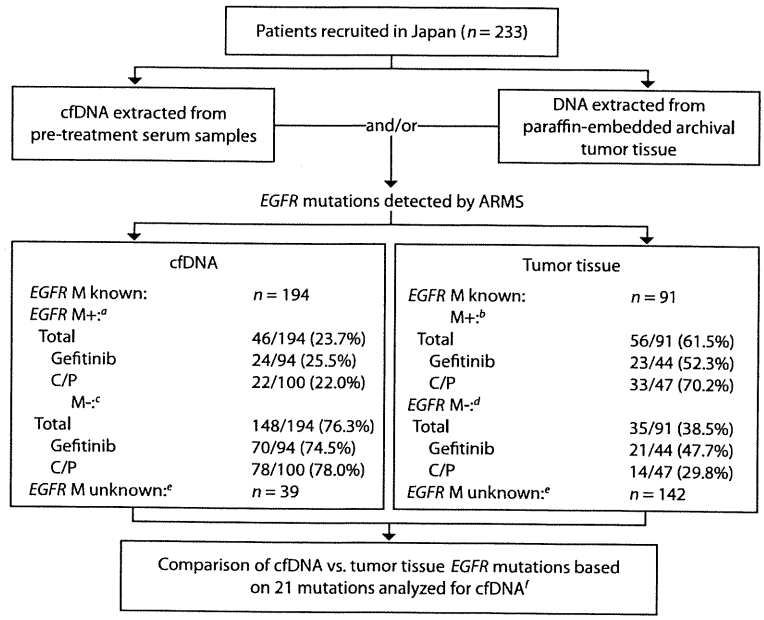

FIGURE 2. Flow and results of *EGFR* mutation analysis. [a]Sample positive for ≥1 of 21 mutations tested; detected 19 deletions in exon 19, L858R, and T790M. [b]Sample positive for ≥1 of 29 mutations tested; detected 19 deletions in exon 19, L858R, T790M, L861Q, G719S, G719A, G719C, S768I; 3 insertions in exon 20. [c]Sample negative for all 21 mutations tested. [d]Sample negative for all 29 mutations tested. [e]Unknown *EGFR* mutations: no sample available or failed analysis. [f]86 patients had known mutation status by both tumor tissue and cfDNA. C/P, carboplatin/paclitaxel; *EGFR*, epidermal growth factor receptor; M, mutation; M+, mutation-positive; M−, mutation-negative.

TABLE 1. Patient Demographics, Baseline Characteristics, and Efficacy (PFS and ORR) for Patients with Samples (cfDNA or Tumor) Evaluable for *EGFR* Mutation Status Compared with the Overall Japanese[a] Study Population (Japanese ITT Population)

	Evaluable for *EGFR* Mutation Status (cfDNA) (n = 194)[b]	Evaluable for *EGFR* Mutation Status (Tumor) (n = 91)[b]	Overall Japanese Study Population (n = 233)
Demography, n (%)			
Female	172 (88.7)	84 (92.3)	204 (87.6)
WHO PS 0/1	185 (95.4)	89 (97.8)	223 (95.7)
Never-smoker	177 (91.2)	83 (91.2)	212 (91.0)
Stage IIIB	66 (34.0)	27 (29.7)	73 (31.3)
Age <65 yr	97 (50.0)	45 (49.5)	121 (51.9)
Efficacy			
PFS HR[c] (95% CI)	0.68 (0.49–0.95)	1.08 (0.68–1.72)	0.69 (0.51–0.94)
ORR OR[d] (95% CI)	1.45 (0.80–2.61)	0.99 (0.41–2.40)[e]	1.34 (0.78–2.30)

[a] Refers to the country of recruitment and not necessarily to racial origin.
[b] Includes both mutation-positive and mutation-negative samples.
[c] HR <1 indicates a difference in favor of gefitinib.
[d] OR >1 indicates a greater chance of response on gefitinib.
[e] These results should be interpreted with caution as the logistic regression model did not converge.
cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; HR, hazard ratio; ITT, intent-to-treat; OR, odds ratio; ORR, objective response rate; PFS, progression-free survival; PS, performance status; WHO, World Health Organization.

believe that this result was due to the high rate of false negative results as described later (i.e., this group included both tumor *EGFR* M+ and M− patients).

In the cfDNA M+ subgroup, ORR was not significantly different in the gefitinib group compared with carboplatin/paclitaxel treatment (75.0% [18/24] and 63.6% [14/22], respectively; odds ratio [OR], 1.71; 95% CI, 0.48–6.09; p = 0.40). In the cfDNA M− subgroup, there were no significant differences in ORR with gefitinib compared with carboplatin/paclitaxel (27.1% [19/70] and 21.8% [17/78], respectively; OR, 1.34; 95% CI, 0.63–2.84; p = 0.45) (Figure 4). Again, this subgroup included both tumor *EGFR* M+ and M− patients as described later.

The results for clinical outcome by *EGFR* mutation status (M+, M−) for the Japanese subset of patients with known tumor *EGFR* mutation status (n = 91) are included in Supplemental Digital Content 2 (Results http://links.lww.com/JTO/A153).

Comparison of *EGFR* Mutation Status in Pretreatment cfDNA and Tumor Tissue

A total of 108 patients had a known mutation result by cfDNA but not by tumor; 5 patients had a known mutation

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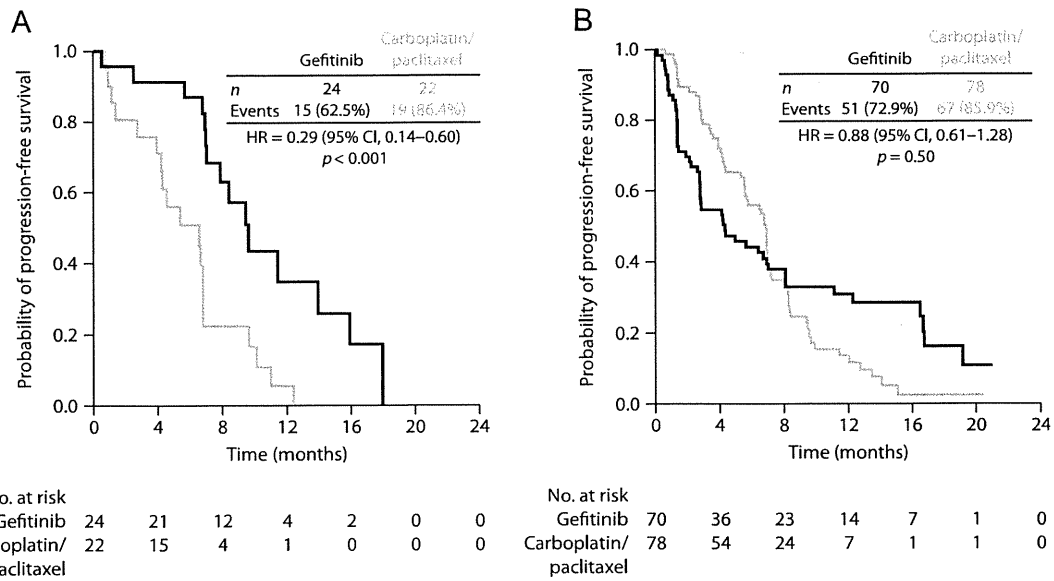

FIGURE 3. Kaplan-Meier curves of progression-free survival in cfDNA *EGFR* mutation-positive (*A*) and cfDNA *EGFR* mutation-negative (*B*) patients in the Japanese subset of IPASS. HR <1 indicates a difference in favor of gefitinib. CI, confidence interval; cfDNA, circulating free DNA; *EGFR*, epidermal growth factor receptor; HR, hazard ratio.

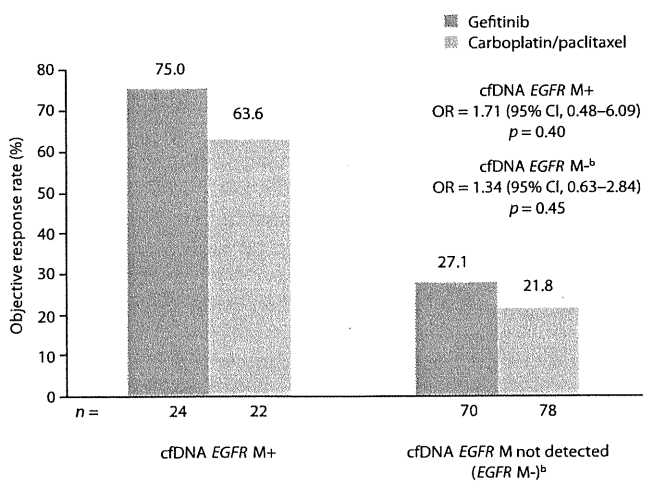

FIGURE 4. Objective response rates by treatment and by cfDNA (serum) *EGFR* mutation status (Japanese ITT population[a]). [a]Refers to the country of recruitment and not necessarily to racial origin. [b]There was a high rate of false-negative results, i.e., this group included both tumor *EGFR* M+ and M− patients. OR >1 implies a greater chance of response on gefitinib. OR, CI, and *p* values from logistic regression. cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat; M+, mutation-positive; M−, mutation-negative; OR, odds ratio.

result by tumor but not cfDNA (no serum sample provided); and 86 patients had a known mutation status by both tumor and cfDNA.

