Clinical Evaluation of HER-2/neu Protein in Malignant Pleural Effusion-Associated Lung Adenocarcinoma and as a Tumor Marker in Pleural Effusion Diagnosis


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ABSTRACT

Purpose: Lung adenocarcinoma presenting as malignant pleural effusion (MPE) is common in Taiwan. Microscopically, the involved pleurae are infiltrated by numerous tumor foci, which suggests that the cancer cells are highly invasive. Overexpression of HER-2/neu has been related to proliferation, antiapoptosis, and the high invasiveness of various cancer cells. We therefore were interested in studying the role of HER-2/neu in MPE-associated adenocarcinoma cell lung cancer (ADCLC).

Experimental Design: The expression of HER-2/neu in pleural effusion was measured by ELISA. The HER-2/neu protein expression on tumor cells was evaluated by immunohistochemical (IHC) staining, and gene amplification was assayed by fluorescence in situ hybridization.

Results: The mean value of HER-2/neu in pleural effusions of patients with ADCLC and other nonmalignant lung diseases was 9.9 and 2.7 ng/ml, respectively. The difference is statistically significant (P < 0.001). Compared with cytokeratin 19 fragment CYFRA 21-1, the performance of HER-2/neu as a tumor marker in pleural effusion diagnosis was better. Overexpression of HER-2/neu in tumor tissues was found in 70% (23 of 32) of patients with MPE-associated ADCLC, 30% (13 of 43) with stage I/II non-small cell lung cancer (NSCLC), and 44% (14 of 32) with stage III NSCLC. The incidence of HER-2/neu overexpression in tumor tissues of patients with MPE-associated ADCLC was significantly higher than that of patients with stage I-III NSCLC without MPE. HER-2/neu gene amplification was uncommon (1.9%). The correlation between the IHC H-score in tumor samples and the pleural effusion level of HER-2/neu was significant (P < 0.01). A higher incidence of HER-2/neu expression beyond the cutoff point (5.5 ng/ml) in pleural effusions was also found in patients whose IHC H-scores were >50.

Conclusions: These findings indicate that HER-2/neu is important in the pathogenesis of MPE-associated ADCLC and is a potential tumor marker for a diagnosis of pleural effusion.

INTRODUCTION

Approximately 15% of lung cancer patients have a pleural effusion at the time of initial diagnosis and 50% develop a pleural effusion later in their courses (1, 2). Patients with MPE have a short life expectancy and are difficult to manage clinically (3, 4). MPE commonly accompanies adenocarcinoma (5, 6). Since 1952, adenocarcinoma has become the most common cell type of lung cancer in Taiwan (7), and subsequently, patients with MPE-associated ADCLC have become common in Taiwan. Microscopically, we found that involved pleural surfaces were diffusely infiltrated by cancer nests. This observation suggested that the neoplastic cells are highly invasive.

HER-2/neu, also referred to as c-erbB-2, is a member of the epidermal growth factor receptor family and has intrinsic tyrosine kinase activities (8). Amplification or overexpression of the HER-2/neu gene is found in ~30% of human breast carcinoma (9) and in a significant fraction of many other types of malignancies, including lung adenocarcinoma (10). The abnormal expression of HER-2/neu in primary tumors from human NSCLC has been correlated with poor clinical outcome (11, 12). One of the underlying mechanisms is that overexpression of HER-2/neu enhances metastasis-related properties (invasion,

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3 The abbreviations used are: MPE, malignant pleural effusion; ADCLC, adenocarcinoma cell lung cancer; NSCLC, non-small cell lung cancer; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, carbohydrate antigens 15-3; CA19-9, carbohydrate antigens 19-9; 72-4, carbohydrate antigens 72-4; CYFRA 21-1, cytokeratin 19 fragments; SCC, squamous cell carcinoma antigen; IHC, immunohistochemical; FISH, fluorescence in situ hybridization; TB, tuberculosis; PPE, parapneumonic effusion; CHF, congestive heart failure; ROC, receiver operating characteristic; CDA, cumulative distribution analysis.
angio genesis, and increased survival) of cancer cells (13). The other is that overexpression of HER-2/neu confers to cancer cells resistance to various cancer therapies (14). Because the poor prognosis of patients with MPE-associated ADCLC is related to the high invasiveness and drug resistance of tumor cells, we were interested in studying the role of HER-2/neu in this special disease category.

