culture, the time-consuming nature of the technique, and a poor correlation with the clinical response.[2,3] To overcome these obstacles, DNA, RNA, and protein-based chemosensitivity tests have been tried, but it remains unknown which gene alteration is well predictive of the clinical drug response. In our previous studies, 80 in vitro chemosensitivity-associated genes were identified in the medical literature,[4] and the association between alterations of these genes and clinical drug responses in lung cancer patients was described.[5] The purpose of this study was to find candidate genes to develop clinically useful chemosensitivity tests for patients with breast cancer.

## Materials and methods

We identified 80 in vitro chemosensitivity-associated genes that met the following definition in the medical literature: (1) their alteration could be identified in human drug-induced resistant solid tumor cell lines; (2) their transfection induced drug resistance; or (3) their downregulation increased drug sensitivity. The genes included transporters: *ABCA2*, *ABCB1*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCG2*, *MVP*, *ATP7A*, *ATP7B*, *SLC29A1*, *SLC28A1*, and *SLC19A1*; drug targets: *TUBB*, *TUBB4*, *TUBA*, *TYMS*, *TOP1*, *TOP2A*, *TOP2B*, and DHFR; target-associated proteins : *MAP4*, *MAP7*, *STMN1*, *KIF5B*, *HSPA5*, *PSMD14*, and *FPGS*; intracellular detoxifiers: *GSTP1*, *GPX*, *GCLC*, *GGT2*, *MT*, *RRM2*, and *AKR1B1*; DNA damage recognition and repair proteins: *HMGB1*, *HMGB2*, *ERCC1*, *XPA*, *XPD*, *MSH2*, *MLH1*, *PMS2*, *APEX1*, *MGMT*, *BRCA1*, and *GLO1*; cell-cycle regulators: *RB1*, *GML*, *CDKN1A*, *CCND1*, *CDKN2A*, and *CDKN1B*; mitogenic signal regulators: *ERBB2*, *EGFR*, *KRAS2*, *HRAS*, and *RAF1*; survival signal regulators: *AKT1* and *AKT2*; integrins: *ITGB1*; transcription factors: *JUN*, *FOS*, *MYC*, and *NFKB1*; and apoptosis regulators: *TP53*, *MDM2*, *TP73*, *BCL2*, *BCL2L1*, *MCL1*, *BAX*, *BIRC4*, *BIRC5*, *TNFRSF6*, *CASP3*, *CASP8*, and *HSPB1*.[4] Papers describing an association between the alteration of the gene and clinical drug response in patients with breast cancer were identified by extensive Medline searches using the name of the gene as a key word. Papers in which the association was evaluated in 25 or more patients were included in this study.

We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and CIs were estimated using the DerSimonian-Laird method, as previously described.[5] The formula used for the combined OR and that for 95% CI were as follows:

$$\text{Combined OR} = \exp\{\Sigma(\text{weight}_i \cdot \ln\text{OR}_i)/\Sigma\text{weight}_i\}$$

$$95\%\ \text{CI of combined OR} = \exp\{\ln \text{combined OR} \pm 1.96\ (1/\Sigma\text{weight}_i)^{1/2}\}$$

where $\text{weight}_i$ is the weight for each study determined by variance of the study, and OR is the OR of each study.

## Results

Clinical drug responses were evaluated in 18 genes from 69 studies, which included a median of 73 patients (range, 29–319 patients) per study to give a total of 6378 patients. The methods used to identify the gene alteration were immunohistochemical protein expression analysis ($n$ = 52), protein activity analysis using tritium-release assay ($n$ = 1), polymerase chain reaction (PCR)-based mRNA expression analysis ($n$ = 8), PCR-based mutation analysis ($n$ = 3), and gene amplification analysis using fluorescence in situ hybridization or chromogenic in situ hybridization ($n$ = 5). The gene alteration was associated with the clinical response in 25 of the 69 (36%) studies.

High expression of *ABCB1* was associated with a poor response to first-line chemotherapy in three of five studies, and the combined OR (CI) in a total of 322 patients was 0.16 (0.05–0.59). Other transporter expressions were not associated with chemotherapy responses (Table 1). Study results showing associations between drug target alterations and clinical responses were promising. The alteration of *TYMS* (thymidylate synthetase), *TUBB* (beta-tubulin class I), and *TUBB4* (beta-tubulin class III) was associated with chemosensitivity, although there was only one study for each gene. The overexpression and amplification of *TOP2A* (topoisomerase II-alfa) were more frequently observed in patients who responded to first-line chemotherapy in four out of five studies with a combined OR (CI) of 2.73 (1.02–7.274) in a total of 323 patients (Table 2). The high expression of the DNA repair gene *BRCA1* (Breast cancer 1) was associated with chemosensitivity in one study (Table 3). The overexpression of *ERBB2* (c-erbB2, Her2, or neu) was associated with favorable responses in patients treated with first-line anthracycline-based chemotherapy, and the combined OR (CI) was 1.60 (1.19–2.17) in a total of 1807 patients (Table 4). This was also true among patients treated with second-line chemotherapy containing taxanes (combined OR [CI], 2.24 [1.06–4.74]; $n$ = 259; Table 5). *TP53* mutations were not associated with clinical drug responses (combined OR [CI],1.09 [0.73–1.62]; $n$ = 1588; Table 6), whereas *BCL2* overexpression was associated with resistance to first-line chemotherapy (combined OR [CI], 0.44 [0.21–0.91]; $n$ = 816; Tables 7 and 8).

## Discussion

Association between a gene alteration and clinical chemosensitivity was evaluated in 18 of the 80 in vitro chemosensitivity-associated genes in patients with breast cancer. Among them, *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for further studies.

*ABCB1* has been extensively studied as a major cellular mechanism of multidrug resistance,[6] but there has been no firm evidence that the expression of this transporter in tumor cells has been associated with a poor response to cytotoxic chemotherapy in patients with breast cancer. A

**Table 1.** Expression of transporter proteins and clinical response to first-line chemotherapy

| Author (year, country) | Drugs | Method | Expression | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| *ABCB1* | | | | | | |
| Ro[19] (1990, USA) | CPA, DOX, VCR | IHC | Low | 20 | 95 | 0.08 |
| | | | High | 20 | 60 | (0.01–0.71) |
| Veneroni[20] (1994, Italy) | DOX ± VCR | IHC | Low | 21 | 86 | 0.02 |
| | | | High | 18 | 11 | (0.0–0.14) |
| Chevillard[21] (1996, France) | CPA, DOX, 5-FU | IHC | Low | 36 | 50 | 0.75 |
| | | | High | 7 | 43 | (0.15–3.84) |
| Bottini[22] (2000, Italy) | CPA, MTX, 5-FU, or EPI | IHC | Low | 99 | 28[a] | 0.51 |
| | | | High | 42 | 17[a] | (0.20–1.27) |
| Burger[23 b] (2003, Netherlands) | CPA, MTX, 5-FU, or | RT-PCR | Low | 47 | 68 | 0.09 |
| | CPA, DOX or EPI, 5-FU | | High | 12 | 17 | (0.02–0.48) |
| Combined odds ratio (95% CI) for ABCB1 ($n$ = 322): 0.16 (0.05–0.59) | | | | | | |
| *ABCC1* (Multidrug resistance-associated protein 1; MRP1) | | | | | | |
| Burger[23 b] (2003, Netherlands) | CPA, MTX, 5-FU, or | RT-PCR | Low | 30 | 60 | 0.82 |
| | CPA, DOX or EPI, 5-FU | | High | 29 | 55 | (0.29–2.31) |
| *ABCC2* (Multidrug resistance-associated protein 1; MRP2) | | | | | | |
| Burger[23 b] (2003, Netherlands) | CPA, MTX, 5-FU, or | RT-PCR | Low | 28 | 64 | 0.48 |
| | CPA, DOX or EPI, 5-FU | | High | 28 | 46 | (0.16–1.41) |
| *ABCG2* (Breast cancer resistance protein; BCRP) | | | | | | |
| Burger[23 b] (2003, Netherlands) | CPA, MTX, 5-FU, or | RT-PCR | Low | 42 | 64 | 0.39 |
| | CPA, DOX or EPI, 5-FU | | High | 17 | 41 | (0.12–1.23) |
| *MVP* (major vault protein, lung resistance-related protein) | | | | | | |
| Burger[23 b] (2003, Netherlands) | CPA, MTX, 5-FU, or | RT-PCR | Low | 37 | 65 | 0.45 |
| | CPA, DOX or EPI, 5-FU | | High | 22 | 45 | (0.15–1.33) |

RR, response rate. Drugs: CPA, cyclophosphamide; DOX, doxorubicin; EPI, epirubicin; 5-FU, 5-fluorouracil; MTX, methotrexate; VCR, vincristine. Methods: IHC, immunohistochemical analysis; RT-PCR, reverse transcriptase-polymerase chain reaction
[a] Complete response rate (%)
[b] In this study 20% of patients had received adjuvant chemotherapy

**Table 2.** Drug targets, intracellular detoxifier, and clinical response to first-line chemotherapy

| Author (year, country) | Drugs | Method | Alteration | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| *TYMS* (thymidylate synthetase) | | | | | | |
| Foekens[24] (2001, Netherlands) | 5-FU- based | TRA | Low expression | 13 | 8 | 12.0 |
| | | | High expression | 108 | 50 | (1.51–95.5) |
| *TUBB* (beta-tubulin class I) | | | | | | |
| Hasegawa[25] (2003, Japan) | DTX | Real-time | Low expression | 19 | 63 | 0.25 |
| | | PCR | High expression | 20 | 30 | (0.07–0.95) |
| *TUBB4* (beta-tubulin class III) | | | | | | |
| Hasegawa[25] (2003, Japan) | DTX | Real-time | Low expression | 19 | 68 | 0.15 |
| | | PCR | High expression | 20 | 25 | (0.04–0.62) |
| *TOP2A* (topoisomerase II-alfa) | | | | | | |
| Jarvinen[26] (1998, Finland) | EPI | IHC | Low expression | 31 | 58 | 0.61 |
| | | | High expression | 24 | 46 | (0.21–1.79) |
| Coon[27] (2002, USA) | Anthracycline-based | IHC | Low expression | 26 | 77 | 2.40 |
| | | | High expression | 9 | 89 | (0.25–23.2) |
| MacGrogan[28] (2003, France) | EPI, MTX, VCR | IHC | Low expression | 68 | 32 | 2.88 |
| | | | High expression | 57 | 58 | (1.38–5.97) |
| Martin-Richard[29] (2004, Spain) | CPA, DOX, 5-F-U or CPA, | IHC | Low expression | 25 | 24 | 5.28 |
| | EPI, 5-FU | | High expression | 16 | 63 | (1.35–20.7) |
| Park[30] (2003, Korea) | DOX | CISH | Normal | 48 | 54 | 15.2 |
| | | | Amplified | 19 | 95 | (1.88–123) |
| Combined odds ratio (95% CI) for TOP2A ($n$ = 323): 2.73 (1.027–7.27) | | | | | | |
| GSTP1 (glutathione S-transferase pi) | | | | | | |
| Wright[31] (1992, UK) | MIT | IHC | Low expression | 30 | 37 | 1.22 |
| | | | High expression | 29 | 41 | (0.43–3.48) |

