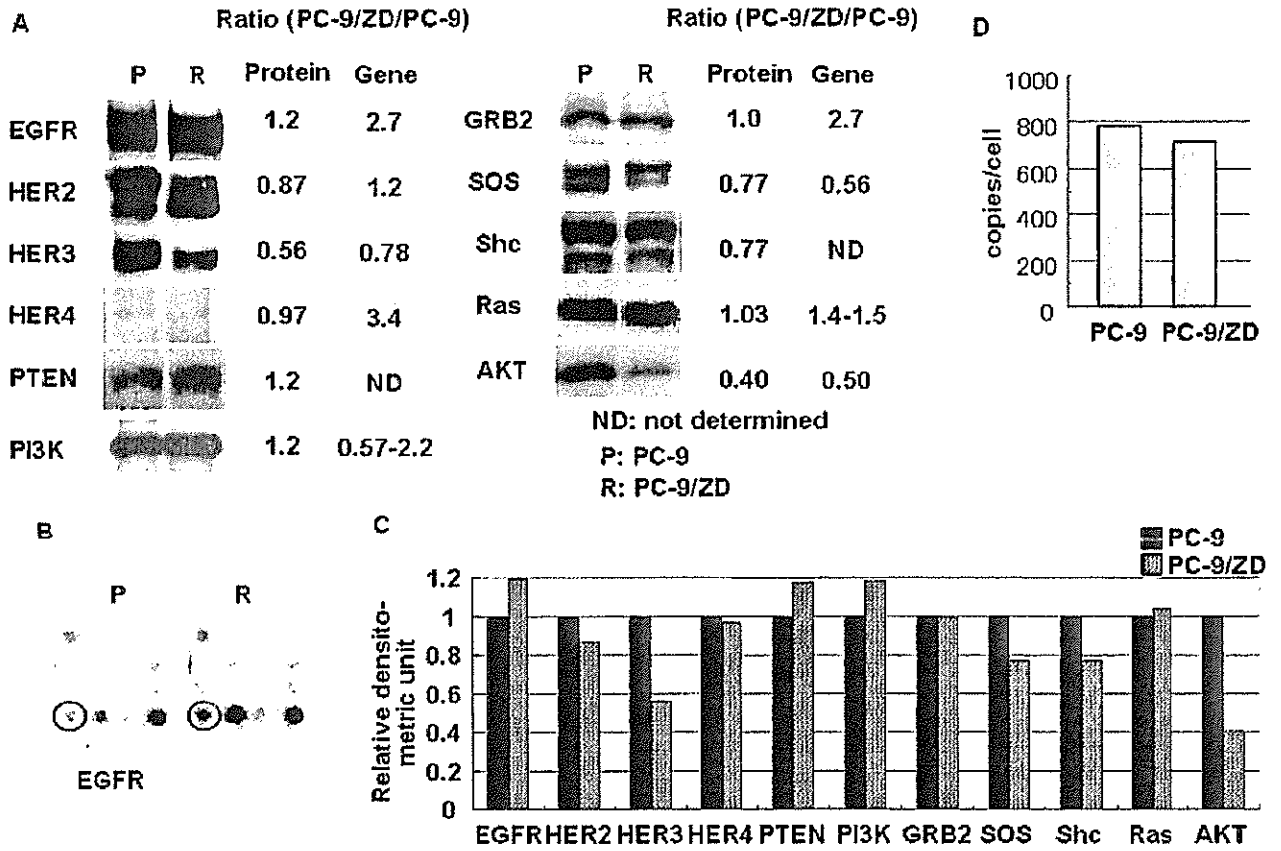


FIGURE 3 – Expresssion of HER family members and related molecules in PC-9 (P) and PC-9/ZD (R) cells. (*a*) Western blot analysis; a 20 μg sample of total cell lysates was separated by SDS-PAGE, transferred to a PVDF membrane, and incubated with a specific anti-human antibody as the first antibody and then with horseradish peroxidase-conjugated secondary antibody. The ratios of the levels of expression of proteins and genes in PC-9 cells to the levels in PC-9/ZD cells are shown. (*b*) cDNA expression array; Poly A RNA was converted into ^{32}P-labeled first-strand cDNA with MMLV reverse transcriptase. The ^{32}P-labeled cDNA fraction was hybridized to the membrane on which fragments of 777 genes were spotted. The close-up view shows *EGFR* mRNA expression. (*c*) Each band was quantified by a densitometry and with NIH image software. The levels of protein expression are shown in a graph. (*d*) Absolute amounts of *EGFR* transcripts of PC-9 cells and PC-9/ZD cells measured by real-time quantitative RT-PCR. The values were calculated back to the initial cell numbers for RNA extraction in Material and Methods.