Malignancy is one of the main causes of pleural effusion. However, cytological examination of pleural fluid fails to detect neoplastic cells in ~40–50% of malignant effusions (15), and a blind pleural needle biopsy adds very little to negative cytology (16); therefore, several investigators have tried to improve diagnosis by measuring tumor markers in pleural fluids. Frequently studied markers include CEA, CA 125, CA 15-3, CA19-9, CA 72-4, CYFRA 21-1, neuron-specific enolase, and SCC (17, 18). Of these, CYFRA 21-1 was found to be the serum marker of choice in the diagnosis and follow-up of patients with NSCLC, especially for SCC (19, 20). In pleural effusion diagnosis, the pleural fluid CYFRA 21-1 did not differ when the histological type of lung cancer was considered, and CYFRA 21-1 was shown to have an advantage over CEA because of its higher specificity (21, 22).

In the current study, we have compared the performance of HER-2/neu with CYFRA 21-1 in pleural effusion diagnosis. The protein expression of HER-2/neu in tumor samples was measured by IHC. FISH was used to detect gene amplification. This study shows the contribution of HER-2/neu to the diagnosis and pathogenesis of MPE-associated ADCLC. We also discuss the correlation of HER-2/neu expression between different tests.

### MATERIALS AND METHODS

#### Patients and Sample Processing.

Pleural effusion samples were collected from 145 patients with various diseases: ADCLC-induced MPE (50); TB pleural effusions (20); empyema (20); PPE (20); and benign pleural effusions induced by CHF (35). Each ADCLC-associated MPE was verified positive by cytological analysis or a pleural biopsy. Patients with other primary sites of malignancies or without any lung parenchymal tumor were excluded. The diagnosis of TB was based on a positive culture from the pleural fluid or a pleural biopsy. All empyema effusions had positive cultures for bacteria. PPE indicates nonempyemic effusion accompanying pneumonia. Effusions of CHF were collected from patients with documented heart failure but without neoplastic or other major disease. Effusions were collected in sterile tubes and centrifuged immediately at 4°C. Cell-free supernatants were collected, and aliquots were stored frozen at −70°C until use.

#### Immunoassay for CYFRA 21-1.

Levels of CYFRA 21-1 in pleural effusions were determined using ECLIA on the Roche Elecsys 2010 immunoassay analyzers (Roche Diagnostics Corp., Indianapolis, IN). The immunoassay of CYFRA 21-1 used a sandwich technology. The sample, a biotinylated monoclonal cytokeratin 19-specific antibody and a monoclonal cytokeratin 19-specific antibody labeled with a ruthenium complex (Tris 2,2-bipyridyl ruthenium), reacted to form a sandwich complex. After the addition of streptavidin-coated microparticles, the complex became bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was then aspirated into the measuring cell, where the microparticles were magnetically captured onto the surface of the electrode. Application of a voltage to the electrode induced chemiluminescent emission, which was measured by a photomultiplier.

#### Immunoassay for HER-2/neu.

Pleural effusion samples were assayed for HER-2/neu using an ELISA kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer’s instructions.

#### Immunohistochemical Staining for HER2/neu.

The expression of HER-2/neu protein in tumor tissue was examined immunohistochemically. Tumor sections 4-μm thick were obtained from 43 patients with stage I/II NSCLC, 32 patients with stage III NSCLC without pleural effusion, and 33 patients with MPE-associated ADCLC. The paraffin-embedded sections were dewaxed in xylene, rehydrated in a decreasing ethanol series, and washed in water. These samples were then incubated for 20 min with 0.3% hydrogen peroxide (H₂O₂) in methanol at room temperature to quench endogenous peroxidase activity. Antigen retrieval was performed in an antigen-retrieval fixative (diluted 1:10; BioGenex, San Ramon, CA) heated in a microwave oven at 700 W (100%) for two 5-min cycles. After being washed with PBS (pH 7.4), the samples were immersed in normal goat serum for 30 min to block nonspecific protein binding. Tissue sections were then incubated with c-neu (Ab-3) OP 15 monoclonal antibody (Oncogene Research Products; 1:100) at 4°C overnight. Bound antibody was detected with biotinylated goat antirabbit IgG secondary antibody and streptavidin-peroxidase complex (MultiLink Supersensitive 500 Detection System; BioGenex), using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer’s hematoxylin.