Drugs: DTX, docetaxel; MTX, methotrexate; MIT, mitoxantrone: CISH, chromogenic in situ hybridization; TRA, tritium-release assay

previous meta-analysis, summarizing the data of 115 patients published between 1990 and 1996, showed only a marginal association between *ABCB1* expression in tumor tissue before treatment and failure of response (relative risk, 1.47; 95% CI, 0.94–2.29; $P$ = 0.088).[7] The present study included recent studies with a total of 322 patients, and showed that the expression of *ABCB1* was significantly associated with a poor drug response. Key anticancer agents in the

**Table 3.** DNA repair gene, cell-cycle regulator and clinical response to first-line chemotherapy

| Author (year, country) | Drugs | Method | Expression | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| *BRCA1* (Breast cancer 1) | | | | | | |
| Egawa[32] (2003, Japan) | CPA, EPI | Real-time PCR | Low | 25 | 32 | 4.01 |
| | | | High | 26 | 65 | (1.25–12.9) |
| *CCND1* (cyclin D1) | | | | | | |
| Bonnefoi[33] (2003, Switzerland) | CPA, EPI ± 5-FU | IHC | Low | 126 | 22[a] | 2.02 |
| | | | High | 52 | 37[a] | (1.00–4.07) |

[a]Complete response rate (%)

**Table 4.** *ERBB2* (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to first-line anthracycline-based chemotherapy

| Author (year, country) | Drugs | Method | Alteration | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| Niskanen[b 34] (1997, Finland) | CPA, EPI, 5-FU | IHC | Low expression | 89 | 33 | 2.07 |
| | | | High expression | 14 | 50 | (0.66–6.45) |
| Rozan[35] (1998, France) | CPA, DOX, 5-FU | IHC | Low expression | 131 | 21 | 1.62 |
| | | | High expression | 36 | 31 | (0.71–3.69) |
| Jarvinen[26] (1998, Finland) | EPI | IHC | Low expression | 36 | 64 | 0.26 |
| | | | High expression | 19 | 32 | (0.08–0.85) |
| Vincent-Salomon[36] (2000, France) | CPA, DOX, 5-FU | IHC | Low expression | 36 | 78 | 0.57 |
| | | | High expression | 18 | 67 | (0.16–2.01) |
| Geisler[37] (2001, Norway) | DOX | IHC | Low expression | 72 | 37 | 1.17 |
| | | | High expression | 17 | 41 | (0.40–3.43) |
| Coon[27] (2002, USA) | Anthracycline-based | IHC | Low expression | 20 | 70 | 2.79 |
| | | | High expression | 15 | 87 | (0.47–16.4) |
| MacGrogan[28] (2003, France) | EPI, MTX, VCR | IHC | Low expression | 102 | 40 | 1.82 |
| | | | High expression | 20 | 55 | (0.69–4.78) |
| Bonnefoi[33] (2003, Switzerland) | CPA, EPI ± 5-FU | IHC | Low expression | 132 | 24[a] | 1.61 |
| | | | High expression | 47 | 34[a] | (0.78–3.32) |
| Zhang[38] (2003, USA) | CPA, DOX, 5-FU | IHC | Low expression | 69 | 78 | 3.61 |
| | | | High expression | 28 | 93 | (0.77–17.0) |
| Martin-Richard[29] (2004, Spain) | CPA, DOX, 5-FU or | IHC | Low expression | 30 | 37 | 1.44 |
| | CPA, EPI, 5-FU | | High expression | 11 | 45 | (0.35–5.84) |
| Burcombe[39] (2005, UK) | Anthracycline-based | IHC | Low expression | 84 | 71 | 1.87 |
| | | | High expression | 34 | 82 | (0.69–5.08) |
| Prisack[40] (2005, Germany) | CPA, EPI | IHC | Low expression | 257 | 10[a] | 2.13 |
| | | | High expression | 62 | 19[a] | (1.01–4.51) |
| Manna Edel[41] (2006, Brazil) | Anthracycline-based | IHC | Low expression | 86 | 63 | 1.11 |
| | | | High expression | 23 | 65 | (0.42–2.91) |
| Park[30] (2003, Korea) | DOX | CISH | Normal | 36 | 47 | 7.54 |
| | | | Amplified | 31 | 87 | (2.19–26.0) |
| Konecny[c 42] (2004, USA) | CPA, EPI | FISH | Normal | 88 | 33 | 1.80 |
| | | | Amplified | 49 | 46 | (0.88–3.68) |
| Bozzetti[43] (2006, Belgium) | Anthracycline-based | FISH | Normal | 86 | 62 | 1.63 |
| | | | Amplified | 29 | 72 | (0.65–4.11) |
| Combined odds ratio (95% CI) for *ERBB2* (anthracyclines; $n$ = 1807): 1.60 (1.19–2.17) | | | | | | |

FISH, fluorescence in situ hybridization
[a]Pathological complete response rate
[b,c]In these studies, 15% and 40%, respectively, of patients had received adjuvant chemotherapy

**Table 5.** ERBB2 (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to second-line taxanes

| Author (year, country) | Drugs | Method | Alteration | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| Taxanes | | | | | | |
| Baselga[44] (1997, USA) | DTX or PTX | IHC | Low expression | 76 | 65 | 3.40 |
| | | | High expression | 46 | 36 | (1.58–7.33) |
| Sjostrom[45] (2002, Finland) | DTX | IHC | Low expression | 36 | 53 | 1.02 |
| | | | High expression | 30 | 53 | (0.39–2.70) |
| Di Leo[46] (2004, Europe) | DTX | FISH | Normal | 50 | 40 | 3.00 |
| | | | Amplified | 21 | 67 | (1.03–8.74) |
| Combined odds ratio (95% CI) for *ERBB2* (taxanes, $n$ = 259): 2.24 (1.06–4.74) | | | | | | |

DTX, docetaxel; PTX, paclitaxel

**Table 6.** Tumor protein *TP53* (p53) mutation and clinical response to first-line chemotherapy

| Author (year, country) | Drugs | Method | Mutation | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| Niskanen[c34] (1997, Finland) | CPA, EPI, 5-FU | IHC | Normal | 86 | 37 | 0.52 |
| | | | Mutated | 17 | 24 | (0.16–1.73) |
| Frassoldati[47] (1997, Italy) | CPA, DOX or CPA, MTX, 5-FU | IHC | Normal | 26 | 42 | 0.68 |
| | | | Mutated | 3 | 33 | (0.05–8.50) |
| Bonetti[d48] (1998, Italy) | CPA, MTX, 5-FU or Anthracycline-based | IHC | Normal | 21 | 30 | 0.94 |
| | | | Mutated | 22 | 27 | (0.25–3.56) |
| Rozan[35] (1998, France) | CPA, DOX, 5-FU | IHC | Normal | 97 | 22 | 1.25 |
| | | | Mutated | 70 | 26 | (0.61–2.58) |
| Jarvinen[26] (1998, Finland) | EPI | IHC | Normal | 37 | 57 | 0.61 |
| | | | Mutated | 18 | 44 | (0.20–1.90) |
| Colleoni[49] (1999, Italy) | CPA, DOX or VNR, 5-FU | IHC | Normal | 59 | 53 | 5.42 |
| | | | Mutated | 14 | 86 | (1.11–26.4) |
| Bottini[22] (2000, Italy) | CPA, MTX, 5-FU or EPI | IHC | Normal | 111 | 72 | 1.16 |
| | | | Mutated | 32 | 75 | (0.47–2.86) |
| Kandioler-Eckersberger[50] (2000, Austria) | CPA, EPI, 5-FU | IHC | Normal | 20 | 85 | 0.01 |
| | | | Mutated | 15 | 7 | (0.00–0.13) |
| Kandioler-Eckersberger[50] (2000, Austria) | PTX | IHC | Normal | 20 | 35 | 3.71 |
| | | | Mutated | 12 | 67 | (0.82–16.8) |
| Bonnefoi[33] (2003, Switzerland) | CPA, EPI ± 5-FU | IHC | Normal | 126 | 29[a] | 0.73 |
| | | | Mutated | 53 | 23[a] | (0.35–1.55) |
| MacGrogan[28] (2003, France) | EPI, MTX, VCR | IHC | Normal | 89 | 40 | 2.38 |
| | | | Mutated | 34 | 62 | (1.06–5.35) |
| Rahko[c51] (2003, Finland) | Anthracycline-based | IHC | Normal | 15 | 33 | 0.73 |
| | | | Mutated | 15 | 27 | (0.15–3.49) |
| Ogston[52] (2004, UK) | CPA, DOX, VCR | IHC | Normal | 65 | 52[b] | 1.25 |
| | | | Mutated | 38 | 59[b] | (0.56–2.81) |
| Prisack[40] (2005, Germany) | CPA, EPI | IHC | Normal | 269 | 11[a] | 2.12 |
| | | | Mutated | 38 | 21[a] | (0.89–5.06) |
| Berns[53] (2000, Netherlands) | CPA, DOX, 5-FU or CPA, MTX, 5-FU | sequencing | Normal | 16 | 63 | 0.34 |
| | | | Mutated | 25 | 36 | (0.09–1.24) |
| Geisler[37] (2001, Norway) | DOX | TTGE, sequencing | Normal | 64 | 36 | 1.31 |
| | | | Mutated | 26 | 42 | (0.52–3.32) |
| Geisler[54] (2003, Norway) | MMC, 5-FU | TTGE, sequencing | Normal | 17 | 41 | 0.55 |
| | | | Mutated | 18 | 28 | (0.13–2.26) |
| Combined odds ratio (95% CI) for *TP53* ($n$ = 1588): 1.09 (0.73–1.62) | | | | | | |

Drugs: MMC, mitomycin C; VNR, vinorelbine. Method: TTGE, temporal temperature gel electrophoresis
[a]Pathological complete response rate
[b]Good pathological response rate
[c,d]In these studies, 15% and 30%, respectively, of patients had received adjuvant chemotherapy

**Table 7.** *BCL2* (B-cell CLL/lymphoma 2) and clinical response to first-line chemotherapy

| Author (year, country) | Drugs | Method | Expression | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| Frassoldati[47] (1997, Italy) | CPA, DOX or CPA, MTX, 5-FU | IHC | Low | 19 | 47 | 0.48 |
| | | | High | 10 | 30 | (0.09–2.42) |
| Bonetti[c48] (1998, Italy) | CPA, MTX, 5-FU or Anthracycline-based | IHC | Low | 32 | 44 | 0.19 |
| | | | High | 23 | 13 | (0.05–0.78) |
| Colleoni[49] (1999, Italy) | CPA, DOX or VNR, 5-FU | IHC | Low | 27 | 52 | 1.58 |
| | | | High | 46 | 63 | (0.60–4.15) |
| Bottini[22] (2000, Italy) | CPA, MTX, 5-FU or EPI | IHC | Low | 48 | 71 | 1.15 |
| | | | High | 95 | 74 | (0.53–2.49) |
| Geisler[37] (2001, Norway) | DOX | IHC | Low | 46 | 37 | 1.12 |
| | | | High | 43 | 40 | (0.47–2.62) |
| Ogston[52] (2004, UK) | CPA, DOX, VCR | IHC | Low | 55 | 71[b] | 0.22 |
| | | | High | 48 | 25[b] | (0.10–0.52) |
| Buchholz[55] (2005, USA) | CPA, DOX, 5-FU | IHC | Low | 33 | 27[a] | 0.11 |
| | | | High | 49 | 4[a] | (0.02–0.57) |
| Prisack[40] (2005, Germany) | CPA, EPI | IHC | Low | 118 | 25[a] | 0.16 |
| | | | High | 124 | 5[a] | (0.06–0.42) |
| Combined odds ratio (95% CI) for *BCL2* ($n$ = 816): 0.44 (0.21–0.91) | | | | | | |