Of the 86 patients who had a known tumor and cfDNA mutation status, no false positives were identified (i.e., no samples were tumor M− but cfDNA M+). All 22 patients identified as cfDNA *EGFR* M+ were tumor *EGFR* M+, i.e., the positive predictive value was 100% (all samples that were cfDNA M+ were tumor M+) and the specificity was 100% (all samples that were tumor M− were cfDNA M−) (Table 2). However, the rate of false negatives was high: 29/51 (56.9%) of patients identified as tumor *EGFR* M+ were cfDNA *EGFR* M− (Table 2).

TABLE 2. Comparison of *EGFR* Mutation Status in cfDNA and Tumor Samples in 86 Patients with a Known *EGFR* Mutation Status Using Both Methods (Japanese[a] ITT Population)

	Mutation Status (Tumor Tissue), *n*		
	M+	M−	Total
Mutation status (cfDNA), *n*			
M+	22	0	22
M−	29	35	64
Total	51	35	86

Sensitivity = 43.1% (22 cfDNA M+ out of 51 tumor M+).[b]
Specificity = 100% (all 35 tumor M- were cfDNA M−).[b]
Positive predictive value = 100% (all 22 cfDNA M+ were tumor M+).[b]
Negative predictive value = 54.7% (35 tumor M- out of 64 cfDNA M−).[b]
Concordance = 66.3% (cfDNA and tumor results agreed in 57 of 86 cases).[b,c]

[a] Refers to the country of recruitment and not necessarily to racial origin.
[b] Those with a known *EGFR* mutation status using both methods.
[c] Kappa coefficient 0.38 (95% CI, 0.24–0.53).
cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat; M+, mutation positive; M−, mutation negative.

EGFR Mutation Types in Pretreatment cfDNA and Tumor Tissue

Of the patients classified as *EGFR* M+ at pretreatment by both tumor and cfDNA, all had the same mutation type in

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TABLE 3. *EGFR* Mutations in Pretreatment cfDNA vs. Tumor Samples (Japanese[a] ITT Population)

	Tumor *EGFR* Mutation[b]						
cfDNA *EGFR* Mutation	**Exon 19 Deletions Only**	**Exon 20 T790M Only**	**Exon 21 L858R Only**	**Exon 20 T790M and Exon 21 L858R**	**Negative**	**Unknown**	**Total**
Exon 19 deletions only	11	0	0	0	0	15	26
Exon 20 T790M only	0	0	0	1	0	1	2
Exon 21 L858R only	0	0	10	0	0	8	18
Exon 20 T790M and exon 21 L858R	0	0	0	0	0	0	0
Negative	18	0	11	0	35	84	148
Unknown	2	1	0	0	2	34	39
Total	31	1	21	1	37	142	233

The categories are mutually exclusive. The categories "Exon 19 deletions and exon 20 T790M" and "Exon 19 deletions and exon 21 L858R" were 0 for both tumor and cfDNA and have been omitted from the table.

[a] Refers to the country of recruitment and not necessarily to racial origin.

[b] Mutations that were tested in tumor tissue samples but not serum included: exon 20 insertion, exon 21 L861Q, exon 18 G719X, and exon 20 S768I. Two patients with tumor samples had these mutations (1 with exon 20 insertion and 1 with exon 21 L861Q). These patients were excluded from the comparative analysis of mutation detection by sample type.

cfDNA, circulating free DNA; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat.

tumor and cfDNA except one patient who had exon 20 T790M and exon 21 L858R by tumor but exon 20 T790M only by cfDNA (Table 3).

DISCUSSION

The feasibility of using cfDNA to detect *EGFR* mutations was assessed in the Japanese subset of patients from the IPASS study. The proportion of patients identified as *EGFR* M+ was lower when assessed in cfDNA (23.7%) compared with tumor tissue (61.5%). Although cfDNA results identified no false positives, a high rate of false negatives (56.9%) was observed, with more than half of the tumor M+ patients not detected by cfDNA testing (of patients with evaluable mutation status from both cfDNA and tumor). Further research into appropriate methods and analysis needs to be performed before it could be accepted as an option in the diagnostic or screening setting. If larger patient series confirmed the absence of false-positive results and demonstrated an improvement or lowering of false-negative results, serum testing may prove useful for patients for whom tumor samples are not available.

Testing of biopsied tumor tissue remains the current recommended method for *EGFR* mutation analysis.[8] However, tumor tissue is often difficult to obtain, particularly from patients with advanced non-small cell lung cancer (NSCLC), and a lack of tumor cells in a given sample and subsequently failure on pathological examination can make *EGFR* mutation analysis very difficult. The increased recognition of the relevance of mutation testing to treatment selection may stimulate efforts to better obtain tissue for *EGFR* mutation testing in the future. In the meantime, detection of *EGFR* mutation status in cfDNA derived from serum/plasma may allow patients without diagnostic tumor material the opportunity to benefit from personalized treatment and also has a use in the clinical trial setting where tumor material is not always available.

Although minimally invasive, the use of serum as a nontumor surrogate sample may be limited by the amount of cfDNA available in the sample, meaning that some positive samples are not detected. In addition, some patients may not have cf tumor DNA as their tumors may not be releasing this material into the bloodstream, giving rise to false-negative results. Because of the limited yields of cfDNA obtained from serum, two changes (in addition to duplicate tests) were made to the *EGFR* mutation ARMS kit used to detect *EGFR* mutations in this study: an increase in the number of PCR cycles and an alteration of the cutoffs used to define M+ samples (dCt values). Further analysis is underway to investigate whether these conditions are the most appropriate and whether less stringent settings could result in more true positives (fewer false negatives) while retaining no false positives.

There have been several reports on the detection of cfDNA *EGFR* mutation status using different methods. A significant correlation between cfDNA *EGFR* mutation status and clinical response to gefitinib was found in two previous small studies that assessed cfDNA *EGFR* mutation status using the ARMS method of detection, a highly sensitive (1% sensitive) targeted technique to detect specific known *EGFR* mutations.[9,11] Other screening techniques detect all *EGFR* mutations, known and novel variants, by PCR amplification followed by sequencing, pyrosequencing, or melt analysis (10–30% sensitivity).[8] However, although these methods are widely used for *EGFR* mutation analysis of DNA derived from tumor tissue, not all of these methods have demonstrated utility for *EGFR* mutation analysis of cfDNA. In a small study that used DNA sequencing to detect *EGFR* mutations in serum, mutations were more frequently observed in patients experiencing partial response or stable disease compared with those whose disease progressed, although the difference did not reach statistical significance.[10] No statistically significant association between cfDNA *EGFR* mutation status and PFS by multivariate analysis (HR, 1.48; 95% CI, 0.93–2.36; $p = 0.09$) was found in the study by Rosell et al.[12] which assessed *EGFR* mutations by PCR-based methods in the presence of a protein nucleic acid (PNA) clamp in the cfDNA extracted from serum of 164 patients

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treated with erlotinib. In another study that used denaturing high-performance liquid chromatography to analyze for mutations in exons 19 and 21 from matched plasma and tumor samples, patients with plasma *EGFR* mutations had significantly higher ORR and prolonged PFS.[7] The present study using ARMS demonstrated that the treatment effect for the Japanese cfDNA *EGFR* M+ subgroup followed the same pattern as the tumor *EGFR* M+ subgroup of the overall IPASS population (i.e., PFS HR significantly in favor of gefitinib and higher ORR with gefitinib versus carboplatin/paclitaxel).[6] There was a significant interaction between cfDNA *EGFR* mutation status and treatment for PFS.

Any variance in concordance rates for mutation results between pretreatment serum versus tumor tissue (66.3% in our study and between 58 and 93% in previously reported studies)[7,9–11] may be attributed to different methods of extraction, detection, run conditions, the size and yield of the DNA fragments, and the fact that cfDNA may not be present in the circulation of all patients with NSCLC. For example, targeted sequences amplified by ARMS are short, at 100–150 bp, leading to decreased assay failure rates (particularly from formalin-fixed paraffin-embedded material or fragments of cfDNA) compared with sequencing methods, which tend to involve the amplification of longer target sequences of 150–250 bp or above.[8,13,14,17,18]

In patients who were cfDNA *EGFR* M− in this study, no significant difference for PFS was seen with gefitinib compared with carboplatin/paclitaxel; however, the HR was not constant over time (as was observed for the overall Japanese study population). These results should be interpreted with caution as there was a high rate of false negatives, and this subgroup is likely to include tumor *EGFR* M+ and M− patients.

In conclusion, these results merit further investigation to determine whether alternative samples, including serum or plasma, may be considered for determining *EGFR* mutation status in future, particularly in cases where diagnostic tumor material is not available. Currently, analysis of tumor material is the recommended method for determining *EGFR* mutation status.

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Laboratory-Clinic Interface

Critical comments for roles of biomarkers in the diagnosis and treatment of cancer

Nagahiro Saijo *

Medical Oncology Division, Kinki University School of Medicine, Japan

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ABSTRACT

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic/pharmacodynamic responses to a therapeutic intervention". Various assays, including immunohistochemistry, gene constitution such as amplification, mutation, and rearrangement, gene and protein expression analysis such as single gene or protein expression, exhaustive analysis and gene or protein signature and single nucleotide polymorphism have been used to identify biomarkers in recent years. No therapeutic effects have yet been predicted based on the results of such exhaustive gene analysis because of low reproducibility although some correlate with the prognosis of patients. Biomarkers such as HER2 for breast cancer or EGFR mutation for lung cancer and KRAS mutation in colon cancer have contributed to identify a patient population that might show a good and bad treatment response, respectively. On the other hand, other biomarkers such as bcr-abl, c-kit gene mutation and CD20 expression, which are positive for CML, GIST and B cell lymphoma, respectively, have crucial biological significance but have not necessarily been used for practical clinical screening since pathological diagnosis coincide with finding of biomarkers. Hence, much work remains to be done in many areas of biomarker research.