#### Table 1 Pleural fluid level of HER-2/neu and Cyfra 21-1 in various groups

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>HER2/neu</th>
<th>P</th>
<th>CYFRA 21-1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCLC</td>
<td>50</td>
<td>8.5 (1.3–22.1)</td>
<td>&lt;0.001</td>
<td>53.5 (2.2–488.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TB</td>
<td>20</td>
<td>3.5 (0.1–7.9)</td>
<td>&lt;0.001</td>
<td>3.0 (0.1–42.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Empyema</td>
<td>20</td>
<td>2.4 (1.2–4.3)</td>
<td>&lt;0.001</td>
<td>4.6 (0.1–37.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPE</td>
<td>20</td>
<td>2.7 (1.1–4.9)</td>
<td>&lt;0.001</td>
<td>2.1 (0.3–52.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CHF</td>
<td>35</td>
<td>2.2 (0.7–6.1)</td>
<td>&lt;0.001</td>
<td>13.9 (2.0–78.1)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* n = number of patients.

* Data are expressed as the median (minimum-maximum values).

* P between ADCLC and other disease groups by ANOVA test.
grade 3 (3+) = a strong cytomembrane staining. In this study, sections with a 2+ staining in >30% of tumor cells or a 3+ staining in >10% of tumor cells were considered as having overexpressed HER-2/neu (23). To study the correlation between IHC and ELISA assays, an H-score system was applied (24). Each tumor sample was scored by the cross-product (H-Score) of the percentage of tumor cells stained and the grades of staining intensity. For example, a particular tumor with 80% of the cells stained at 2+ would be scored as 80*2 = 160.

**FISH Assay.** FISH study for erbB2 gene copy number was performed using Pathvysion dual color probes (Vysis, Downers Grove, IL). Hybridization occurred with the orange erbB2 probe and green CEP17 (staining chromosome 17 centromere) probe. At least 60 cells were counted, and amplification of the erbB2 gene was defined as the orange: green signal >2.

** statistical Analysis.** Differences between two independent groups were determined by the Mann-Whitney U test. Differences between more than two groups were determined by one-way ANOVA-Bonferroni multiple comparisons. ROC curves were calculated by logistic regression, considering the malignant/nonmalignant condition as a dependent variable and the different tumor markers as independent variables. Sensitivity and specificity patterns were studied by CDA (25). The two-sided Fisher’s exact test was used to assess associations between categorical variables. Significance testing of correlations was performed with the Spearman rank correlation analysis. Statistical significance was set at P < 0.05.

**RESULTS**

**Expression of HER-2/neu and CYFRA 21-1 in Pleural Effusion.** The median and range of HER-2/neu and CYFRA 21-1 in five disease categories are summarized in Table 1. Individual and mean levels of HER-2/neu and CYFRA 21-1 in the five subgroups are shown in Figs. 1 and 2, respectively. By one-way ANOVA-Bonferroni multiple comparisons, the pleural levels of HER-2/neu and CYFRA 21-1 in the ADCLC subgroup
were significantly higher than those in the other four subgroups. We then combined the four benign disease subgroups into a nonmalignant group. Fig. 3 shows the individual and mean values of HER-2/neu in malignant and nonmalignant groups, and Fig. 4 shows the individual and mean values of CYFRA 21-1 in malignant and nonmalignant groups. For both markers, the distribution of the values appeared significantly higher in malignant than in nonmalignant groups (both \(P < 0.0001\)).

Comparison of CYFRA 21-1 and HER2/neu as a Diagnostic Marker. The CDA curves (Figs. 5 and 6) indicate that the maximum sensitivity and specificity expectable from pleural fluid was 84% for a HER-2/neu assay and 65% for a CYFRA 21-1 assay. To achieve 95% specificity, the cutoff points for HER-2/neu and CYFRA 21-1 were 5.5 and 60 ng/ml, respectively. The sensitivity of detecting malignant effusion induced by ADCLK was 72% for HER-2/neu and 40% for CYFRA 21-1. The ROC curves for HER-2/neu and CYFRA 21-1 are shown in Fig. 7. The area under the ROC curve was 0.84 for HER-2/neu, significantly larger than the 0.65 for CYFRA 21-1.