[a]Pathological complete response rate
[b]Good pathological response rate
[c]In this study, 30% of patients had received adjuvant chemotherapy

**Table 8.** Other apoptosis regulators and clinical response to chemotherapy

| Author (year, country) | Drugs | Method | Expression | No. of pts | RR (%) | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| *BCL2L1* (Bcl2-like 1, Bcl-xL) | | | | | | |
| Sjostrom[56] (2002, Finland) | DTX or MTX, 5-FU (second-line) | IHC | Low | 59 | 36 | 1.32 |
| | | | High | 64 | 42 | (0.64–2.73) |
| *BAX* (Bcl2-associated X protein ) | | | | | | |
| Krajewski[57] (1995, Finland) | CPA, EPI, 5-FU (first-line) | IHC | Low | 39 | 21 | 2.84 |
| | | | High | 65 | 43 | (1.13–7.13) |
| Sjostrom[56] (2002, Finland) | DTX or MTX, 5-FU (second-line) | IHC | Low | 59 | 39 | 1.03 |
| | | | High | 53 | 39 | (0.48–2.20) |
| Buchholz[55] (2005, USA) | CPA, DOX, 5-FU (first-line) | IHC | Low | 12 | 58[a] | 0.04 |
| | | | High | 69 | 6[a] | (0.01–0.20) |
| *TNFRSF6* (tumor necrosis factor receptor superfamily, member 6, FAS, CD95) | | | | | | |
| Sjostrom[56] (2002, Finland) | DTX or MTX, 5-FU (second-line) | IHC | Low | 53 | 42 | 0.83 |
| | | | High | 70 | 37 | (0.40–1.73) |

[a] Pathological complete response rate

treatment of breast cancer, such as anthracyclines, vinca alkaloids, and taxanes, are substrates of ABCB1 protein, and its expression must therefore be an important determinant for chemosensitivity. The association between the expression and clinical drug responses of other transporters is also worth evaluating, although no statistically significant association has been obtained due to the too-small sample size.

Qualitative and quantitative alterations of the drug's target are another important mechanism involved in classical drug resistance. DNA topoisomerase II enzymes pass one double-stranded DNA segment through a transient, enzyme-mediated break in another strand to relax a highly twisted superhelical DNA.[8] One isoform of these enzymes, TOP2A, is the target of most active anticancer agents, including anthracyclines, because its expression levels are tightly linked to the proliferative state of the cell, and are higher in tumor tissue than in adjacent normal tissue.[8] Although there have been many attempts to correlate *TOP2A* status with anthracycline efficacy in breast cancer patients, the results have been controversial.[9] The present study showed that *TOP2A* gene amplification and protein overexpression were associated with a higher response rate in a total of 323 patients. *TYMS* and beta-tubulins are also important targets for fluoropyrimidines and taxanes, respectively. Further studies are needed before the association can be definitively established between alteration of these gene expressions and clinical chemotherapy responses.

*ERBB2* is a member of the human epidermal growth factor receptor family, which plays an important role in regulating cell growth, survival, adhesion, migration, and differentiation, by forming heterodimers within the family. The ERBB2 receptor is the most potent oncoprotein, and amplification and overexpression of *ERBB2*, noted in about 30% of breast cancers, are associated with a poor prognosis.[10,11] The predictive value of *ERBB2* overexpression for poor responses to endocrine therapy and trastuzumab therapy has been well documented, but the association between *ERBB2* status and chemosensitivity remains controversial.[11,12] This issue has been evaluated mainly in the adjuvant setting after surgery, and the association between *ERBB2* status and difference in progression-free survival can therefore be attributable to the overall prognosis as well as the efficacy of chemotherapy. The *ERBB2* status and responses to chemotherapy in patients with locally advanced or the metastatic breast cancer have been evaluated in small studies. Few studies, however, showed any significant difference in the response rates between ERBB2-normal and ERBB2-overexpressed patients.[12] The present study showed that patients with overexpression or amplification of *ERBB2* responded significantly better to anthracycline-based chemotherapy than patients with a normal *ERBB2* status. This was explained by the correlation between the expressions of the *ERBB2* and *TOP2A* genes; high expression of the *TOP2A* gene was detected in 30%–60% of breast cancer tissue with *ERBB2* overexpression, while it was detected in only 5%–10% of breast cancer tissue without *ERBB2* overexpression. The mechanism of this correlation remains unclear. The *ERBB2* and *TOP2A* genes were previously thought to be coamplified, because both the genes are located on chromosome 17q12-21. Recent studies, however, showed that when these genes were amplified, they were located in different amplicons. In other studies, the number of copies of the *ERBB2* and *TOP2A* genes were not identical.[13] The present study also showed that the overexpression or amplification of *ERBB2* was significantly associated with better responses to taxanes. Other genetic events on the 17q12-21 and other chromosomal regions that occur when *ERBB2* is amplified may be involved in its mechanisms.[14]

TP53 preserves genome integrity as the "guardian of the genome" in response to various cellular stresses by invoking cell-cycle arrest and allowing the repair system to eliminate mutations, or by inducing apoptosis when the correct DNA repair is not accomplished.[15] Because most chemotherapeutic agents induce apoptosis through either DNA damage or microtubule disruption, the *TP53* status may affect the sensitivity of tumor cells against these agents. Animal and in vitro studies, however, failed to show general trends of associations between *TP53* status and drug sensitivity.[15,16] The present study also showed inconsistent results in clinical studies. This is probably because only *TP53* gene mutations and mutated TP53 protein accumulation have been

examined, but many mechanisms regulating TP53 protein activity have never been evaluated, which include post-translational modification and interaction with other upstream and downstream molecules.[15]

The Bcl-2 family of proteins plays a central role in regulating apoptosis by balancing expression between pro- and anti-apoptotic family members. Cytotoxic stimuli that promote apoptosis, including DNA damage or microtubule disruption by chemotherapy, can be prevented by *BCL2* expression. An in vitro study consistently showed that overexpression of *BCL2* increased the resistance of MCF-7 cells to doxorubicin, and this resistance was positively correlated with *BCL2* expression levels of individual MCF/BCL2 clones.[17] In clinical studies, however, the association between the expression of *BCL2* and chemosensitivity was not conclusive, mostly due to the small sample size of each study. The present study showed that patients with *BCL2*-positive breast cancer were twice as likely to be resistant to chemotherapy.

The methodological limitations of studies on the association between gene alterations and clinical drug sensitivity are summarized as follows: (1) all the studies were retrospective subgroup analyses; (2) the endpoint of these studies was the response rate in the metastatic or neoadjuvant setting, which is not as objective an endpoint as survival; (3) the sample size of these studies was relatively small; and (4) the majority of the studies assessed the alterations by immunohistochemistry using monoclonal antibodies, but no international standard criteria of positivity and negativity have been defined.[18] In addition, the present study had major problems, such as large heterogeneity among studies; publication bias; and a selection bias, in that studies with incomplete information were excluded from this study. In spite of these limitations, the exploratory analyses in this study will help select genes for future confirmatory studies of molecular markers associated with the clinical response to cytotoxic chemotherapy.

In conclusion, *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for future clinical trials of predictive chemosensitivity tests in patients with breast cancer.

## Conflict of Interest

The authors indicate no potential conflicts of interest.

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ORIGINAL REPORT

# *EGFR* Mutations Predict Survival Benefit From Gefitinib in Patients With Advanced Lung Adenocarcinoma: A Historical Comparison of Patients Treated Before and After Gefitinib Approval in Japan

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## ABSTRACT

### Purpose
This study evaluated whether the presence of *epidermal growth factor receptor* (*EGFR*) mutations is a predictive marker for survival benefit from gefitinib and/or a prognostic marker in patients with advanced lung adenocarcinoma.

### Patients and Methods
Overall survival (OS) was compared between patients with advanced lung adenocarcinoma who began first-line systemic therapy before and after gefitinib approval in Japan (January 1999 to July 2001 and July 2002 to December 2004, respectively). Deletional mutations in exon 19 or the L858R mutation in exon 21 of *EGFR* were evaluated using high-resolution melting analysis.

### Results
*EGFR* mutations were detected in 136 (41%) of the 330 patients included in this study. OS was significantly longer among the *EGFR*-mutant patients treated after gefitinib approval compared with the OS of patients treated before gefitinib approval (median survival time [MST], 27.2 *v* 13.6 months, respectively; $P < .001$), whereas no significant survival improvement was observed in patients without *EGFR* mutations (MST, 13.2 *v* 10.4 months, respectively; $P = .13$). A significant interaction between the presence of *EGFR* mutations and a survival improvement was seen ($P = .045$). Among patients treated before gefitinib approval, those with *EGFR* mutations lived longer than those without *EGFR* mutations (MST, 13.6 *v* 10.4 months, respectively; $P = .034$). The response rates to first-line cytotoxic chemotherapy were not significantly different between patients with and without *EGFR* mutations (31% *v* 28%, respectively; $P = .50$).

### Conclusion
*EGFR* mutations significantly predict both a survival benefit from gefitinib and a favorable prognosis in patients with advanced lung adenocarcinoma.

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## INTRODUCTION

Gefitinib (Iressa; AstraZeneca, Osaka, Japan) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). Gefitinib was approved for the treatment of patients with advanced non–small-cell lung carcinoma (NSCLC) in Japan in July 2002, after its antitumor activity had been demonstrated in two phase II studies.[1,2] The response rate to gefitinib was higher among women, patients with adenocarcinoma, never-smokers, and Japanese or East Asians.[1-3] In April 2004, somatic mutations in the kinase domain of *EGFR*, mainly in-frame deletions including amino acids at codons 747 to 749 (DEL) in exon 19 and a missense mutation at codon 858 (L858R) in exon 21, were suggested to be determinants of gefitinib sensitivity.[4,5] Since then, retrospective studies have consistently revealed a strong association between *EGFR* mutations and clinical outcomes in NSCLC patients treated with gefitinib.[6-9] Although these studies showed that overall survival (OS) was much longer among patients with *EGFR* mutations, they did not intrinsically prove a survival benefit of gefitinib in patients with *EGFR* mutations because there remained the possibility that the differences in OS were merely caused by prognostic differences independent of gefitinib treatment.