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Introduction

Most cancer patients treated with chemotherapy will suffer severe toxicity, because response rates to a single therapy with anticancer drug are much lower than that to therapy for other diseases and also effective dose levels of anticancer drugs are often close to or overlap the toxic dose level. Thus, it is important to identify a patient population that is likely to be responsive to treatment with anticancer drugs. To address this challenge, various biomarkers have recently been studied. In addition, molecular-targeted agents have been extensively developed by many pharmaceutical companies and some of these agents are currently available in clinical practice. One group of molecular-targeted agents exert their antitumor activity by modification of a tumor cell-specific target. Development of biomarkers is necessary for predicting the effects of these agents on the relevant targets. The goal of the development of biomarkers will be to design ways to predict efficacy of molecular-targeted agents including response rate, progression-free survival (PFS) and overall survival (OS). If biomarkers allow us to select a patient population that might show a good treatment response, they are believed to be beneficial to both patients and physicians. Furthermore, biomarkers are expected to provide valuable information for developing new drugs, thereby reducing development costs and duration as well as the number of patients enrolled in clinical studies while no reliable biomarkers have yet been identified for tumor-environment-specific molecular targeted agents such as antiangiogenic drugs. Recently, biomarker research has become complicated because of the emergence of molecular targeted agents with multiple targets. In this review, I will attempt to discuss current trends and the clinical significance of biomarker research.

Definition of biomarkers

A biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic/pharmacodynamic responses to a therapeutic intervention".[1,2] In routine clinical oncology practice, patients' characteristics and findings such as performance status (PS), disease stage, histological type, X-ray, MRI, CT, scintigram and other laboratory examinations have been assessed as biomarkers. With recent progress in molecular biological research, various advanced technologies, including pharmacogenomics, such as transcriptomics, proteomics, metabolomics and molecular imaging, have been introduced in clinical settings to analyze factors regulating both the effects and the adverse events of treatments. Furthermore, the following attempts have been made: understanding the effects of the cancer and the drug actions on DNA, RNA, proteins and their metabolites, and assessment of the significance of surrogate endpoint biomarkers as alternatives for true clinical endpoints. The main purpose of using anticancer drugs

* Tel.: +81 4 7134 6902; fax: +81 4 7131 3212.
E-mail address: nsaijo@med.kindai.ac.jp

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is to increase total survival time and the complete remission rate. Under these circumstances, it is important for clinicians to identify a biomarker that correlates directly with these parameters. In other words, clinicians need to determine whether the effect on a biomarker correlates directly with the effect on a true endpoint.

Necessity of biomarkers and rationale for biomarker research

In general, response rates to anticancer drugs are much lower than those to drugs for other diseases. Even with limits on the approved products, the rate of anticancer drug responsiveness is as low as approximately 20% according to RECIST criteria. These criteria are not strict for evaluating the antitumor activity, because, in particular, a partial response is defined as at least a 30% decrease in tumor size sustained for at least 4 weeks with one direction measurement.[3]

Success rates in anticancer drug development remain low or have gradually declined in recent years. Moreover, the price of newly approved anticancer drugs has been exorbitant due to increases in their development costs. If a patient population that is expected to show a good response to an anticancer drug can be selected by a reliable biomarker, these problems will be resolved. For instance, it is known that in trastuzumab-based therapy, HER2-positive patients had a good response rate of 50%; while those without screening for HER-2 had a poor response rate of only 10%.[4]

Positioning of biomarkers (diagnostic, prognostic and effect predictive markers)

Biomarkers basically include tumor markers (including diagnostic function tests) and are a characteristic that is evaluated using pharmacogenomic methods reflecting PK/PD reaction when administering anticancer drugs. Biomarkers can be divided into the following three types: (1) diagnostic markers (such as tumor markers), (2) prognostic markers and (3) predictive markers. Special care should be taken to determine whether a certain biomarker is a prognostic or predictive marker. When a biomarker correlates with anti-tumor effects including response rate, progression free survival, time to progression and overall survival, it will be regarded as a surrogate endpoint of clinical effect. Such a correlation between an identified biomarker and clinical effect supports molecular targeted agents exerting an anti-tumor effect via molecular target modulation. This approach is thought to be translational studies in United States. Application to biomarker research in anticancer drug development based on pharmacogenetics can generally be divided into three steps: target identification in the drug discovery process, elucidation of the action mechanism and identification of the biomarker. In the process of this research, pre-clinical studies focus on elucidating the in vitro/vivo mechanisms of action and pharmacogenomic reasons for the toxicities of drugs. While clinical studies aim to elucidate the in vivo mode of action and to develop and validate biomarkers. Diligent and intensive research activities are required and validation of biomarker analysis methods is the most important aspect on these processes.

Conditions of biomarkers

A new biomarker will become beneficial to patients, when newer and more important information becomes available as compared with the old prognostic and/or predictive markers used in current clinical practice. A new technology, regardless of its cost-saving and user-friendly properties, will be meaningless unless there is true clinical significance.

Practical questions that clinicians commonly face are as follows: (1) can data from molecular biomarkers, genomics or proteomics provide more correct information than those from prognostic and/or predictive markers routinely used in clinical practice? (2) Can the gene panel or molecular signature be an independent prognostic and/or predictive marker rather than a surrogate marker for a factor previously used? and (3) why do results from gene prediction differ among researchers?

It is well known that substantial efforts in biomarker validation are required to use a new biomarker in clinical practice. In such biomarker validation research, a prospective study design using high quality tumor samples from patients, who are enrolled in a controlled clinical study, should be adopted.[5,6] A sufficient sample size is also necessary to assess the specificity, sensitivity and predictive values (positive and negative) before developing a hypothesis and the endpoints introduced for biomarker validation.[7] However, high quality tumor samples are not consistently collected from all patients enrolled, suggesting that data from tumor samples of some cases may not reflect data that would be obtained from the entire patient population. Patients treated with adjuvant chemotherapy after surgery would be the best subjects for biomarker validation, because great amount of tumor samples can be collected from all such patients.

Henceforth, the clinical significance of new biomarkers should be evaluated by comparing the predictability between molecular biomarkers and clinical prognostic factors, therapeutic gain factors and predictive factors, such as age, sex, clinical stage and type of tissue, which are routinely used in clinical settings, in such a patient population.

Classification of biomarkers

In light of the clinical significance, biomarkers could be classified into the following three groups[8] (Table 1). First, biomarkers in Group 1 are known as valid markers which are well known to correlate with clinical response. This biomarker group includes expressions of human epidermal growth factor receptor 2 (HER2) expression for trastuzumab (Herceptin)[9,10] epidermal growth factor receptor (EGFR) expression[11] and K-RAS mutation[12,13] for cetuximab (Erbitux), EGFR mutation for epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI).[14,15] These biomarkers are thought to be essential for deciding indication of anticancer drugs and now used to optimize patient selection when administering Herceptin, Erbitux EGFR-TK I in clinical settings. When using EGFR-TKI, previous data suggest that patients with EGFR mutations have high response rates of 70–80% with extremely high predictability. While approximately 10% of patients without this mutation also show a good response.[16–18] Recent IPASS trial, however showed that response rate of EGFR-wild type patient was only 1% if EGFR mutation was analysed by highly sensitive Scolpion Arms method.[19,20] In addition patient with EGFR mutation showed an amazingly longer progression free survival when treated by

Table 1
Classification of biomarkers.

Known Valid Biomarkers: Test required
– Accepted by scientific community at large to predict clinical outcome
Her2(Herceptin), EGFR mt(EGFR-TKI), EGFR, KRASmt(Erbitux),
BRCA1&2 mt,def. PARP-inhibitor,
EML4-ALK(ALK-inhibitor),Mamma Print, Oncotype Dx (Chemotherapy)
Probable Valid Biomarkers: Test recommended
– Appears to have predictive value but not yet replicated or widely accepted
UGT1A1*28,*6, (irinotecan), Cytidine deaminase*3(gemcitabine)
Exploratory Biomarkers: (Valid, non Valid) Information only
– Supported by initial identification data
Genomic & Proteomic predictors
(Single gene:ERCC1,RRM1,MSH2, TS,
Exhaustive Analysis : Gene/Protein Signatures)

EGFR-TK I compared with standard chemotherapy.[21] Thus, there are following advantages to tests for these biomarkers in clinical practice: (1) high predictability of cases with high response rates and (2) optimizing patient selection when administrating EGFR-TKI in a high risk group. Second, biomarkers in Group 2 are probable valid biomarkers, which can probably predict good clinical response or adverse events. This group includes UGT1A1*28 or *6 for irinotecan.[22,23] These marker tests should be conducted before using these drugs to avoid severe adverse events. The biomarker test for UGT1A1*28, however, may not be reliable in clinical use, because it is based on data that only three of six patients homozygous for this genotype had neutropenia of grade 4.[24,25] Another problem is that it's frequency is very low. Finally, biomarkers in Group 3 are still in the process of evaluation, and some have not been validated as yet. This group includes ERCC1 and MSH-2 for platinum-based drugs[26,27], RRM1 for gemcitabine[28], and thymidylate synthetase (TS) for pemetrexed and fluorinated pyrimidines.[29] Also, majorities of expression profiles of genes and proteins signature have not been validated.[31] Other biomarkers such as CD20 in B-cell lymphoma, bcr-abl in chronic myeloid leukemia (CML)or c-kit in gastro intestinal stromal tumors (GIST) are not always essential for optimizing patient selection when administering rituximab or imatinib because expression of markers and the presence of its gene mutation in tumor cell coincide with results of cytopathological diagnosis. These biomarkers however, will play an important role in identifying the second mutation with resistance and finding a compound for a new molecular target with a mutation in addition to a crucial role to understand biology of each disease.[33]