Expression of HER-2/neu by Immunohistochemistry. The expression of HER-2/neu in tumor tissues was studied in 108 patients with NSCLC, comprising 43 patients with stage I/II NSCLC, 32 patients with stage III NSCLC but without MPE, and 33 patients with MPE-associated ADCLK. Only four tumor tissues from the 108 patients showed a pure membranous staining pattern of HER-2/neu. The cell type, disease stage, and correlation with FISH assay of the four cases with membranous staining are summarized in Table 2. All of the other positive stains were found in the cytoplasms, although it was impossible to distinguish between membrane staining and strong cytoplasmic reactivity in some instances (26). The membranous staining pattern is not restricted to any particular disease stage or histological type. Positive HER-2/neu expression (Fig. 8) was observed in 13 patients (30%) in the stage I/II NSCLC group, 14 patients (44%) in the stage III NSCLC group, and 23 patients (70%) in the MPE-associated ADCLK group. The differences between the stage I/II NSCLC group versus the stage III NSCLC group versus the MPE-associated ADCLK group (\(P = 0.0046\)) were statistically significant. There was, however, no statistical difference (\(P = 0.336\)) between the stage I/II and the stage III NSCLC groups.

Correlation between FISH and IHC. There were only two specimens in which the HER-2/neu gene amplification was \(\geq 2\) by FISH (Fig. 8). As shown in Table 2, both had 3+ HER-2/neu membranous staining. Therefore, the correlation between HER-2/neu gene amplification and 3+ membranous staining was good, but there was no gene amplification in specimens with positive cytoplasmic staining.
Correlation of HER-2/neu Expression between Tumor Tissue and Pleural Effusion. Tumor tissues and pleural effusions had been collected simultaneously from 27 patients with MPE-associated ADCLC. The expression of HER-2/neu by the H-score system in tumor tissues was compared with the concentration of HER-2/neu in paired pleural effusion samples (Fig. 9). According to the H-scores, patients were separated into three groups (group A 0–50; group B 60–150; and group C 160–240). By one-way ANOVA-Bonferroni multiple comparisons, the differences of pleural levels of HER-2/neu in group B versus group A and in group C versus group A were statistically significant (P < 0.05). The percentage of pleural HER-2/neu expression above the cutoff point (5.5 ng/ml) was 25 and 84%, respectively, in patients with an IHC H-scores ≤50 and >50. The difference was also statistically significant (P = 0.003). Therefore, there is a correlation between tumor-tissue expression and pleural fluid values of HER-2/neu.

DISCUSSION
Membranous staining of HER-2/neu has been demonstrated in 2–40% of NSCLC cases (11, 12). Although only membranous expression of HER-2/neu was considered functionally important in breast cancers, several studies from lung cancers have shown that cytoplasmic HER-2/neu staining in 10–60% of NSCLC cases, especially in adenocarcinoma, were associated with a poor prognosis (10, 27–29). The enormous variations in frequencies and patterns of HER-2/neu expression in NSCLC might be because of different assay methods and scoring systems. Therefore, to ensure a good correlation between the IHC and ELISA tests, we used testing materials purchased from the same manufacturer. Because membranous and cytoplasmic expressions of HER-2/neu have all been related to the prognosis of patients with NSCLC, we interpreted either membranous or cytoplasmic staining as positive in IHC experiments. In our series, the majority of tumor specimens with positive HER-2/neu staining was cytoplasmic (46 of 50); only 4 were purely membranous. One of the membranous stainings was scored as 2+, and the other three scored as 3+, resulting in an overall 3% (3 of 108) of 3+ membranous staining. The 3+ membranous staining was also found to be uncommon (4%) in NSCLC by Hirsch et al. (30). HER-2/neu gene amplification >2, assayed by FISH in our study, was present in only 1.9% (2 of...
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The incidence ranges from 2 to 6% in the literature (30) of breast cancer (9), appears to be infrequent in lung carcinoma. Gene amplification, as has been described in breast cancer (31), but other studies have found opposite results (32). The mechanism resulting in HER-2/neu overexpression in lung cancer is not yet clear. However, the findings of (a) a high HER-2/neu mRNA expression in 34.9% of NSCLC patients by quantitative real-time PCR assay (33) and (b) changes in 92% of tumors by cDNA microarray assay (34) imply that transcriptional or posttranscriptional regulatory mechanisms are involved. Therefore, the rarity of purely membranous staining and gene amplification of HER-2/neu in the current study is compatible with observations from other investigators. Whether cytoplasmic HER-2/neu has functions or just represents a background awaits additional research. In our study, a tumor with a high IHC score tended to generate a pleural effusion with high expression of HER-2/neu protein, suggesting that the cytoplasmic staining represented protein overexpression rather than background staining. Overexpression of HER-2/neu has frequently been linked to a poor prognosis for patients with NSCLC (10, 12, 27–29), but other studies have found opposite results (35, 36). In our study, cytomembranous staining of HER-2/neu was found in 30% of stage I/II NSCLC cases, 44% of stage III cases, and 70% of stage IIB/IV MPE-associated ADCLC cases. The correlation of HER-2/neu overexpression with advanced stage NSCLC in this study supports a linkage between HER-2/neu expression and a poor prognosis. The high percentage (70%) of HER-2/neu staining on tumor tissue from patients with MPE-associated ADCLC may be because of cells with overexpressed HER-2/neu in a tumor are more likely to invade pleurae than others or because ADCLC with HER-2/neu overexpression tends to include pleural invasion and develop MPE. We were able to obtain tumor tissue from involved pleurae via thoracoscopy-guided biopsy, but we had difficulties in obtaining tissue from the primary lung tumors because either surgical resection was not indicated for these patients or a bronchoscopic biopsy was unable to reach a peripherally located primary tumor. We therefore do not yet have a definite answer to this question. One recent study (37) found that discordance in HER-2/neu status between the primary breast tumor and involved axillary lymph node metastases was small (5%), which suggests that HER-2/neu-overexpressed ADCLC does in fact tend to involve the pleurae.