Eight large-scale, randomized, phase III trials were conducted to evaluate the survival benefits of



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gefitinib or erlotinib (Tarceva; OSI Pharmaceuticals Inc, Melville, NY), another EGFR-TKI, in patients with advanced NSCLC. The Iressa NSCLC Trial Assessing Combination Treatment (INTACT) –1, INTACT-2, Tarceva Responses in Conjunction with Paclitaxel and Carboplatin (TRIBUTE), and Tarceva Lung Cancer Investigation (TALENT) trials tested the concurrent combination of platinum-based chemotherapy and EGFR-TKIs in a first-line setting but failed to show a survival benefit from the addition of the EGFR-TKIs.[10-13] The Iressa Survival Evaluation in Lung Cancer (ISEL) trial tested the role of second- or third-line gefitinib monotherapy but also failed to show a significant survival benefit over a placebo,[14] whereas the BR.21 trial showed a significant survival benefit of second- or third-line erlotinib monotherapy.[15] The Iressa NSCLC Trial Evaluating Response and Survival against Taxotere (INTEREST) and V15-32 trials compared OS after second-line gefitinib monotherapy and docetaxel monotherapy, which is a standard second-line treatment; the former study proved the noninferiority of gefitinib to docetaxel, whereas the latter study failed to do so.[16,17]

In subgroup analyses of some of these trials, significant survival benefits were observed for never-smokers[12,14] and Asian patients.[14] In the BR.21 trial, no history of smoking was a significant predictor of a survival benefit from erlotinib.[15] Because never-smokers and Asian patients are known to have higher frequencies of *EGFR* mutations,[4-9,18,19] these results suggested an association between *EGFR* mutations and a survival benefit from EGFR-TKIs. However, in all of these trials, mutational analyses failed to show a significant survival benefit from EGFR-TKIs in *EGFR*-mutant patients,[20-23] partly because of the small sample sizes that were used.

In the INTACT and TRIBUTE trials, patients with *EGFR* mutations lived longer than those without *EGFR* mutations, irrespective of treatment with EGFR-TKIs[20,21]; this result suggested that *EGFR* mutations may have prognostic value in patients with advanced NSCLC who were treated with standard chemotherapy. However, these trials were inconclusive regarding this point because of the small number of *EGFR*-mutant patients who were examined. As for early-stage NSCLC patients, several large-scale retrospective studies have been reported; some studies showed no significant association between the presence of *EGFR* mutations and OS after surgery,[19,24] whereas others showed that the presence of *EGFR* mutations was associated with a favorable prognosis in a univariate analyses, but the association disappeared when adjustments for patient characteristics like sex and smoking history were made.[25,26]

To evaluate whether gefitinib provides a survival benefit to patients with lung adenocarcinoma and whether the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib and/or a prognostic factor, we analyzed data obtained on patients with advanced lung adenocarcinoma who were treated before and after gefitinib approval.

## PATIENTS AND METHODS

### Patients

We performed all the analyses in this study using a protocol approved by the institutional review board of the National Cancer Center Hospital (NCCH; Tokyo, Japan). Consecutive patients with advanced lung adenocarcinoma who had been pathologically diagnosed at NCCH and began first-line systemic therapy without thoracic radiotherapy between July 2002 and December 2004 (after gefitinib approval; group A) or between January 1999 and July 2001 (at least 1 year before gefitinib approval; group B) were identified using the databases of NCCH. Patients for whom appropriate pathologic samples were available and a mutational analysis could be successfully performed were included in this study.

### Mutational Analysis

DNA was extracted from archived paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides, and the two major hotspots of *EGFR* mutations, DEL and L858R, were analyzed using high-resolution melting analysis according to a previously described method.[9,27] Briefly, polymerase chain reaction (PCR) was performed using primers designed to amplify a region containing E746-I759 or L858 of *EGFR* and the dye LCGreen I (Roche Diagnostics, Indianapolis, IN). Melting curves were obtained using HR-1 (Idaho Technology, Salt Lake City, UT), and the curves of the samples and controls were compared. All of the mutational analyses were performed in a blinded fashion.

### Clinical Outcomes

OS was defined as the time from the start of first-line systemic therapy until death. In patients with measurable lesions, tumor response to first-line cytotoxic chemotherapy, including second-line therapy after first-line gefitinib therapy, was evaluated using standard bidimensional measurements.[28] The response rate was defined as the proportion of complete and partial responses compared with the total number of patients.

### Statistical Analysis

The differences in OS for the patients in group A and those in group B were compared using Kaplan-Meier curves and log-rank tests. To assess the interaction between the groups and the mutational status of *EGFR*, interaction terms were included in the Cox proportional hazards models. The interaction was considered significant if $P < .10$. The impact of *EGFR* mutations on tumor response to chemotherapy and prognosis was assessed using a $\chi^2$ test and a log-rank test, respectively. These analyses were performed with or without adjustments for the following baseline characteristics: age, sex, smoking history (never-smokers *v* others), performance status (PS), and disease stage (recurrence after surgery *v* stage III/IV). All the statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

## RESULTS

### Mutational Analysis

Medical and pathologic records were reviewed for 414 clinically eligible patients (255 in group A and 159 in group B), and the mutational status was successfully determined in 330 patients (200 in group A and 130 in group B). Appropriate pathologic samples were not available in 68 patients (49 in group A and 19 in group B), and indeterminate results were obtained because of incomplete PCR in 16 patients (six in group A and 10 in group B). Of the 330 successfully analyzed patients, 193 were analyzed using only cytology samples, 106 were analyzed using only tissue samples, and 31 were analyzed using both samples. DEL and L858R mutations were detected in 77 (23%) and 59 patients (18%), respectively, and these mutations were mutually exclusive.

### Patient Characteristics

The patient characteristics of the 330 patients are listed in Table 1. All of the patients were Japanese except for one Korean patient and one Chinese patient. When groups A and B were compared, group A had a significantly higher percentage of patients with recurrence after surgery and patients with a poor PS. Age, sex, and smoking history were similar between the two groups. In group A, most of the patients were treated with EGFR-TKIs. However, 15 patients (8%) were not treated with EGFR-TKIs, and in 12 patients (6%), the EGFR-TKI



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**Table 1.** Patient Characteristics

| Characteristic | Group A: July 2002 to December 2004 (n = 200) | | Group B: January 1999 to July 2001 (n = 130) | | |
|---|---|---|---|---|---|
| | No. of Patients | % | No. of Patients | % | *P* |
| Age, years | | | | | .47 |
| Median | 62 | | 62 | | |
| Range | 27-84 | | 37-84 | | |
| Sex | | | | | .52 |
| Female | 84 | 42 | 50 | 38 | |
| Male | 116 | 58 | 80 | 62 | |
| Smoking history* | | | | | .70† |
| Never-smoker | 92 | 46 | 57 | 44 | |
| Former smoker | 42 | 21 | 33 | 25 | |
| Current smoker | 66 | 33 | 40 | 31 | |
| Histologic diagnosis | | | | | — |
| Adenocarcinoma | 200 | 100 | 130 | 100 | |
| Other | 0 | 0 | 0 | 0 | |
| Performance status | | | | | .049‡ |
| 0 | 70 | 35 | 46 | 35 | |
| 1 | 113 | 57 | 80 | 62 | |
| 2 | 13 | 7 | 4 | 3 | |
| 3 | 4 | 3 | 0 | 0 | |
| Stage | | | | | .001§ |
| IIIB | 37 | 19 | 29 | 22 | |
| IV | 79 | 40 | 70 | 54 | |
| Recurrence after surgery | 84 | 42 | 31 | 24 | |
| First-line cytotoxic chemotherapy‖ | | | | | — |
| Platinum + third-generation drug¶ | 140 | 70 | 88 | 68 | |
| Other platinum-based regimen | 0 | 0 | 8 | 6 | |
| Non–platinum-based regimen | 14 | 7 | 34 | 26 | |
| No cytotoxic chemotherapy | 46 | 23 | 0 | 0 | |
| EGFR-TKI therapy | | | | | — |
| First line | 81 | 41 | 0 | 0 | |
| Second line | 63 | 32 | 9 | 7 | |
| Third or more line | 29 | 15 | 10 | 8 | |
| Never | 15 | 8 | 111 | 85 | |
| Unknown | 12 | 6 | 0 | 0 | |
| *EGFR* mutation status | | | | | |
| DEL | 46 | 23 | 31 | 24 | |
| L858R | 32 | 16 | 27 | 21 | |
| Wild type | 122 | 61 | 72 | 55 | |

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; DEL, deletional mutations in exon 19.
*Never-smokers were defined as patients who had never had a smoking habit, and former smokers were defined as patients who had stopped smoking at least 1 year before diagnosis.
†Never-smokers *v* others.
‡0 or 1 *v* 2 or 3.
§IIIB or IV *v* recurrence after surgery.
‖Including second-line therapy after first-line gefitinib therapy.
¶Third-generation drug indicates paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan.

treatment history was unknown because the patients had been transferred to another hospital and the subsequent treatment data was not available. In group B, all but 19 patients (15%) had no history of EGFR-TKI treatment; six patients had been treated with gefitinib in clinical trials before gefitinib approval, one patient had been treated with erlotinib in a phase II trial, and 12 patients had been treated with gefitinib in a clinical practice setting after gefitinib approval.

### Historical Comparison Before and After Gefitinib Approval

The median follow-up time for 46 survivors in group A was 30.8 months (range, 10.7 to 49.8 months), and the follow-up times for two survivors in group B were 65.7 and 85.0 months. OS was significantly longer in group A than in group B (median survival time [MST], 18.1 *v* 12.5 months, respectively; hazard ratio [HR] = 0.66; 95% CI, 0.52 to 0.84; $P < .001$; Fig 1A). In group A versus group B, a significant improvement in survival was observed in patients with *EGFR* mutations (MST, 27.2 *v* 13.6 months, respectively; HR = 0.48; 95% CI, 0.32 to 0.71; $P < .001$; Fig 1B), whereas no significant improvement in survival was observed in patients without *EGFR* mutations (MST, 13.2 *v* 10.4 months, respectively; HR = 0.79; 95% CI, 0.59 to 1.07; $P = .13$; Fig 1C). The improvement in survival was similar among patients with DEL (Fig 1D) and those with L858R (Fig 1E). A significant interaction between the mutational status of *EGFR*