Prognostic versus predictive factors

Prognostic factors are defined as patient- and tumor-side factors that provide information about the natural histories of diseases, such as survival time after surgery, with no relationship to treatment. Predictive factors represent patient- and tumor-side factors that allow clinicians to assess clinical effects of chemotherapy and molecular targeted agents on response rate and survival time. For example, if survival times of patients without treatment, whose performance status (PS) is 0/1 or 2/3, are 6 and 3 months, respectively, PS will be a prognostic factor. If the survival times of patients, with PS of 0/1 or 2/3, increase from 6 to 8 months and from 3 to 4 months with treatment, respectively, both hazard ratios (HRs) will be 0.75. In this case PS would not be a predictive factor for clinical effect. Recent studies suggested that gene signature and proteomics may become prognostic factors for lung cancer, breast cancer and colon cancer[34,35], although there is no evidence showing either to be a predictive factor for clinical effects.

ERCC1 is known to be a nucleotide excision repair (NER) enzyme associated with the repair of DNA damage caused by platinum-based drugs.[36] In lung cancer field, considerable research on ERCC1 has been conducted in recent years.[37,38] Simon reported that ERCC1 expression is a prognostic factor of survival in patients with resected non-small cell lung cancer (NSCLC) and that those with resected NSCLC with high ERCC1 expression show better survival than patients with low ERCC1 expression.[37] Lord et al. demonstrated response rate and prognosis to be better in IV-stage NSCLC patients with low ERCC1 expression than in those with high expression after cisplatin plus gemcitabine chemotherapy.[38] They also concluded that ERCC1 is a predictive factor for tumor response to platinum-based chemotherapy. This finding was supported by results of the IALT study, which demonstrated a correlation between ERCC1 protein expression and adjuvant chemotherapy response. This analysis indicated significant effect of adjuvant chemotherapy in patients with ERCC1-negative tumors; however, the survival time of patients with ERCC1-positive tumors treated by chemotherapy tended to be shorter.[26] In a comparison between patients without adjuvant chemotherapy, survival was longer in the ERCC1-positive than in ERCC1-negative group.[26] On the basis of these results, that ERCC1 is a tumor cell biomarker has been established in western countries. Caution, however, is necessary when interpreting the results of these subgroup analyses. In such subgroup data, biomarkers can be interpreted as both prognostic and predictive factors. If a specified subgroup shows response to chemotherapy, subgroup interaction is considered to be present in the statistical sense of the term. For example, if the patient subgroup with ERCC1-negative tumors shows response to adjuvant chemotherapy while a subgroup with ERCC1-positive tumors does not show tumor response[39], ERCC1 is interpreted as a predictive factor. This is statistically called "treatment with baseline covariate interaction".

These interactions include quantitative and qualitative interactions. The former interactions originally had the same trend but different intensities. Thus, each treatment group shows a tumor response without notable problems. For interactions between response with some drug and marker A, the subgroup with marker A has a higher response rate while another subgroup without marker A also shows a response to the agent although the response rate is low. On the other hand, the latter interactions may differ in mechanism between subgroups. Careful attention is required when such a mechanism is observed. Examples include interaction concerning pemetrexed for subgroups with squamous cell carcinoma and non-squamous cell carcinoma[40], interaction concerning platinum-based chemotherapy for ERCC1-positive and negative subgroups[38], and interaction in cetuximab therapy for KRAS mutation positive and negative subgroups in colon cancer.[41,42] It must be determined whether or not these interactions are statistically significant by testing differences in two hazard ratios. However, this test power will not be sufficiently robust for the following reasons: (1) there are two sources of variation and (2) sample sizes are small in subgroups. Thus, tumor responses with treatment in each subgroup evaluated are likely to be obtained by chance. Data including ERCC1, KRAS-mutation and histological type should be evaluated keeping this mind. A recent prospective randomized trial (COIN trial) in patients with KRAS type could not demonstrate the survival benefit of cetuximab if combined with FOLFOX regimen. (Press release)

RRM1 is associated with nucleoside metabolism and is a molecular target of gemcitabine. It has been reported that this RRM1 is a positive prognostic factor for early-stage lung cancer. In advanced-stage lung cancer patients with high RRM1 expression, the tumor response to gemcitabine + cisplatin combination is low, thereby showing that RRM1 is a predictive factor for advanced lung cancer.[37,38] A relationship between these factors including the recent results of MSH2 is similar to that with ERCC1.

A report demonstrated that the KRAS mutation is a predictive factor for increased survival with adjuvant chemotherapy. In spite of the relationship between the KRAS mutation and responses to cetuximab in colon cancer[41,42], KRAS mutation was identified not as a predictive factor of cetuximab treatment in lung cancer.[43] Recent topic is the effect of PARP inhibitor in BRCA1&2 mutant/deficient population. It has been demonstrated that PARP inhibitors inhibit repair of damaged DNA. PARP inhibitors has showed activity in those populations of breast and ovarian cancer by itself. PARP inhibitor has also been identified to be active against triple negative breast cancer by the combination-with carboplatin and gemcitabine, both of which are DNA damaging agents.[44–46]

Molecular targeted agent development and biomarkers

To establish molecular targeted therapies, the following are required: (1) validated molecular target; (2) assay that can deter-

mine expression and activity of target and signaling pathway; (3) expression in targeted tumors; (4) potent and specific inhibitor with high pharmacological activity; and (5) proved suppression targeting human tumors. It is more essential that a target with over-expression or a mutation should be present in tumor cells, or that a target is associated with cell proliferation, cell death or metastatic capacity and target suppression inhibits cell proliferation or promotion. Biomarkers play a role in monitoring such targets; however, various challenges remain, including tumor proliferation, the clinical significance of target related to survival, the reliability of assay methods for the target (sensitivity, specificity and precision), tissue specificity in targeted expression, heterogeneity, availability of tissue (sample) and clinical significance. In recent years (2001–2006), 67% of FDA approved anticancer drugs have been molecular targeted agents while only 33% were cytotoxics. Among anticancer drugs approved between 2000 and 2005, 37% have a filed application of pharmacogenetic data showing a rapid increase in the proportion of molecular targeted agents.

To use molecular targeted agents more effectively, clinicians should consider not only pharmacokinetics (PK) and pharmacodynamics (PD) but also pharmacogenomics (PGx) which lies in the background of PK/PD. In PK analysis, whether or not a clearance saturation or protein binding present when C_{max} or trough value reaches to effective blood concentration should be considered. In PD analysis, general observations include side effects or tumor reduction. In addition, elimination of target molecular and expression changes in downstream molecules can be assessed by PGx methods.

PGx markers are also used to determine whether or not a target molecule is expressed and to estimate the intensity of tumor response to anticancer drugs. Thus, it is essential to evaluate proof-of-principles (POP), for setting an optimal dosage and its schedule. For anticancer drugs, in particular, PGx analysis must be performed in cancer cells themselves, even if excellent PK analysis results are obtained. This is because such responses to drugs differ between tumors due to their features.

Application of biomarkers to non-clinical studies can provide the following advantages: (1) identifying targets for drugs; (2) clarifying the action mechanisms of drugs; and (3) predicting possible toxicity in humans. At this time, there are very few reliable methods (biomarkers) to identify responders, and biomarker development is needed in the future. The advantages in clinical studies include: (1) identifying responders and non-responders; (2) designing a clinical study for only responders; and (3) excluding patients with possible serious adverse events. Although application to routine clinical practice has not yet been achieved, identification of a biomarker that can predict serious a pulmonary fibrosis caused by EGFR-TKI, such as gefitinib or erlotinib, may lead to the prevention of side effects of the drug. In addition, the advantages of developing promising biomarkers are: (1) facilitating patient entry into clinical studies and decreasing the development period; (2) decreased sample size and development cost; (3) simplifying GO/NO GO decision; and (4) decreasing new drug approval period. The disadvantage of developing biomarkers include: (1) complicated clinical study plan; (2) segmentalizing the market; and (3) costly study for application of biomarkers.

Design for clinical studies with individualized therapeutics using biomarkers

Order-made or tailor-made therapy, i.e. individualized therapeutics based on pharmacogenomic information, became popular several years ago. However, only a few individualized therapies have been applied in clinical practice and there are no comprehensive data from genes and proteins useful for optimizing patient selection. In this section, several clinical study designs are discussed for evaluating tailor-made therapies that can optimize patient selection by using biomarkers.[47,48]

All comer's design

The all comer's design has been adopted by most of clinical studies using biomarkers. This design should be selected, when a biomarker has not been established or validated. In the study design, the relationship between presence/absence or higher/lower levels of biomarkers and the response rates or survival times are analyzed retrospectively, although positive or negative marker status could not be used to randomize patients even if biomarker levels are determined in all the patients enrolled. It is difficult to conclude that optimizing patient selection on the basis of study results will lead to increased responses to treatment.