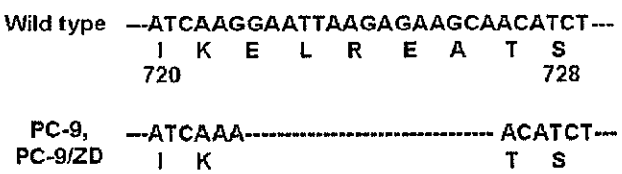


FIGURE 4 – Detection of a deleted position of EGFR. Direct sequencing of a PC-9 and PC-9/ZD-derived, amplified cDNA fragment containing the ATP-binding site of EGFR. *Top*, wild-type EGFR; *bottom*, PC-9 and PC-9/ZD.

linking assay showed that in the absence of gefitinib the amount of high molecular weight complexes (~400 kDa) that are recognized by anti-EGFR antibody (EGFR dimers), including formations of homodimers and heterodimers (EGFR-EGFR, EGFR-HER2 or EGFR-HER3), was almost the same in PC-9 and PC-9/ZD cells, whereas HER2 dimerization detected by anti-HER2 antibody was remarkably lower in PC-9/ZD cells (Fig. 7*a*). Increased EGFR/HER2 (and EGFR/HER3) heterodimer formation was detected in PC-9/ZD cells by immunoprecipitation analysis (Fig. 5*a*). The proportion of EGFR heterodimer to homodimer is increased significantly in PC-9/ZD (Fig. 7*b*). When exposed to gefitinib at a concentration of 0.2 μM for 6 hr the amount of dimer-formation increased similarly in PC-9 and PC-9/ZD cells (Fig. 7*a*), whereas marked induction of hetero-dimerization of EGFR-HER2 was observed only in PC-9 cells (Fig. 5*a*). These results suggest that a difference in hetero- or homo-dimerization is a possible determinant factor of gefitinib sensitivity.

AKT and MAPK pathways in PC-9/ZD cells

Because phosphorylation at Tyr 1068 of EGFR plays an important role for transduction of the signal to downstream of MAPK and AKT pathway,[28,29] we examined the difference between PC-9 and PC-9/ZD cells in downstream signaling. The basal level of phosphorylated AKT is higher in PC-9 cells than in PC-9/ZD cells, and although gefitinib inhibited AKT phosphorylation in a dose-dependent manner (Fig. 8*a*), the inhibitory effect of gefitinib on phosphorylation of AKT in PC-9/ZD cells was significantly less than in PC-9 cells (Fig. 8*a*). This difference in the inhibitory effect of gefitinib on AKT phosphorylation between PC-9 and PC-9/ZD cells is very similar to the difference in effect on EGFR autophosphorylation. No inhibition of phosphorylation of MAPK by gefitinib was observed in either cell line (Fig. 8*b*). These results suggest that downregulation of activated AKT is closely correlated with the cellular sensitivity to gefitinib, but that inhibition of the MAPK pathway does not contribute to drug sensitivity.