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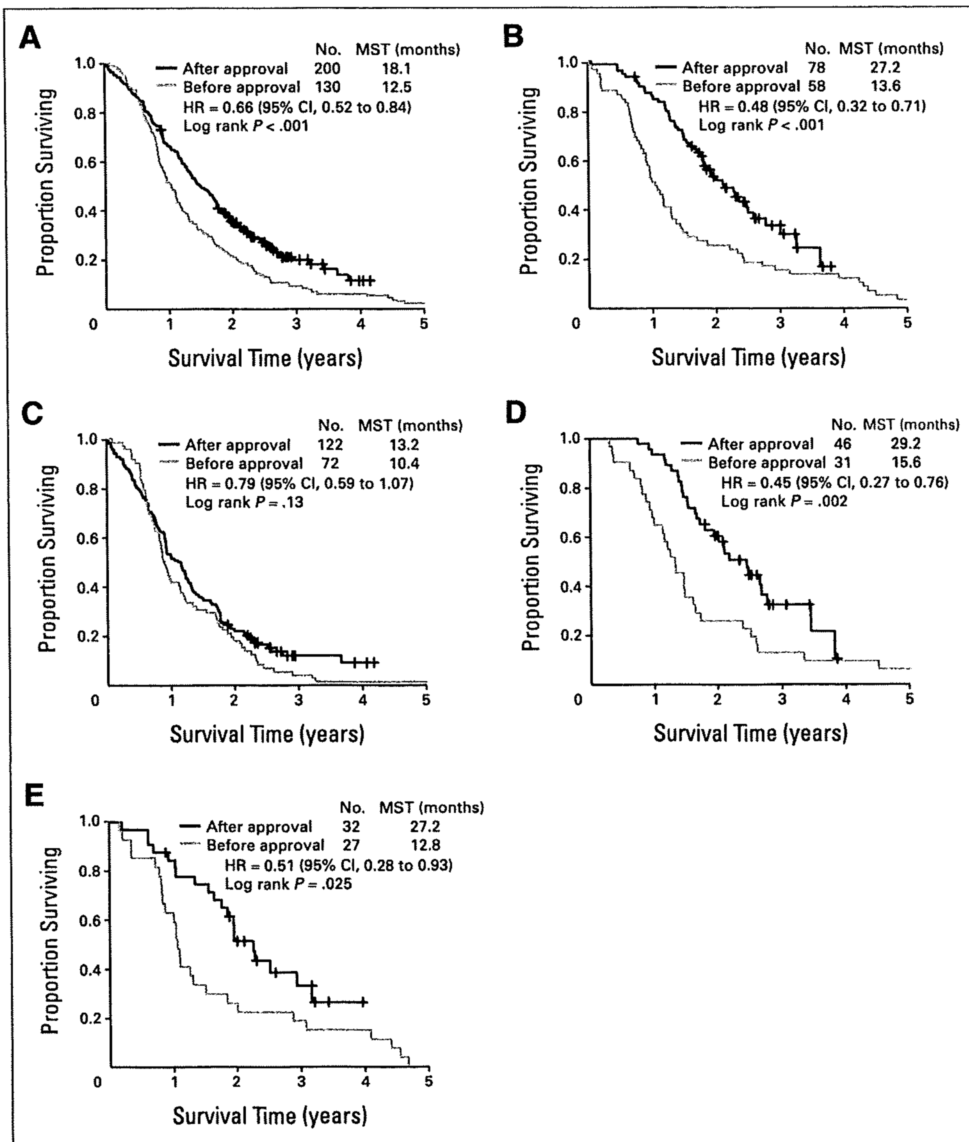


**Fig 1.** Comparison of overall survival between patients who began first-line systemic therapy after gefitinib approval and patients who began treatment before gefitinib approval. (A) All patients included in the current study. (B) Patients with epidermal growth factor receptor (*EGFR*) mutations. (C) Patients without *EGFR* mutations. (D) Patients with deletional mutations in exon 19. (E) Patients with L858R mutation. MST, median survival time; HR, hazard ratio.

(mutant *v* wild type) and the improvement in survival was observed ($P = .045$). After adjusting for age, sex, smoking history, PS, and disease stage, the HR of after to before gefitinib approval was 0.47 (95% CI, 0.31 to 0.70; $P < .001$) among patients with *EGFR* mutations and 0.76 (95% CI, 0.55 to 1.04; $P = .088$) among patients without *EGFR* mutations. The interaction was also significant after the adjustment ($P = .035$).

### Prognosis in Patients Before Gefitinib Approval

When patients with and without *EGFR* mutations were compared in group B (patients treated before gefitinib approval), the patients with *EGFR* mutations lived significantly longer than patients without *EGFR* mutations (MST, 13.6 *v* 10.4 months, respectively; HR = 0.68; 95% CI, 0.48 to 0.97; $P = .034$; Fig 2A), and this finding persisted after adjustments for age, sex, smoking history, PS, and disease stage (HR = 0.65; 95% CI, 0.44 to 0.96; $P = .028$). However, this result may be affected by EGFR-TKI treatment administered to 19 patients (12 with *EGFR* mutations and seven without *EGFR* mutations). When the start of EGFR-TKI administration in the 19 patients was treated as a censoring event to exclude the effect, the difference in OS was not significant (HR = 0.74; 95% CI, 0.50 to 1.08; $P = .12$; Fig 2B). Between patients with DEL and those with L858R, the difference in OS was not significant (MST, 15.6 *v* 12.8 months, respectively; HR = 0.86; 95% CI, 0.51 to 1.46: $P = .58$).

### Response to Cytotoxic Chemotherapy

The response to cytotoxic chemotherapy was evaluated in 279 of the 330 patients. The other 51 patients were excluded because no chemotherapy other than gefitinib was administered (n = 46) or they had no measurable lesions (n = 5). As shown in Table 2, the total response rate was 29%, and the response rates were not significantly different between patients with and without *EGFR* mutations (31% *v* 28%, respectively; $P = .50$). These findings were similar for patients with DEL and with L858R (29% *v* 35%, respectively; $P = .49$). *EGFR*


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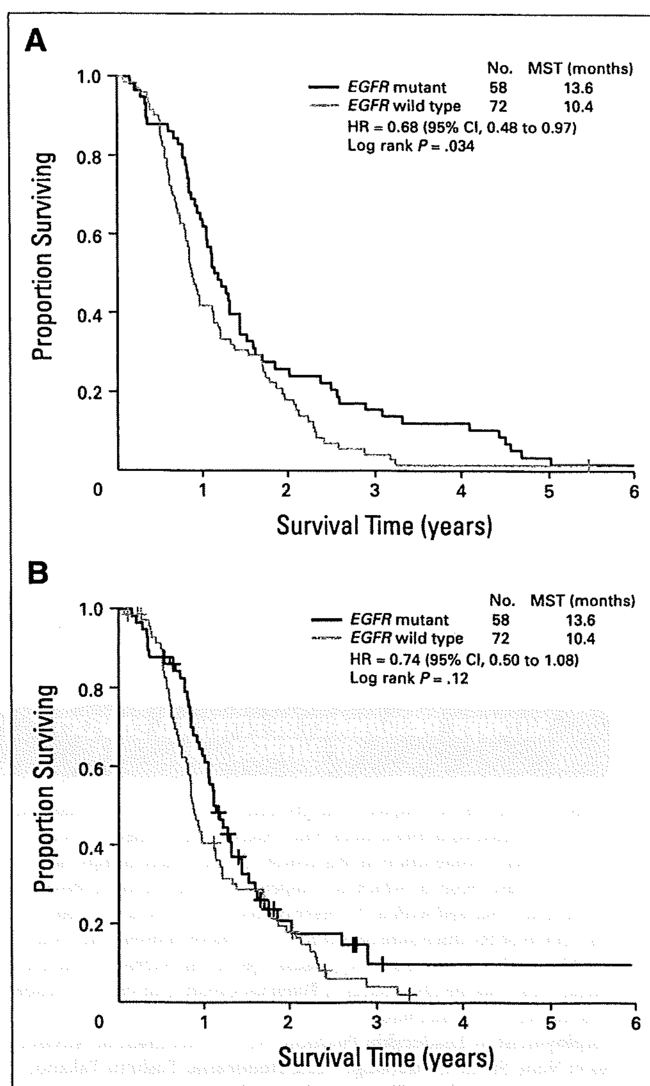


**Fig 2.** (A) Comparison of overall survival between patients with and without epidermal growth factor receptor (*EGFR*) mutations among patients treated before gefitinib approval; and (B) the same comparison when the start of EGFR tyrosine kinase inhibitor administration is treated as a censoring event. MST, median survival time; HR, hazard ratio.

mutations were not significantly associated with response to any specific regimen, although the response rate to taxane monotherapy tended to be higher among patients with *EGFR* mutations than in patients without *EGFR* mutations (31% *v* 13%, respectively; $P = .17$).

## DISCUSSION

To assess the survival benefit of gefitinib in patients with lung adenocarcinoma, we compared the OS of patients treated after gefitinib approval (group A) with a historical control (group B). As the historical control, we selected patients treated between January 1999 and July 2001 because most of these patients routinely received a combination of platinum and a third-generation drug and were also administered second-line cytotoxic chemotherapy, if indicated; thus, their cytotoxic chemotherapy regimens were similar to those of the patients in group A. Actually, fewer cytotoxic chemotherapy regimens were used in group A because some cytotoxic chemotherapy options were replaced with gefitinib therapy. Because the most essential difference between the two groups was the availability of gefitinib, the survival improvement observed in this historical comparison can be interpreted as reflecting a survival benefit from the addition of gefitinib monotherapy or the replacement of cytotoxic chemotherapy with gefitinib monotherapy. Although there was a small number of patients who were not treated with EGFR-TKIs in group A or who were treated with EGFR-TKIs in group B, we included all consecutive patients in the analysis to avoid biases. Some imbalances in the baseline patient characteristics of the two groups were noted; however, all of the results described in the present study were similar even after adjustments were made for the baseline patient characteristics.

In this study, we clearly showed an improvement in the survival of patients with *EGFR* mutations after gefitinib approval. In fact, the MST doubled (13.6 to 27.2 months), a feat that has never before been achieved in the history of NSCLC treatment. Even in patients without *EGFR* mutations, a nonsignificant improvement in survival was obtained (MST, 10.4 to 13.2 months); this result might be a result of the efficacy of gefitinib, period effects other than the approval of gefitinib therapy, or selection biases. Nevertheless, a significant interaction between the presence of *EGFR* mutations and an improvement in survival was obtained, meaning that the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib.

To our knowledge, this is the first study to show a significant interaction between *EGFR* mutations and a survival benefit from EGFR-TKI therapy. Although this study was a retrospective historical comparison conducted only in East Asian patients and some biases could not be excluded, the number of patients with *EGFR* mutations analyzed in this study ($n = 136$) was much larger than those in phase III trials (INTACT, $n = 32$; TRIBUTE, $n = 29$; ISEL, $n = 26$; BR.21, $n = 34$),[20-22,29] and we believe that the results of this study have a certain amount of importance to clinical practice.

The current study also showed that, among the patients treated with chemotherapy before gefitinib approval (group B), the OS was significantly longer in the patients with *EGFR* mutations than in those without *EGFR* mutations. As with the INTACT and TRIBUTE trials,[20,21] this result suggested that the presence of *EGFR* mutations was a favorable prognostic factor in patients with advanced NSCLC. However, this result is not conclusive because the difference was marginal when the effects of EGFR-TKIs, which were used in a small number of patients, were excluded.

As for the patients who were treated after gefitinib approval (group A), the difference in OS between the patients with and without *EGFR* mutations can be partly explained by the prognostic value of the *EGFR* mutations themselves. However, this study indicated that the difference was mainly caused by the mutations' predictive value for a survival benefit from gefitinib.