Marker + design

The marker + design has been applied to clinical studies of Herceptin for breast cancer[10], standard chemotherapy versus imatinib for CML[31] and rituximab for B cell lymphoma.[30] In a study for B cell lymphoma, this design is not intended to optimize patient selection, because the pathologic disease entity is almost always CD20-positive. Biomarkers used in this study design must be established and validated. In the IPASS trial using gefitinib, clinical characteristics are used as biomarkers to optimize patient selection.[19] The WJOG and the North East Japan Gefitinib Study Group conducted a randomized controlled study with selecting patients with EGFR mutations.[21] When both biomarker positive and negative patients are randomized into treatment and controlled groups, interactions between the biomarker and response to treatment can be evaluated.

Marker strategy design

The marker strategy design is an important approach that can determine whether or not individualized therapeutics based on a biomarker has clinical significance. Generally, patients are allocated into two groups: the A group is treated with standard chemotherapy without biomarker measurement and the B group with biomarker measurement includes B1 (marker-positive) and B2 (marker-negative) subgroups which are treated with new agents and standard chemotherapy, respectively. In this study design, when survival time is significantly longer in the B (B1 + B2) group than in the A group, the significance of individualized therapeutics can be confirmed. Rosell et al. conducted a clinical study for the clinical significance of platinum-based chemotherapy based on the ERCC1 expression level, using this marker strategy design. In this study, the control group received CDDP + DTX. In the selection group, however, patients with low ERCC1 mRNA and patients with high ERCC1 mRNA received CDDP + DTX and DTX + GEM (excluding CDDP), respectively. The results showed patients who were selected based on their ERCC1 mRNA level to show a higher response rate, although survival times were similar in the two groups.[49] In conclusion, the selection of patients based on their ERCC1 expressions did not provide a good prognosis with chemotherapy for advanced-stage lung cancer, although response rate was significantly better than control group.

Conclusion

The clinical significance of biomarkers has been recognized among clinicians. Considerable research on biomarkers has been conducted in recent years; however established findings are as

yet limited. Limitations include validation of methods, the feasibility of obtaining tumor samples and low sensitivity. For examples, samples could not always be collected from all patients, samples for determination were not consistently of adequate volume, and the detection methods were not available at all facilities participating in the study. The most important issue is whether or not a biomarker is validated; if not, various data obtained in clinical studies will be minimally useful in clinical practice. In order to improve the results of cancer treatment, reliable biomarkers fully validated in relation to specific drugs must be established.

Conflict of interest

None declared.

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ORIGINAL PAPER

Usefulness of the serum cross-linked N-telopeptide of type I collagen as a marker of bone metastasis from lung cancer

Motohiro Tamiya · Hidekazu Suzuki · Masashi Kobayashi · Shinji Sasada · Norio Okamoto · Naoko Morishita · Tomomi Yasue · Yuka Matsuura · Tomonori Hirashima · Ichiro Kawase

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Abstract Bone metastasis is an important factor for determining the appropriate treatment for patients with lung cancer. The cross-linked N-terminal telopeptide of type I collagen (NTx) is a metabolite of type I collagen, the main constituent of the bone matrix. Urinary NTx is recognized as a useful marker of bone metastasis, but the application of serum NTx and its cutoff value for determining bone metastasis from lung cancer have not been characterized. We measured serum NTx by enzyme-linked immunosorbent assay of individuals who underwent staging during hospitalization for initial treatment of lung cancer in our department and compared the NTx levels with the presence of bone metastasis in staging. The study included 166 patients with lung cancer (128 men and 38 women), including 85 adenocarcinoma, 42 squamous cell carcinoma, 32 small-cell carcinoma, and 7 other cancer types. Bone metastasis was present in 73 cases. The average/median serum NTx of bone metastasis (+) and bone metastasis (−) was 27.8/23.8 and 17.1/16.5 nmol bone collagen equivalents/L, respectively. There was an intentional difference with $P < 0.001$. The cutoff value of the serum NTx level indicating bone metastasis from lung cancer was estimated using the receiver operating characteristics curve. The optimal cutoff value was found to be 22.0 (sensitivity: 61.6%, specificity: 89.2%). The results of univariate and multivariate analysis revealed that the serum NTx levels were significantly related to bone metastasis from lung cancer ($P < 0.001$). Measurement of serum NTx levels provides a simple diagnostic marker of bone metastasis from lung cancer.

Keywords Cross-linked N-telopeptide of type I collagen · Bone metastasis · NTx · Lung cancer

M. Tamiya (✉) · H. Suzuki · M. Kobayashi · S. Sasada · N. Okamoto · N. Morishita · Y. Matsuura · T. Hirashima · I. Kawase
Department of Thoracic Malignancy, Osaka Prefectural Hospital Organization, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino 3-7-1, Habikino, Osaka 583-8588, Japan
e-mail: moto19781205@yahoo.co.jp

T. Yasue
Department of Laboratory Medicine, Osaka Prefectural Hospital Organization, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino 3-7-1, Habikino, Osaka 583-8588, Japan

Introduction

Lung cancer is the leading cause of cancer-related deaths in Japan and several other countries. Bone metastasis is one of the most common complications of cancer metastasis, particularly in lung, breast, and prostate cancer, with rates of 30–60, 73, and 68%, respectively [1, 2]. Bone metastasis is an important factor that guides the selection of the most appropriate treatment for patients with lung cancer, and as a result, it affects the prognosis as well as the patients' quality of life.

Bone metastasis is primarily evaluated using bone scintigraphy, bone radiography, and magnetic resonance imaging (MRI). More recently, ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) has been used for further evaluation. Although bone scintigraphy has high sensitivity, its specificity is inadequate due to false-positive results caused by inflammation and traumatic fractures [3, 4]. Furthermore, MRI and FDG-PET are costly and time-consuming. Therefore, we sought to identify a diagnostic marker of bone metastasis for convenient clinical use.

A number of studies have evaluated the urine and blood levels of bone biochemical markers, including urinary deoxypyridinoline (D-PYD), serum pyridinoline cross-linked C-telopeptide of type I collagen (1CTP), and urinary pyridinoline cross-linked N-telopeptide of type I collagen (NTx). It has been demonstrated that these molecules are useful as markers for detecting bone metastasis by various cancers [5–7]. However, an appropriate serum NTx level and cutoff value for the determination of bone metastasis from lung cancer have not been defined. In this study, we determined the cutoff value of the serum NTx for bone metastasis from lung cancer and investigated the relationship between baseline serum NTx levels and bone metastasis from patients with lung cancer.

Patients and methods

The subjects included 166 patients who underwent staging for the initial treatment of lung cancer during hospitalization in our department between September 2008 and September 2010. For each patient, a blood specimen for the assessment of serum NTx levels was collected early in the morning on the day after admission. For comparison, serum alkaline phosphatase (ALP), lactate dehydrogenase (LDH), calcium (Ca), carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYF), neuron-specific enolase (NSE), blood urea nitrogen (BUN), creatinine (Cr), total protein (TP), and albumin (ALB) were simultaneously measured. We excluded patients with poor renal function, for which the eGFR (estimated glomerular filtration rate) value at the time was estimated to be 60 mL/min or less. The serum NTx level was measured by ELISA.

Bone scintigraphy was used to determine the presence or absence of bone metastasis. When it was necessary to obtain a more detailed evaluation for bone metastasis, we added contrast-enhanced computed tomography (CT) from the thorax to pelvis, MRI, and FDG-PET.

The "R, ver2.8.1" software program (available at: http://www.R-project.org; R Foundation for Statistical Computing, Vienna, Austria) was used to perform the analysis. The optimal cutoff value was selected by performing a receiver operating characteristic (ROC) curve analysis of relationship between abnormal serum NTx values and bone metastasis from lung cancer, and its cutoff value was used for the evaluations in the subsequent analyses. Univariate and multivariate analyses were performed using a logistic regression. Only the variables that were found to have a p value of less than 0.10 in univariate analysis were included in the multiple logistic regression analysis. These variables included performance status (PS), ALB, Ca, ALP, LDH, and NTx. A p value of less than 0.05 was considered statistically significant.

Results

A total of 166 patients (128 men and 38 women) were included in the study. The median age was 68 years (range: 23–85 years). The histological classification of the lung cancers was as follows: adenocarcinoma in 85 cases, squamous cell carcinoma in 42 cases, small-cell lung cancer in 32 cases, and large cell lung cancer in 7 cases. Bone metastasis was present in 73 of the 166 cases. PS was 0 in 17 cases, 1 in 86 cases, 2 in 30 cases, and ≥ 3 in 33 cases. According to the staging system (TNM Stage Ver. 6), there were 9 stage Ia–IIb cases, 11 stage IIIa cases, 45 stage IIIb cases, and 101 stage IV cases (Table 1).

The mean/median serum NTx values according to the presence/absence of bone metastasis were 27.8/23.8 nmol bone collagen equivalents (BCE)/L in the bone metastasis-positive group and 17.1/16.5 nmol BCE/L in the bone metastasis-negative group. The differences were significant ($P < 0.001$ for both groups).

The ROC curve plotted to determine the relationship between serum NTx values and bone metastasis revealed an optimal cutoff value of 22.0 nmol BCE/L and yielded a sensitivity of 61.6% and a specificity of 89.2%. We also plotted ROC curves for LDH and ALP. The values of these parameters are thought to increase in bone metastasis. These parameters were compared with the ROC curve for serum NTx. Compared to ALP and LDH levels, the serum NTx level was found to have a clearly superior correlation with bone metastasis (Fig. 1).