HER-2/neu protein is produced by an oncogene. Its extracellular domain is solubilized and may shed into the culture medium of tumor cell lines and sera of advanced breast carcinoma patients (38). In this study, we investigated expression of HER-2/neu in the pleural fluids of patients with MPE-associated ADCLC, pulmonary TB, empyema, pneumonia, and CHF. The pleural levels of HER-2/neu were significantly higher in the malignant group than in the nonmalignant group. The 72% sensitivity of HER-2/neu in diagnosing MPE is very close to the 70% positive cytomembrane staining of HER-2/neu on pleural tumor cells. Furthermore, the correlation between grades of HER-2/neu expression on pleural tumor cells and concentrations of HER-2/neu protein in pleural fluids was significant in our study. In their study of serum specimens, Osaki et al. (39) found that circulating HER-2/neu levels in patients with tissue overexpression were higher than those in patients without overexpression. On the contrary, Ardizzoni et al. (40) did not find a good correlation between circulating HER-2/neu levels and tissue expression. In a very recent study of 568 cases of NSCLC (41), circulating shed receptor was not found to be correlated with either FISH or IHC, and gene amplification of HER-2/neu was found in only 8% of patients. The positive correlation between pleural HER-2/neu levels and tumor tissue expression in the present study may be because of a direct shedding of HER-2/neu from tumor cells into pleural effusion and, therefore, suggesting that measuring pleural HER2/neu may be an acceptable alternative to direct IHC staining on tumor cells.

Although CYFRA 21-1 is one of the most reliable tumor markers in pleural effusion diagnosis, when compared with HER-2/neu, we found that the latter had higher sensitivity to detect malignancies and had a larger area under the ROC curve. In addition to its good performance in plural effusion diagnosis, HER-2/neu is also an indicator for aggressive tumor biology, so it has the potential to be an ideal tumor marker in pleural effusion diagnosis and a good biomarker of MPE-inducing cancers. The findings in lung cancer are consistent with experience from a breast cancer study (42) that showed serum HER-2/neu to be more sensitive than conventional tumor markers in predicting tumor relapse. This study focused only on MPE-associated ADCLC and did not investigate the positive rate of HER-2/neu elevation in MPE caused by other malignancies. Because the rates of HER-2/neu overexpression vary widely among different malignancies, attempting to study expression rates with multiple cancers would make it difficult to interpret the data. Although the frequencies of HER-2/neu elevation in pleural fluids caused by other malignancies and the significance of cytoplasmic staining of HER-2/neu await additional studies, a malignant origin should be highly suspected in patients with pleural effusion in which the HER-2/neu level is >5.5 ng/ml, and an invasive diagnostic procedure like thoracoscopy-guided biopsy may have to be used.

In conclusion, HER-2/neu may play an important role in the pathogenesis of MPE-associated ADCLC, and HER-2/neu in pleural fluid can be used as an ideal tumor marker for differential diagnosis and prediction of tumor biology.
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