The difference in OS according to the mutational status of *EGFR* in group B can also be explained by the predictive value for chemotherapy efficacy other than the pure prognostic value. In INTEREST and V15-32, which were phase III trials comparing docetaxel and gefitinib, the HRs for OS were almost the same between patients with and without *EGFR* mutations,[16,30] suggesting that *EGFR* mutations might be a predictive factor for a survival benefit from both docetaxel


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**Table 2.** *EGFR* Mutations and Tumor Response to Cytotoxic Chemotherapy

| Therapy | Mutant *EGFR* | | Wild-Type *EGFR* | | *P* | Total | |
|---|---|---|---|---|---|---|---|
| | No. of Patients | Response Rate (%) | No. of Patients | Response Rate (%) | | No. of Patients | Response Rate (%) |
| Total | 112 | 31 | 167 | 28 | .50 | 279 | 29 |
| Regimens | | | | | | | |
| Platinum + taxane | 54 | 37 | 97 | 34 | .71 | 151 | 35 |
| Platinum + other third-generation drug* | 35 | 26 | 39 | 26 | .99 | 74 | 26 |
| Taxane† monotherapy | 16 | 31 | 23 | 13 | .17 | 39 | 21 |
| Other regimen | 7 | 14 | 8 | 0 | .27 | 15 | 7 |
| Treatment line | | | | | | | |
| First line | 95 | 33 | 147 | 27 | .37 | 242 | 29 |
| Second-line therapy after first-line gefitinib therapy | 17 | 24 | 20 | 30 | .66 | 37 | 27 |

Abbreviation: *EGFR*, epidermal growth factor receptor.
*Other third-generation drug indicates gemcitabine, vinorelbine, or irinotecan.
†Taxane indicates paclitaxel or docetaxel.

and gefitinib. In the current study, response rate to taxane monotherapy tended to be higher in patients with *EGFR* mutations, although the number of patients was small. These results are inconclusive, and further investigation is needed.

We detected no significant difference in the predictive and prognostic values of DEL and L858R in the current study. Some researchers, including ourselves, have reported that patients with DEL had better outcomes after EGFR-TKI treatment than those with L858R[9,31,32]; however, the current study showed that gefitinib yielded almost the same survival benefit to both patients with DEL and patients with L858R, and we think that the two *EGFR* mutations should be treated equally when making clinical decisions.

In the ISEL and BR.21 trials, the *EGFR* copy number (evaluated using fluorescence in situ hybridization), rather than the *EGFR* mutation status, was suggested to predict a survival benefit from EGFR-TKIs,[22,23,29] and the authors concluded that a mutational analysis was not necessary to select patients for treatment with EGFR-TKIs. In contrast, the current study indicated that the *EGFR* mutation status was a determinant of a survival benefit from gefitinib, although *EGFR* copy numbers were not evaluated in this study. Our previous study showed that the *EGFR* copy number, as evaluated using quantitative PCR, was associated with a response to gefitinib; however, an increased *EGFR* copy number tended to be seen in patients with *EGFR* mutations and was not an independent predictor of response or OS in gefitinib-treated patients.[6] These discrepancies may be a result of the ethnic difference, the methodologic difference between fluorescence in situ hybridization and quantitative PCR, or the accuracy of biomarker analyses. Although controversy still remains, we believe that the *EGFR* mutation status is the most useful biomarker for patient selection, at least in East Asian patients who have *EGFR* mutations more frequently than non-Asian patients.

In conclusion, gefitinib yielded a survival benefit among Japanese patients with lung adenocarcinoma, and the survival benefit was significantly greater in patients with *EGFR* mutations than in those without *EGFR* mutations. The presence of *EGFR* mutations may also be a favorable prognostic factor in advanced lung adenocarcinoma independent of gefitinib treatment. We need to consider appropriate treatment strategies for patients with NSCLC based on their *EGFR* mutation status.

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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# Prospective Study of the Accuracy of *EGFR* Mutational Analysis by High-Resolution Melting Analysis in Small Samples Obtained from Patients with Non-Small Cell Lung Cancer

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**Abstract** **Purpose:** Epidermal growth factor receptor (*EGFR*) mutations, especially in-frame deletions in exon 19 (DEL) and a point mutation in exon 21 (L858R), predict gefitinib sensitivity in patients with non-small cell lung cancer (NSCLC). In this study, we verified the accuracy of *EGFR* mutation analysis in small samples by high-resolution melting analysis (HRMA), which is a rapid method using PCR amplification with a dye to analyze the melting curves in NSCLC.

**Experimental Design:** We designed a prospective study to compare the sensitivity and specificity of HRMA and DNA sequencing with laser capture microdissection. Eligible patients with lung lesions were screened by bronchoscopy or percutaneous needle biopsy to histologically confirm the diagnosis, followed by surgical resection of the NSCLC. Small diagnostic specimens were analyzed for *EGFR* mutations by HRMA, and the surgically resected specimens were examined for mutations by HRMA and DNA sequencing.

**Results:** The analyses for *EGFR* mutations were conducted in 52 eligible cases of the 92 enrolled patients. *EGFR* mutations were detected in 18 (34.6%) patients. The results of HRMA from surgically resected specimens as well as DNA sequencing revealed 100% sensitivity and specificity. On the other hand, the sensitivity and specificity of HRMA from the small diagnostic specimens were 83.3% and 100%, respectively.

**Conclusions:** In this study, we showed that HRMA is a highly accurate method for detecting DEL and L858R mutations in patients with NSCLC, although it is necessary to consider the identification of patients with a false-negative result when the analysis is conducted using small samples.

Somatic mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) have been reported in patients with non-small cell lung cancer (NSCLC; refs. 1-3). Although many types of *EGFR* mutations have been identified, they seem to be concentrated in exons 18 to 21 of *EGFR*; ~85% to 90% of *EGFR*-mutant patients have mutations in two hotspots: a short in-frame deletion in exon 19 (DEL) and a point mutation at codon 858 in exon 21 (L858R; ref. 4). Several studies have revealed that *EGFR* mutations are strongly associated with the tumor response and clinical outcome in patients with NSCLC receiving treatment with EGFR tyrosine kinase inhibitors, such as gefitinib (Iressa, AstraZeneca; refs. 5-7). The mutational status of *EGFR*, especially the presence/absence of DEL and L858R, is a strong predictor of the sensitivity to EGFR tyrosine kinase inhibitor, and the detection of *EGFR* mutations is useful for decision-making by both patients and physicians (4, 8). Recently, a laboratory test for *EGFR* mutations has become clinically available for guiding treatment decisions.

Until now, screening for these mutations has most commonly been conducted using DNA sequencing methods. In our previous study, we used methanol-fixed, paraffin-embedded surgical specimens and performed direct sequencing and pyrosequencing with laser capture microdissection (LCM) to ensure high-quality genetic analysis of archived tissues (5, 9). However, these approaches are not useful in clinical practice for two reasons. First, although the sequencing methods require a high ratio of tumor-to-normal tissue DNA for optimal results, the diagnostic specimens obtained from cases of advanced NSCLC may contain only a small amount of tumor cells and

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are highly contaminated with normal cells. Secondly, *EGFR* mutation analysis based on DNA sequencing requires special instruments and is also time-consuming and expensive. Therefore, some simple and highly sensitive nonsequencing methods to detect *EGFR* mutations have been reported (10–22). However, the accuracy of these methods for clinical use have not been assessed in prospective studies.

High-resolution melting analysis (HRMA) using the LCGreen I (Idaho Technology) dye was introduced as an easy, quick, and inexpensive method for the screening of mutations (23), and we established and validated the HRMA method to detect DEL and L858R mutations in cases of NSCLC (9, 10). Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% of mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA retrospectively using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (9). Furthermore, it was shown that among patients treated with gefitinib, the response rate (78% versus 8%), time-to-progression (median, 9.2 versus 1.6 months), and overall survival (median, 21.7 versus 8.7 months) were significantly better in patients with *EGFR* mutations than with wild-type *EGFR* ($P < 0.001$), as detected by HRMA (9). These results suggest that this easy, quick, and inexpensive method which was done using diagnostic small samples of advanced NSCLC tumors is one of the most useful and precise methods to detect *EGFR* mutations in clinical practice.

In this study, we designed a prospective study to detect two major *EGFR* mutations by HRMA using small diagnostic cytologic or biopsy specimens and surgically resected specimens, and the results were compared with the results of DNA sequencing methods combined with LCM, which we consider as the "gold standard" for such detection, applied to methanol-fixed, paraffin-embedded surgically resected specimens. We evaluated the diagnostic sensitivity, specificity, predictive values, and accuracy of the detection of *EGFR* mutations using HRMA and revealed that this method is feasible for clinical use to detect *EGFR* mutations in small samples obtained from patients with NSCLC.

## Patients and Methods

***Patients and materials.*** Patients with lung lesions, which were suspected clinically to be operable NSCLC, were enrolled in this prospective study. The patients were scheduled for bronchoscopy or percutaneous needle biopsy to establish the histologic diagnosis, and informed consent was obtained from each of the patients prior to these diagnostic procedures. Thereafter, the patients diagnosed with NSCLC underwent lung surgery at our hospital. In this study, mutational analysis of *EGFR* was done by HRMA or DNA sequencing methods combined with LCM in all the patients in which both the preoperatively obtained diagnostic specimens and the resected specimens were histologically confirmed by a certified pathologist to contain malignant cells.

Based on a protocol approved by the Institutional Review Board of the National Cancer Center, we did mutational analyses of *EGFR* to detect DEL and L858R in the eligible patients. The Papanicolaou-stained cytologic slides ($n = 35$), formalin-fixed, paraffin-embedded transbronchial or percutaneous needle biopsy specimens ($n = 34$), and methanol-fixed, paraffin-embedded surgically resected specimens subjected to LCM using a PixCell II LCM system (Arcturus Engineering, Inc.; $n = 52$) were collected prospectively. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen), as described in our previous report (10).

***HRMA.*** PCR was done to amplify exons 19 or 21 of *EGFR* using LCGreen I (Idaho Technology) on a LightCycler (Roche Diagnostics) and primers designed as previously described (10). If the first PCR products were not available for the mutational analyses of the melting curves, we did a second PCR using the same primers. These PCR products were denatured at 95°C for 10 min and cooled to 40°C to promote the formation of heteroduplexes. The LightCycler capillary was transferred to an HR-1 (Idaho Technology), an HRMA instrument, and heated at a transition rate of 0.3°C/s. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature-adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between the tumor samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as the negative control sample with wild-type *EGFR*. Samples revealing skewed or left-shifted curves as compared with the control samples were judged to have mutations without positive controls (9, 10). All analyses were done in a blinded fashion by two researchers (T. Fukui and T. Takano). After independent evaluation by the two researchers, the final judgment was arrived at by consensus after joint viewing of the melting curves from both.