In this study, we performed a univariate analysis and multivariate analysis based on the optimal cutoff values (22.0 nmol BCE/L) of serum NTx on the ROC curves. The

Table 1 Patients characteristics ($n = 166$)

Age (years)	Median (range)	68 (23–85)
Sex	Males	128 (77.1%)
	Females	38 (22.9%)
Histological type	Squamous cell carcinoma	42 (25.3%)
	Adenocarcinoma	85 (51.2%)
	Small-cell carcinoma	32 (19.3%)
	Other tumor	7 (4.2%)
Stage	Ia–IIb	9 (5.4%)
	IIIa	11 (6.6%)
	IIIb	45 (27.1%)
	IV bone metastasis positive	73 (44.0%)
	IV bone metastasis negative	28 (16.9%)
Performance status (PS)	0	17 (10.2%)
	1	86 (51.8%)
	2	30 (18.1%)
	3	24 (14.5%)
	4	9 (5.4%)

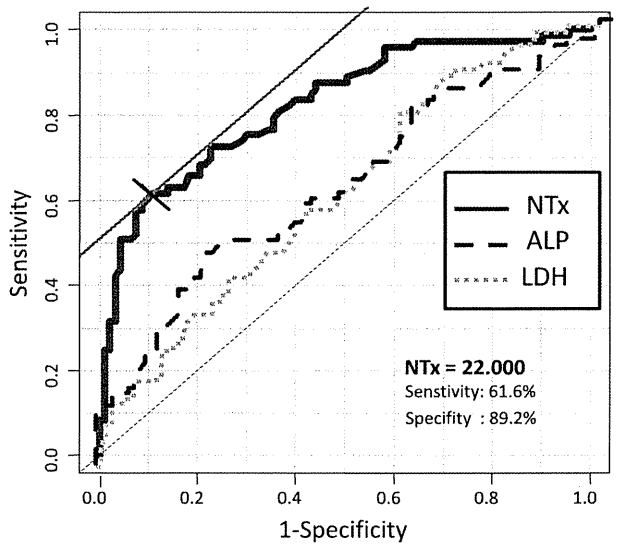

Fig. 1 The optimal value was selected by a receiver operating characteristic (ROC) curve analysis of relationship between abnormal serum NTx values and bone metastasis of lung cancer and compared to ROC curve of ALP and LDH

parameters of the analysis were age, sex, PS, histological type, serum NTx value, CEA, CYF, NSE, Ca, ALP, and LDH.

The univariate analysis revealing albumin, LDH, and Ca tended to relate to bone metastasis. Furthermore, PS, ALP, and NTx were found to be significantly related to bone metastasis. It is especially noteworthy that the NTx values were closely associated with the presence or absence of bone metastasis from lung cancer ($P < 0.0001$) (Table 2). PS, albumin, ALP, LDH, Ca, and NTx were included in the multivariate analysis. The results showed that ALP tended to be associated with bone metastasis, and a particularly close association was found between serum NTx levels and bone metastasis from lung cancer ($P < 0.0001$) (Table 3).

Table 2 Univariate analysis in the bone metastasis-positive and bone metastasis-negative group

Bone metastasis from lung cancer	Negative (93)	Positive (73)	*P* value
Age			
≦70	60	39	0.581
70<	33	34	
Sex			
Males	74	54	0.395
Females	19	19	
Pathology			
Non-small-cell lung cancer	74	60	0.671
Small-cell lung cancer	19	13	
PS			
0, 1	67	36	**0.00114**
2, 3, 4	26	37	
Total protein			
≦6.6 [g/dL]	29	28	0.3346
6.6<	64	45	
Albumin			
≦3.4 [g/dL]	16	21	0.0782
3.4<	77	52	
CEA			
≦5 [ng/ml]	76	43	0.3786
5 < [ng/ml]	17	30	
NSE			
≦12 [ng/ml]	67	36	0.433
12<	26	39	
Cytokeratin 19 fragment			
≦3.5 [ng/ml]	16	20	0.7916
3.5<	72	52	
ALP			
≦359 [IU/L]	76	43	**0.00151**
359<	17	30	
LDH			
≦229 [IU/L]	63	40	0.0891
229<	30	33	
Ca			
≦10.2 [mg/dL]	92	68	0.0842
10.2<	1	5	
NTx			
<22.0 [nmol BCE/L]	82	28	**<0.0001**
22.0≦	11	45	

Discussion

The initial diagnosis of bone metastasis is usually made on the basis of the results of bone scintigraphy, but the use of this technique is limited because it is costly and has a high rate of false positives. Furthermore, it is inconvenient to require the use of radioactive substances. Thus, the development of a convenient and useful marker of bone metastasis is imperative. Izumi et al. evaluated bone metabolism markers, such as D-PYD, 1CTP, and NTx, and concluded that urinary NTx is the most useful biomarker for the detection of bone metastasis in patients with lung cancer [8].

The major collagen in bone is type I collagen, and it accounts for 90% of the organic chemical components of bone [9]. NTx is one of the degradation products of collagen, formed as a result of bone resorption. It is highly bone specific and reflects bone resorption by osteoclast-derived cathepsin K during physiological bone remodeling [10]. NTx is also produced under conditions such as bone metastasis by malignant tumors and reflects bone resorption by matrix metalloproteinase as well. When bone is

Table 3 Multivariate analysis in the bone metastasis-positive and bone metastasis-negative group

Bone metastasis from lung cancer	Negative	Positive	*P* value
PS			
0, 1	67	36	0.2274
2, 3, 4	26	37	
Albumin			
≦3.4 [g/dL]	16	21	0.7916
3.4<	77	52	
ALP			
≦359 [IU/L]	76	43	0.062
359<	17	30	
LDH			
≦229 [IU/L]	63	40	0.1761
229<	30	33	
Ca			
≦10.2 [mg/dL]	92	68	0.6943
10.2<	1	5	
NTx			
<22.0 [nmol BCE/L]	82	28	<0.0001
22.0≦	11	45	

resorbed, NTx is released into the blood and is subsequently excreted in the urine. A difference between urinary NTx levels and serum NTx levels is that urinary markers are usually impacted by fluctuations in metabolism to a greater extent than serum NTx because bone metabolism follows a pattern of being very active during the night and decreasing in the afternoon. Therefore, bone metabolism marker values are high at night and low during the day. Furthermore, the analysis of urinary NTx requires caution with regard to the timing of specimen collection because the specimen must be obtained during the first void of the morning [11, 12]. We therefore thought that serum NTx levels might be easier to evaluate for an indication of bone metastasis.

Many clinical studies in which urinary NTx levels were used as a marker have been reported in the past [13–15]; however, we were unable to find examples of studies of serum NTx. In our study, the results clearly indicate that serum NTx levels are more closely related to bone metastasis than to ALP or LDH, which have been said to become elevated during bone metastasis [16, 17].

The results of this study suggest that an appropriate cutoff value of serum NTx is 22.0 nmol BCE/L and that the analysis of serum NTx levels will provide a simple and effective diagnostic marker of bone metastasis from lung cancer.

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Conflict of interest The authors have no financial arrangements or relationships with any individuals or organizations that could potentially influence this work.

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Clinical features of unresectable high-grade lung neuroendocrine carcinoma diagnosed using biopsy specimens☆

Yoshihisa Shimada[a,*], Seiji Niho[a], Genichiro Ishii[b], Tomoyuki Hishida[a], Junji Yoshida[a], Mitsuyo Nishimura[a], Kiyotaka Yoh[a], Koichi Goto[a], Hironobu Ohmatsu[a], Yuichiro Ohe[a], Kanji Nagai[a]

[a] Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan
[b] Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan

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ABSTRACT

Background: The overall clinicopathological features or the optimal therapy for large cell neuroendocrine carcinoma (LCNEC) have yet to be defined, because LCNEC has not been studied in the same depth as had small cell lung carcinoma (SCLC) in both clinical and biological standpoints. The aim of this study was to elucidate the clinical features of high-grade neuroendocrine carcinoma (HGNEC)-probable LCNEC diagnosed by biopsy, and compare therapeutic efficacy with patients with SCLC.
Methods: We retrospectively examined the chart of total of 25 patients who underwent chemotherapy or chemoradiotherapy as initial therapy for a histologic diagnosis of HGNEC-probable LCNEC, using biopsy samples and compared their data with those of 180 patients with SCLC. We analyzed their responses to chemotherapy and/or radiation therapy and survival outcomes.
Results: In 25 patients with HGNEC-probable LCNEC, 18 patients initially received chemotherapy (17 (94%) of whom received platinum-based chemotherapy) with an overall response rate (ORR) of 61%. The remaining 7 patients received chemoradiotherapy with an ORR of 86%, and 12 of the 25 patients who received second-line chemotherapy had an ORR of 17%. A total of 101 patients with SCLC who initially received chemotherapy had an ORR of 63%, and 79 patients who initially received chemoradiotherapy had an ORR of 98%, and 102 of the 180 patients who received second-line chemotherapy had an ORR of 45%. The 1-year overall survival rate for patients with stage IV HGNEC-probable LCNEC ($n = 13$) and those with ED-SCLC ($n = 80$) was 34% and 49%, respectively ($p = 0.84$).
Conclusion: The overall response rate to initial treatment and the survival outcomes of HGNEC-probable LCNEC were comparable to those of SCLC, but the effectiveness of second-line chemotherapy appeared to differ between the 2 groups.