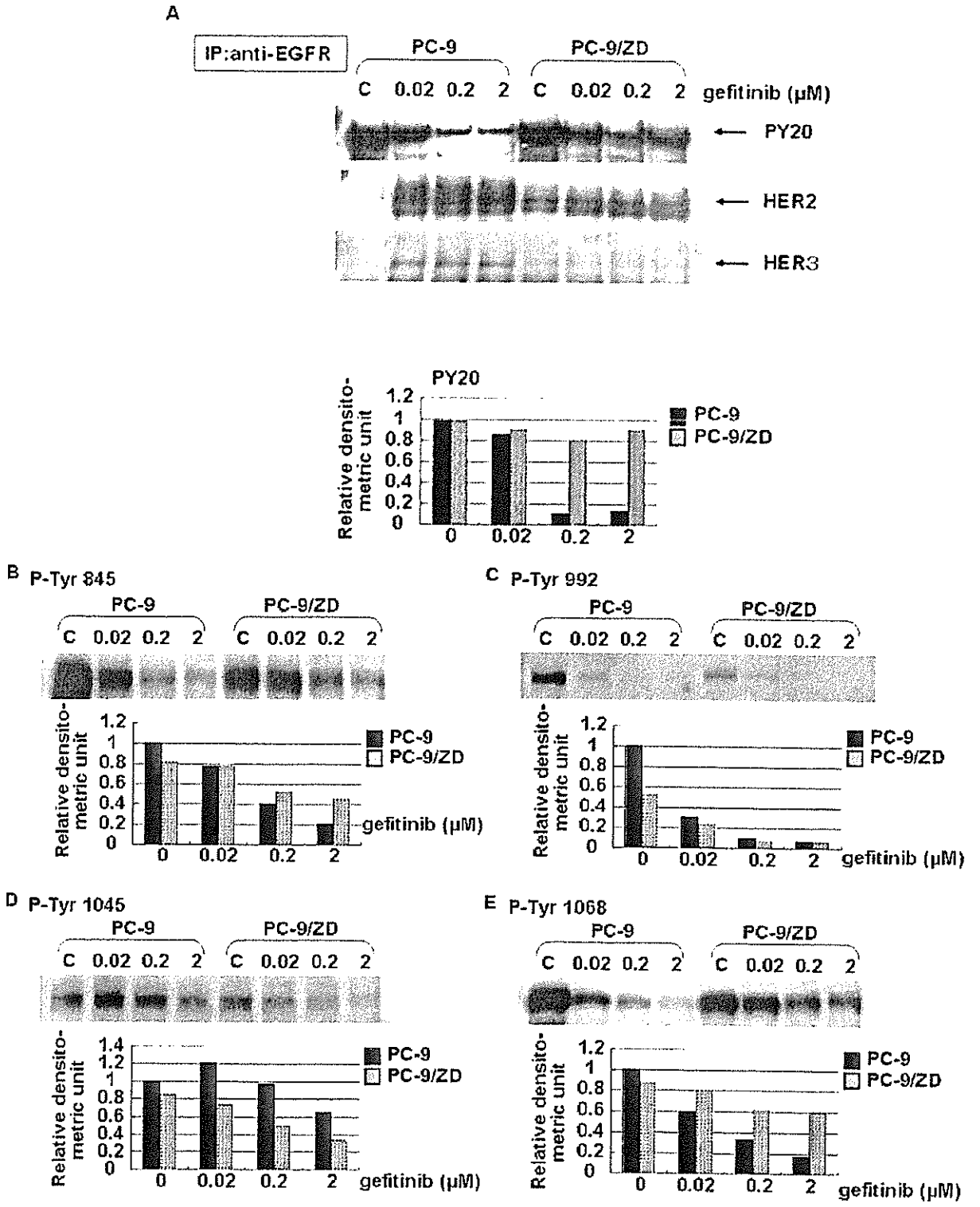


FIGURE 5 – Effect of gefitinib on autophosphorylation of EGFR. (*a*) PC-9 and PC-9/ZD cells (5×10^6) were exposed to 0.02, 0.2 or 2 μM gefitinib for 6 hr. The 1,500 μg of total cell lysate was immunoprecipitated with an anti-EGFR antibody. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-phosphotyrosine, anti-HER2 and anti-HER3 antibodies. Tyrosine-phosphorylated EGFR was determined with an anti-phosphotyrosine antibody. Heterodimer formation of EGFR was analyzed with anti-HER2 and anti-HER3 antibodies. The expression levels have been plotted in a graph. (*b–e*) PC-9 and PC-9/ZD cells were exposed to 0.02, 0.2 and 2 μM gefitinib for 6 hr. A 20 μg of protein of each sample was analyzed by Western blotting by using anti phospho-EGFR (Tyr845, Tyr992, Tyr 1045, Tyr 1068) antibodies.