***DNA sequencing methods with LCM.*** In our previous study, we did a direct sequencing or pyrosequencing of *EGFR* in patients with recurrent NSCLC after primary surgery (5). Based on the results of our previous study, we consider direct sequencing with LCM for the detection of DEL and pyrosequencing with LCM for the detection of L858R as the gold standard in relation to *EGFR* mutational analysis. DNA was extracted from methanol-fixed, paraffin-embedded surgical specimens by LCM, according to a previously described method (24). Direct sequencing of the PCR products for DEL was done using ABI PRISM3700 and 3100 DNA sequencers (Applied Biosystems). Pyrosequencing to analyze L858R was done using Pyrosequencing PSQ 96MA (Pyrosequencing; refs. 5, 25). The *EGFR* mutational analysis using DNA sequencing methods was done in a blinded fashion by a researcher (H. Sakamoto) according to a previously described method (5), and then compared with the corresponding results obtained using HRMA.

***Statistical analysis.*** The primary end point of this study was the sensitivity and specificity of the results obtained using HRMA as compared with those of the results obtained using DNA sequencing with LCM. The sample size was calculated using a statistical power level of 0.80 and two-sided $\alpha$ level of 0.1 on the basis of an estimated sensitivity of at least 0.80 and an expected value of 0.95 for HRMA, a minimum of 20 patients with *EGFR*-mutated tumors were required. Because the percentage of NSCLC patients with *EGFR* mutations was expected to be 40% in this study population composed of only Japanese, approximately 50 patients with NSCLC were needed. Therefore, considering a specificity of at least 0.80 and the expected value of 0.95 for HRMA, 30 patients with wild-type tumors showed a statistical power level of 0.90 using a two-sided $\alpha$ level of 0.1.

The associations between mutational status and patient characteristics were assessed by a $\chi^2$ test using the SPSS statistical package (SPSS version 11.0 for Windows; SPCC, Inc.).

## Results

***Patient characteristics.*** From December 2005 to December 2006, 92 patients with clinically suspected operable NSCLC were enrolled in this study. The following diagnostic procedures were done preoperatively in 90 patients: bronchoscopy ($n = 57$), percutaneous needle biopsy ($n = 27$), or bronchoscopy followed by percutaneous needle biopsy ($n = 6$). The patient characteristics are shown in Table 1. All the patients were Japanese. Among the patients, a definitive diagnosis was established in 85 patients by bronchoscopy in 43 of 59 patients

**Table 1.** Patient characteristics

**(A) Characteristics of all the patients enrolled in this study ($n$ = 92)**

| | All ($n$ = 92) | BF ($n$ = 64) | PNB ($n$ = 34)* |
|---|---|---|---|
| Age, year, median (range) | 64 (34-84) | 64 (38-84) | 62 (41-79) |
| Gender (male/female) | 58/34 | 41/23 | 23/11 |
| Smoking history (N/F/C) | 29/30/33 | 23/19/22 | 7/14/13 |
| Tumor size, mm, average (range) | 27.2 (10.2-73.4) | 28.3 (13.8-56.6) | 24.5 (10.2-73.4) |
| Accuracy of the diagnostic procedure (%) | 66/85 (77.6) | 43/59 (72.9) | 25/31 (80.6) |
| Accuracy of the cytologic slides (%) | 54/85 (63.5) | 31/59 (52.5) | 23/30 (76.7) |
| Accuracy of the biopsy specimens (%) | 42/62 (67.7) | 35/54 (64.8) | 7/9 (77.8) |

**(B) Characteristics of the patients who underwent analysis of the EGFR mutations in this study ($n$ = 52)**

| | All ($n$ = 52) | BF ($n$ = 38) | PNB ($n$ = 17)† |
|---|---|---|---|
| Age, year, median (range) | 64.5 (34-84) | 64.5 (34-84) | 64 (47-78) |
| Gender (male/female) | 36/16 | 25/13 | 14/3 |
| Smoking history (N/F/C) | 16/17/19 | 15/11/12 | 1/7/9 |
| Tumor size, mm, average (range) | 27.0 (11.0-56.6) | 28.3 (20.6-56.6) | 24.1 (11.0-48.8) |
| Postoperative diagnosis (Ad/Sq/LCNEC) | 45/5/2 | 34/4/0 | 12/3/2 |
| Pathologic stage (IA/B, IIA/B, IIIA/B) | 19/13, 3/5, 9/2 | 15/8, 3/2, 8/2 | 7/5, 0/2, 3/0 |

NOTE: Never smokers were defined as patients who had never smoked, former smokers were defined as patients who had stopped smoking at least 1 y before the diagnosis, and current smokers were defined as patients who were still smoking at the time of the diagnosis.
Abbreviations: BF, bronchoscopy; PNB, percutaneous needle biopsy; N, never smoker; F, former smoker; C, current smoker; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.
*Including six patients in whom bronchoscopy was done followed by percutaneous needle biopsy.
†Including three in whom bronchoscopy was done followed by percutaneous needle biopsy.

(72.9%) and by percutaneous needle biopsy in 25 of 31 patients (80.6%); in 18 of the 85 (21.2%) patients, the histologic diagnosis could not be established preoperatively by bronchoscopy and/or percutaneous needle biopsy, the patients underwent lung surgery for suspicious malignant lung lesion, and examination of the resected specimens revealed the diagnosis of primary NSCLC in 17 and malignant lymphoma in 1 of the 18 patients. Among the 76 patients diagnosed to have primary NSCLC, 73 consented to undergo lung surgery. Finally, the analysis for *EGFR* mutations was done on 52 patients with a definitive histologic diagnosis of primary NSCLC, established both by examination of the preoperative diagnostic specimens and of the corresponding resected specimens (Fig. 1).

*Mutational analyses.* We analyzed 35 cytologic samples and 34 biopsy specimens obtained from 52 patients by HRMA, and

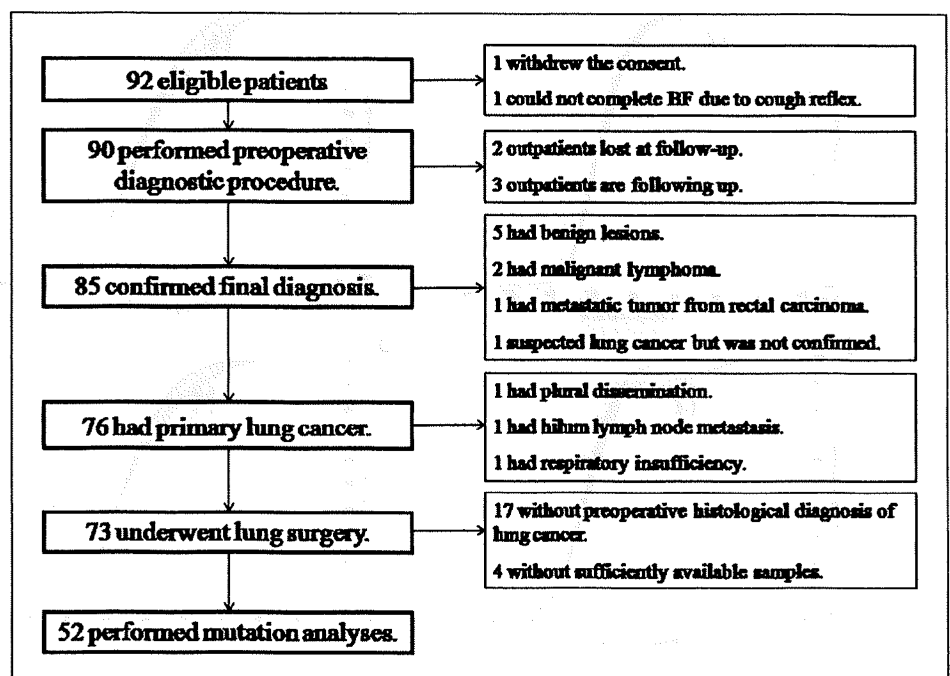


Fig. 1. Flowchart of the analyses conducted in 92 enrolled patients with lung tumors in this study.

**Table 2.** EGFR mutation status among the patient subgroups

| | *n* | *EGFR* mutations* | | | | *P* |
|---|---|---|---|---|---|---|
| | | DEL | L858R | Total | % | |
| Total | 52 | 5 | 13 | 18 | 34.6 | — |
| Gender | | | | | | |
| Women | 16 | 2 | 9 | 11 | 68.8 | 0.001 |
| Men | 36 | 3 | 4 | 7 | 19.4 | |
| Smoking history | | | | | | |
| Never | 16 | 3 | 8 | 11 | 68.8 | 0.001 † |
| Former | 17 | 2 | 4 | 6 | 35.3 | |
| Current | 19 | 0 | 1 | 1 | 5.3 | |
| Histology | | | | | | |
| Ad | 44 | 5 | 13 | 18 | 100 | 0.025 ‡ |
| Sq | 6 | 0 | 0 | 0 | 0 | |
| LCNEC | 2 | 0 | 0 | 0 | 0 | |

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.
*The EGFR mutations were analyzed by DNA sequencing with LCM.
†Comparison between never smokers and others.
‡Comparison between adenocarcinoma and others.

did both HRMA and DNA sequencing with LCM in the 52 resected specimens corresponding to the 52 patients. Among the 52 surgically resected specimens analyzed by DNA sequencing with LCM, there were 18 (34.6%) samples with *EGFR* mutations, 5 with DEL mutations, and 13 with L858R mutations. As shown in Table 2, the *EGFR* mutations were detected more frequently in women, never-smokers, and patients with a histologic diagnosis of adenocarcinoma. All results from HRMA done in a blinded fashion by two researchers (T. Fukui and T. Takano) were consistent.

HRMA could be conducted using small diagnostic samples from all 52 patients, although the analysis needed to be conducted using the second PCR product in 15 cases. In the analysis of exon 19, 5 samples revealed different curves from the control and 47 samples revealed almost the same curves as the control; therefore, we judged that the five former patients had DEL mutations (Fig. 2A). In the analysis of exon 21, 10 samples revealed a left-shift from the control and 42 samples revealed almost the same curves as the control; therefore, we judged that the 10 former patients had L858R mutations

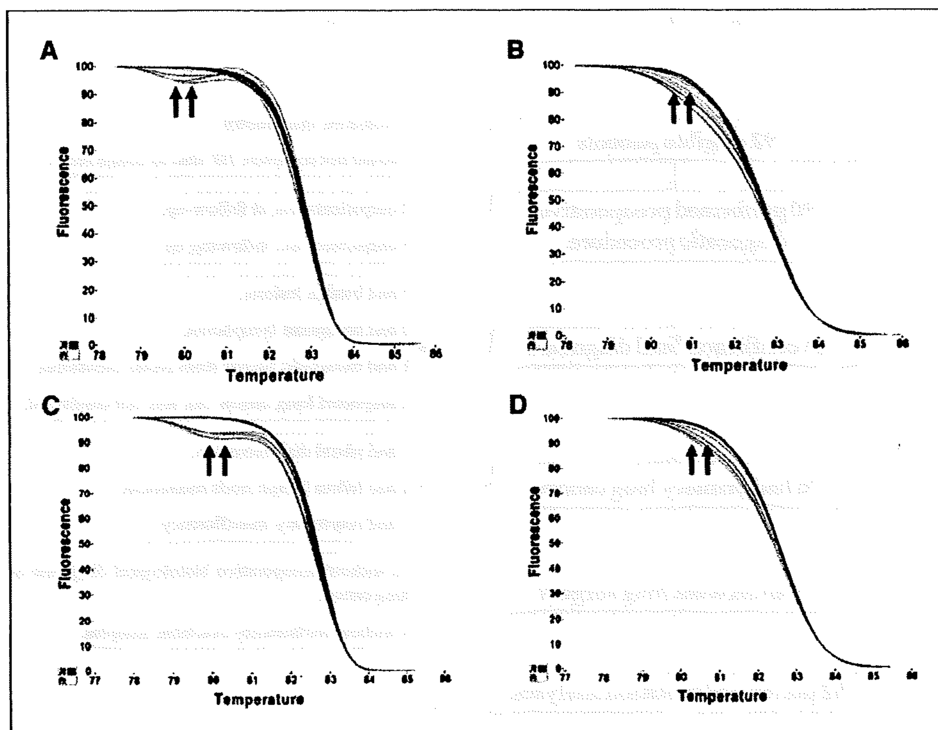


Fig. 2. Adjusted melting curves obtained by HRMA of the samples in this study to detect *EGFR* mutations (↑), in-frame deletions in exon 19 (*A*, small samples; *C*, resected specimens) and a point mutation in exon 21 (*B*, small samples; *D*, resected specimens). Each sample that revealed a skewed or left-shifted curve from those of the control sample was judged to have a mutation.