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1. Introduction

Large cell neuroendocrine carcinoma (LCNEC) of the lung and small cell lung carcinoma (SCLC) are both now considered to be high-grade neuroendocrine carcinomas arising in the lung. Travis et al. [1] were the first to propose the term LCNEC in 1991, to describe cancer which exhibits neuroendocrine morphologic features such as rosette formation, organoid nesting, and palisading, large tumor cells (typically 3 times larger in diameter than a small resting lymphocyte) with a low nuclear/cytoplasmic ratio, numerous nucleoli, a high mitotic rate (>10 in 10 high-power fields), a large degree of necrosis, and immunohistochemical positive staining findings for 1 or more neuroendocrine markers [2]. The tumor cells of SCLC are round, oval, or spindle-shaped; usually less than the size of three small resting lymphocytes, and have scant cytoplasm, finely granular chromatin, and absent or inconspicuous nucleoli [2]. The morphologic features of LCNEC differ distinctly from those of SCLC by definition, however, distinguishing LCNEC from SCLC based on the tumor cell size and chromatin morphology may be difficult in some cases.

SCLC has poorer outcome, despite its marked chemosensitivity, enabling temporary remission in most SCLC patients because most tumors relapse after chemotherapy or chemoradiotherapy. The standard therapeutic strategy for SCLC has already been

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* Corresponding author at: Division of Thoracic Oncology, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa-shi, Chiba 277-8577, Japan.
Tel.: +81 0 4 7133 1111; fax: +81 0 4 7131 4724.
E-mail address: zenkyu@za3.so-net.ne.jp (Y. Shimada).

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Table 1
Proposed criteria for diagnosis of pulmonary HGNEC-probable LCNEC using biopsy specimens.

1.	Solid tumor nesting without either acinar or squamous differentiation
2.	Moderate or marked cellular atypia
3.	Large cell size with low nuclear to cytoplasmic ratio or moderate to abundant eosinophilic cytoplasm
4.	Vesicular and/or coarsely granular nuclear chromatin
5.	Prominent nucleoli
6.	Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A, and synaptophysin)
7	Ki-67/MIB1 labeling index >40%

NCAM, neural cell adhesion molecule; HGNEC, high-grade neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma.

established, and second-line chemotherapy has been recognized to be well-tolerated and effective in patients with chemotherapy-sensitive SCLC [3–7]. In contrast, the overall clinicopathological features or the standard treatment for LCNEC have yet to be defined, because LCNEC has not been studied in the same depth as had SCLC in both clinical and biological standpoints. Moreover, the incidence of the pre-therapeutic diagnosis of LCNEC in unresectable cases is unknown. Although obtaining a definitive diagnosis of LCNEC using small biopsy specimen is difficult, there is an urgent need to establish the diagnostic criteria for LCNEC. Therefore, instead of diagnosing LCNEC, we usually use the term "high-grade neuroendocrine carcinoma (HGNEC)-probable LCNEC" based on the proposed criteria (Table 1).

The aim of this study was to elucidate the clinical features of unresectable HGNEC-probable LCNEC (HG-pLCNEC) with those of SCLC, and compare their outcomes.

2. Patients and methods

2.1. Patient enrollment

From January 2002 through December 2009, we retrospectively examined the charts of total of 25 patients with a histologic diagnosis of HG-pLCNEC, using biopsy specimens. Diagnoses of HG-pLCNEC were all confirmed by pathological examination on biopsy specimens according to the modified criteria for the diagnosis of high-grade non-small cell neuroendocrine carcinoma using biopsy specimens proposed by Igawa et al. [8] (Table 1). All patients had undergone a minimum of 1 course of chemotherapy or chemoradiotherapy as initial therapy. Furthermore, the data of a total of 180 patients with histologically confirmed SCLC who had completed a minimum of 1 course of chemotherapy or chemoradiotherapy were examined as a control group. We used these criteria because the diagnostic criteria for LCNEC in the third edition of the World Health Organization (WHO) guidelines, which have been mainly established for cases of surgical specimens, and fulfilling the diagnostic criteria for LCNEC according to the WHO classification system is often difficult with biopsy specimens. We extracted the clinical data of patients from their medical records, all of whom had been given diagnoses of unresectable HG-pLCNEC or SCLC based on the results of pre-therapeutic evaluation including physical examination, chest radiography, computed tomography (CT) of the chest and abdomen, magnetic resonance imaging (MRI) of the brain, isotopic bone scan, and positron emission tomography (PET) or combined PET-CT. Their clinical disease staging was then reassessed according to the 7th edition of the International Union Against Cancer TNM classification system [9]. Data collection and analyses were approved by the institutional review board in December 2010, and the need to obtain informed consent from patients was waived due to the retrospective nature of the study.

2.2. Histopathology

We reviewed all the available pathology slides of biopsy specimens in this study. After fixing the specimens with 10% formalin and embedding them in paraffin, serial 4 μm sections were stained with hematoxylin–eosin (HE). The sections were reviewed by 2 observers (Y.S. and G.I.) and we classified HG-pLCNEC if they fulfilled all the relevant criteria as described above (Table 1). Immunohistochemical analysis was performed to confirm the neuroendocrine features of the specimens. Formalin-fixed paraffin sections were stained for a panel of neuroendocrine markers, including a polyclonal anti-chromogranin A antibody (Ventana, Arizona), anti-neural cell adhesion molecule (NCAM) antibody (Nippon Kayaku, Tokyo, Japan), and monoclonal anti-synaptophysin antibody (DAKO, Glostrup, Denmark). Immunohistochemically, neuroendocrine differentiation was considered to be positive if the tumor cells exhibited focal, patchy, or diffuse staining in the intracellular areas for one or more of these 3 antibodies. The anti-human Ki-67 antigen was identified by use of a monoclonal mouse anti-human Ki-67 (clone MIB1) antigen (DAKO, Glostrup, Denmark). Only nuclear immunostaining was considered to be positive. The labeling index of Ki-67/MIB1 in each tumor was estimated as a percentage of positive cells by counting from 100 to 1000 tumor cells.

2.3. Evaluation

Response criteria were evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST) guidelines [10]. Patients were evaluated to confirm disease progression or relapse by physical examination, chest radiography, and CT of the chest and abdomen. In some patients, we used PET-CT, MRI or bone scintigraphy to detect the extent of disease progression.

2.4. Statistical analysis

Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. Overall survival (OS) was measured from the first day of treatment to the date of death from any cause or the date on which the patient was last known to be alive. All tests were two-sided, and p-values less than 0.05 were considered to be represent statistically significant difference. We used Statview 5.0 software (SAS Institute Inc., Cary, NC) to perform statistical analysis.

3. Results

Overall, 25 patients were recognized to have tumors with histological characteristics consistent with HG-pLCNEC based on biopsy specimens. The typical microscopic appearances of the transbronchial biopsy specimens in the current study are shown in Fig. 1. The tumor cells showed a proliferation of polygonal cells, and a low nuclear–cytoplasmic ratio, with no differentiation of acinar or squamoid features (A). Positive immunostaining findings for NCAM antibody were observed (B), but findings for chromogranin A and synaptophysin were negative (data not shown). The diagnoses of 17 of 25 patients were obtained by transbronchial lung biopsy, and the diagnoses of the remaining 8 patients were obtained by CT-guided needle biopsy.

The characteristics of all the patients examined in this study are shown in Table 2. Among the 25 patients with HG-pLCNEC, the median age was 67 years (range 48–83 years), and 22 patients (88%) were men. Of the 25 patients, all (100%) were current or former smokers. Stage III B was noted in 7 patients (28%), and 13 patients (52%) had stage IV. In the patients with HG-pLCNEC, 18

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Table 2
Patient characteristics.

Characteristics	Category	HGpL	%	SCLC	%
No. of patients		25		180	
Age	Median (range)	67 (48–83)		68 (28–84)	
Gender	Male	22	88	148	82
	Female	3	12	32	18
Smoking status	Ever	25	100	172	96
	Never	0	0	8	4
Clinical stage	I	0	0	2	1
	II	1	4	12	7
	IIIA	4	16	37	21
	IIIB	7	28	39	22
	IV	13	52	90	50
Initial therapy	CT	18	72	101	56
	CRT	7	28	79	44
Tumor marker					
NSE	Median (NL: <16 ng/ml) (range)	30 (10–273)		29 (3–585)	
ProGRP	median (NL: <46 pg/ml) (range)	234 (10–20,000)		488 (7–18,000)	

HGpL, high-grade neuroendocrine carcinoma probable large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; CT, chemotherapy; CRT, chemoradiotherapy; NL, normal level; NSE, neuron-specific enolase; ProGRP, pro-gastrin-releasing peptide.

(72%) received chemotherapy, and 7 (28%) received chemoradiotherapy as initial treatment.

Among the 180 patients with SCLC, there were 99 patients with limited disease SCLC (LD-SCLC), and the number with extensive disease SCLC (ED-SCLC) was 81. The median age was 68 years (range 28–84 years), and 148 patients (82%) were men. Of 180 patients, 172 (96%) were current or former smokers. In the SCLC patients, 101 (56%) patients initially received chemotherapy, and 79 (44%) patients received chemoradiotherapy.