**Table 3.** Results of the EGFR mutation analyses in patients with *EGFR* mutation-positive tumors

| No. of patients | Small samples | Surgically resected specimens | |
|---|---|---|---|
| | HRMA | HRMA | Sequence with LCM |
| 13 | DEL | DEL | DEL1* |
| 26 | DEL | DEL | DEL1* |
| 32 | DEL | DEL | DEL2 † |
| 40 | DEL | DEL | DEL2 † |
| 47 | DEL | DEL | DEL1* |
| 5 | L858R ‡ | L858R | L858R |
| 6 | Wild-type | L858R | L858R |
| 12 | L858R | L858R | L858R |
| 18 | L858R | L858R | L858R |
| 21 | L858R | L858R | L858R |
| 23 | L858R ‡ | L858R | L858R |
| 25 | Wild-type | L858R | L858R |
| 27 | L858R ‡ | L858R | L858R |
| 28 | L858R | L858R | L858R |
| 31 | Wild-type ‡ | L858R | L858R |
| 41 | L858R ‡ | L858R | L858R |
| 53 | L858R | L858R | L858R |
| 54 | L858R ‡ | L858R | L858R |

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21.
*DEL1: del E746-A750 (del 2235-2249).
†DEL2: del E746-A750 (del 2236-2250).
‡The analyses by HRMA were done using second PCR products.

(Fig. 2B). All the 52 surgically resected specimens analyzed by DNA sequencing with LCM could also be analyzed by HRMA, although the analysis needed to be conducted using the second PCR product in two cases. DEL mutations were detected in 5 patients (Fig. 2C) and L858R mutations in 13 patients (Fig. 2D) among the 52 patients. Of the 52 specimens, both cytologic slides and biopsy specimens were analyzed in 17 cases. Discrepant results were obtained by HRMA in one of the cases, with L858R mutation being detected in the cytologic slides but not in the biopsy specimens. We included this patient in the population with L858R mutations.

The results of HRMA were consistent with the results of DNA sequencing with LCM in all the surgically resected specimens analyzed by the two methods. On the other hand, HRMA using small diagnostic specimens revealed the wild-type curve in three cases, although analysis of the corresponding surgically resected specimens analyzed by pyrosequencing with LCM revealed the L858R mutation (Table 3). Thus, the results for these samples obtained by HRMA were considered as false-negative results. Neither method of analysis yielded any false-positive cases. The results of the *EGFR* mutational analysis by HRMA compared with DNA sequencing with LCM using surgically resected specimens were shown in Table 4. The sensitivity, specificity, and accuracy of HRMA using small diagnostic specimens were 83.3%, 100%, and 94.2%, respectively. Using surgically resected specimens, those of HRMA were all 100%.

## Discussion

In this prospective study, we showed the high accuracy of the HRMA method for detecting two major *EGFR* mutations, DEL and L858R in patients with NSCLC. The accuracy of HRMA was clearly equal to that of DNA sequencing with LCM for the detection of mutations in surgically resected specimens. On the other hand, the sensitivity and specificity of HRMA were 83.3% (90% confidence interval: 68.9-97.7%) and 100%, respectively, when the small diagnostic samples were analyzed. Although the sensitivity of HRMA which was estimated to be at least 0.80 did not reach statistical significance, we consider HRMA as one of the available methods for the detection of *EGFR* mutations in clinical practice because the specificity, which is important for clinical decision-making, of HRMA was 100% and the *EGFR* mutation rate was less than the expected 40% to secure enough statistical power in this study.

Recently, many researchers reported establishing simple and highly sensitive nonsequencing methods for detecting *EGFR* mutations using small tumor samples (11-22), and the results of several mutation analyses were correlated with the clinical outcome of EGFR tyrosine kinase inhibitor treatment (17-19). Using serial dilution studies, some researchers have reported methods that are able to detect mutations in samples containing ~0.1% to 10% mutated DNA (13, 14, 16-18, 20-22), as opposed to direct DNA sequencing which requires the presence of at least 10% to 30% of mutated DNA in the samples (18, 20). Additionally, several novel methods offered higher sensitivity and specificity than DNA sequencing to identify the mutations in clinical samples. But almost none of the methods were validated for diagnostic accuracy in a prospective study, and we therefore consider these methods to still be unsuitable for routine clinical examination. Although these nonsequencing methods were not mutually compared, based on our previous results of retrospectively verifying the accuracy of HRMA (9, 10), we thought to develop in this prospective study an easy, quick (PCR for ~1 hour and HRMA for 2 to 3 minutes), and inexpensive (at a running cost per sample of approximately $7.50, which consisted of $5.50 for the DNA extract and less than $2.00 for PCR using LCGreen I dye) method that might be useful in clinical practice with a great advantage over DNA sequencing, which requires the

**Table 4.** Comparison of the sensitivity, specificity, predictive values, and accuracy between HRMA and DNA sequencing with LCM ($n$ = 52)

| | HRMA using small samples | HRMA using surgically resected specimens |
|---|---|---|
| True-positive | 15 | 18 |
| True-negative | 34 | 34 |
| False-positive | 0 | 0 |
| False-negative | 3 | 0 |
| Sensitivity | 83.3 (68.9-97.8) | 100 |
| Specificity | 100 | 100 |
| NPV | 91.9 (84.5-99.3) | 100 |
| PPV | 100 | 100 |
| Accuracy | 94.2 (88.9-99.5) | 100 |

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). Data are presented as % or % (90% confidence interval). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

**Table 5.** Results of HRMA using cytologic slides or biopsy specimens

| | Cytologic slides ($n$ = 35) | | Biopsy specimens ($n$ = 34) | |
|---|---|---|---|---|
| | **First PCR** | **Second PCR** | **First PCR** | **Second PCR** |
| Successfully analyzed | 29 (83.0%) | 35 (100%) | 5 (15.0%) | 34 (100%) |
| True-positive | 7 | 11 | 1 | 10 |
| True-negative | 19 | 21 | 4 | 22 |
| True-negative | 0 | 0 | 0 | 0 |
| False-positive | 3 | 3 | 0 | 2 |
| Sensitivity | 70.0% (7/10) | 78.6% (11/14) | 100% (1/1) | 83.3% (10/12) |
| Specificity | 100% (19/19) | 100% (21/21) | 100% (4/4) | 100% (22/22) |
| NPV | 100% (7/7) | 100% (11/11) | 100% (1/1) | 100% (10/10) |
| PPV | 86.4% (19/22) | 87.5% (21/24) | 100% (4/4) | 91.2% (22/24) |
| Accuracy | 89.7% (26/29) | 91.4% (32/35) | 100% (5/5) | 94.1% (32/34) |

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

extraction of high-quality DNA from an adequate amount of pure tumor cells, takes a long time, and is expensive.

In this study, the three patients with L858R detected by DNA pyrosequencing with LCM using the surgically resected specimens were labeled as having the wild-type *EGFR* in the analyses conducted using the small diagnostic samples. With regard to these false-negative results, the following three points need to be discussed: first, our previous study, conducted using human lung cancer cell lines, showed that HRMA can detect the mutations, even when samples contain only a small proportion (DEL, 10%; L858R, 0.1%) of mutant cells (10). In this study, the sensitivity of HRMA was also considered to be sufficiently high for the detection of *EGFR* mutations, especially L858R, even when the analysis was conducted using small samples after evaluation by a clinical pathologist to determine if they contained benign or malignant cells. Thus, we assume a higher accuracy of HRMA when using small samples in clinical practice. Although it still needs to be comparatively analyzed with the previously reported non-sequencing methods, HRMA can be considered as one of the sensitive methods available for the detection to *EGFR* mutations in clinical practice.

Second, high-quality DNA should be preserved in clinical samples to obtain the best results. There always remains the risk of an indeterminate or false-negative result because the DNA might have degenerated during sampling or during the preservation of clinical samples. In a comparison between the cytologic slides and biopsy specimens, better results were obtained from analyses of the first PCR products using the cytologic slides rather than the results obtained using the biopsy specimens, regardless of the amount of tumor cells examined (Table 5). This could probably be explained by the differences in the method of sample fixation between the two types of specimens. It has been suggested by a previous report that DNA is preserved better in the methanol-fixed samples than in the formalin-fixed specimens (26). Therefore, if we used methanol for specimen fixation of biopsy specimens, the results of HRMA using the first PCR products from small biopsy samples might improve. Hereafter, we propose to perform mutation analyses using methanol-fixed specimens, if possible.

Finally, we need to consider the possibility of intratumoral heterogeneity, and small diagnostic samples and surgically resected specimens may each represent overlapping but different populations of these tumor cells. A lack of association in the immunohistochemical expression profile between lung biopsy specimens and the corresponding resected tumor specimens has been reported (27). Furthermore, intratumoral heterogeneity was shown not only in terms of microheterogeneity of the tumor cell phenotype (28), but in terms of genetic heterogeneity in cancer (29, 30). In particular, the intratumoral genetic heterogeneity of *EGFR* mutations may explain the variable clinical response of NSCLC to gefitinib. It is also possible that the small diagnostic samples contain only wild-type cells, even if the tumor, overall, shows mutations, because the small samples yield only small part of the tumor. It is always necessary to consider the possibility of a false-negative result of mutational analyses conducted using the small samples.

In the current prospective study, we showed the feasibility and high accuracy of using HRMA for detecting two major *EGFR* mutations, DEL and L858R, in patients with NSCLC. Although HRMA showed high accuracy, the possibility of indeterminate or false-negative results, and because of the sensitivity of this method, the quality of DNA preservation in the clinical samples or intratumoral genetic heterogeneity, must be borne in mind to a certain extent when this analysis is conducted using small diagnostic samples. Therefore, HRMA should not be used to exclude patients from EGFR tyrosine kinase inhibitor treatment on the basis of the negative results only. Based on the results of this prospective study, we suggest that this method is very useful for clinical decision-making, especially in patients with a positive result.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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