Of the 25 patients with HG-pLCNEC, 12 patients (48%) received second-line chemotherapy. The remaining 13 patients did not receive chemotherapy due to death from disease, adverse events caused by initial treatment, or no active treatment determination. Of the SCLC patients, 104 (58%) received second-line chemotherapy. A diagram of the tumor types and management in patients in this study is shown in Fig. 2.

Treatments and clinical response are summarized in Table 3. The regimens of initial treatment chemotherapies are listed in Table 3(a). Of 18 patients with HG-pLCNEC who initially received chemotherapy, 17 (94%) received platinum-based chemotherapy, and the 7 patients who had chemoradiotherapy received platinum-based chemotherapy and concurrent radiation of 45–60 Gy. Of the 101 patients with SCLC who underwent chemotherapy, the most frequently administered chemotherapy regimen was carboplatin and etoposide ($n = 42$), and the second most frequent was cisplatin and etoposide ($n = 23$).

Among the 18 patients with HG-pLCNEC initially receiving chemotherapy, 1 achieved a complete response (CR) and 10 achieved a partial response (PR), with an overall response rate (ORR) of 61% (Table 3(b)). One patient with CR and 4 patients with PR received cisplatin and irinotecan. There were 2 PRs observed in the patients treated with carboplatin and paclitaxel, and 1 PR was observed in each group of patients treated with either cisplatin and vinorelbine, cisplatin and docetaxel, cisplatin and amrubicin, or irinotecan alone. Among the 7 patients with HG-pLCNEC initially receiving initially chemoradiotherapy, 6 achieved PR, with an ORR of 86%. In the patients treated with cisplatin and vinorelbine, 3 PRs were observed while 2 PRs were observed in the patients treated with cisplatin and etoposide, and 1 patient achieved PR with carboplatin and etoposide.

Among the 101 patients with SCLC initially receiving chemotherapy, 2 achieved CR and 62 patients achieved PR, with an ORR of 63%. Among the 79 patients with SCLC initially receiving chemoradiotherapy 21 achieved CR and 56 patients achieved PR, with an ORR of 98%.

The regimens of second-line chemotherapies are listed in Table 3(c). The following chemotherapy regimens were used in 12 patients with HG-pLCNEC: amrubicin alone ($n = 4$), docetaxel alone ($n = 3$), cisplatin and irinotecan ($n = 3$), carboplatin and etoposide ($n = 1$), and cisplatin and irinotecan and etoposide ($n = 1$). In 102 patients with SCLC who received second-line chemotherapy, the frequent administered chemotherapy regimen was cisplatin and irinotecan ($n = 34$), and the second most frequently administered was amrubicin alone ($n = 18$).

Among the 12 patients with HG-pLCNEC receiving second-line chemotherapy, 2 patients achieved PR, with an ORR of 17% (Table 3(d)). One patients achieved PR in each group of patients treated with cisplatin and irinotecan, and carboplatin and paclitaxel. Among the 102 patients with SCLC receiving second-line chemotherapy, 4 achieved CR and 41 patients achieved PR, with an ORR of 45%. These results indicate that the effectiveness of second-line chemotherapy appeared to differ between HG-pLCNEC and SCLC patients in the present study, but the difference was statistically not significant ($p = 0.12$).

Fig. 3 shows the OS curves for the stage IV HG-pLCNEC and ED-SCLC groups. The 1-year OS rate for patients with stage IV HG-pLCNEC was 34%, and that for patients with ED-SCLC was 49%, with no statistically significant difference ($p = 0.84$).

4. Discussion

We set out to determine the clinical features of HG-pLCNEC and other related tumors diagnosed by biopsy specimens and compare these with those of SCLC. We also examined the efficacy of chemotherapy or chemoradiotherapy between HG-pLCNEC and that of SCLC. Little is known about the optimal treatment strategy of LCNEC because most publications concerning LCNEC are based on surgical materials, with limited cohort data [11–13]. From a treatment point of view, it is imperative to establish the appropriate definitive diagnostic criteria based on the examination of biopsy or cytologic specimens, and then evaluate the efficacy of chemotherapy or chemoradiotherapy for those patients with unresectable tumors.

It is often difficult to diagnose LCNEC with small biopsy specimens, because of the possibility of crushed remnants of tissue artifacts due to insufficient specimen size, and some morphological overlap regarding cell size or nucleus size between SCLC and LCNEC [14]. In order to resolve this histological ambiguity in neuroendocrine carcinoma cases with regard to a diagnosis of LCNEC

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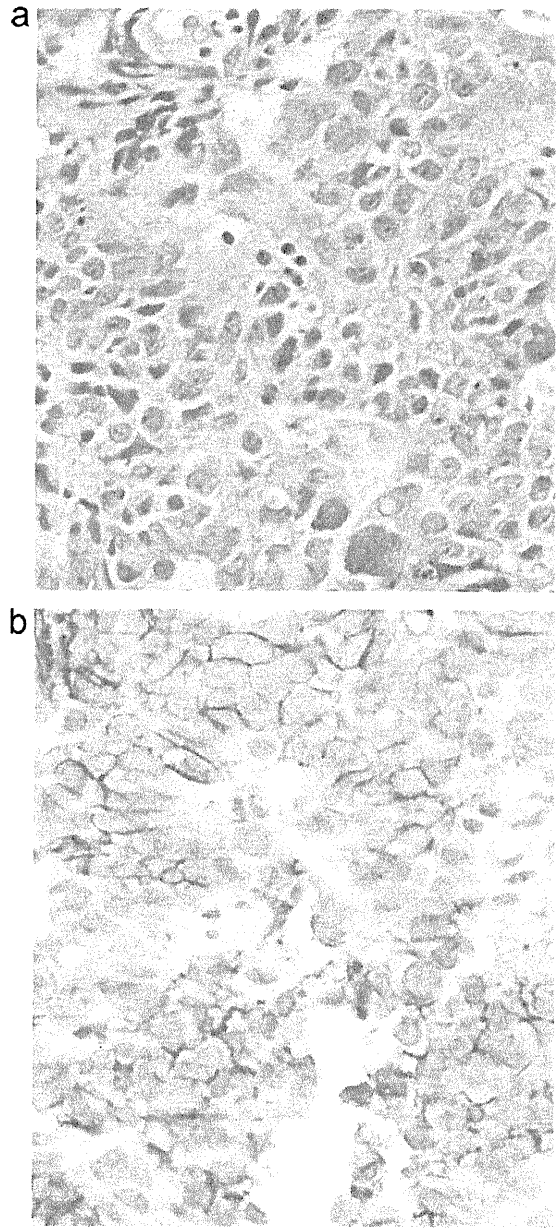
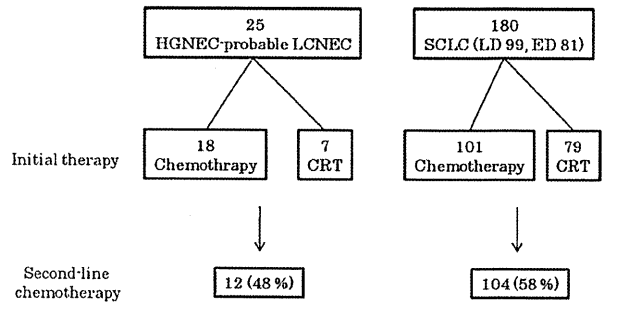


Fig. 1. A biopsy specimen diagnosed as HGNEC-probable LCNEC. (A) The histological features of HGNEC-probable LCNEC are shown with hematoxylin–eosin (HE) staining. The tumor cells are large, with a proliferation of polygonal cells, and have a low nuclear–cytoplasmic ratio, with no differentiation of acinar and squamoid features (400×). (B) Positive staining for neural cell adhesion molecule (NCAM).



HGNEC: high-grade neuroendocrine carcinoma, LCNEC: large cell neuroendocrine carcinoma, SCLC: small cell lung carcinoma, LD: limited disease, ED: extensive disease, CRT: chemoradiotherapy

Fig. 2. The characteristics of patients enrolled in this study.

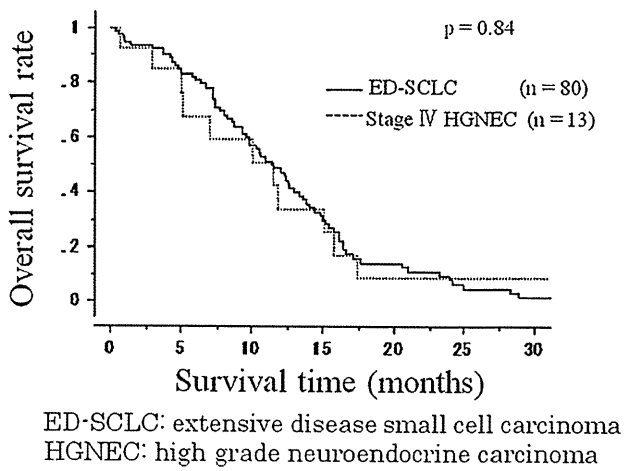


Fig. 3. Overall survival (OS) curve for stage IV HGNEC-probable LCNEC and ED-SCLC groups. The 1-year overall survival rate for patients with stage IV HGNEC-probable LCNEC was 34%, and that for patients with ED-SCLC was 49% ($p = 0.84$).

or SCLC, many researchers have performed immunohistochemistry or molecular analysis [15–18]. Hiroshima et al. reported that the frequencies of the expression of CD56, mASH1, TTF-1, and p16 were higher and that of NeuroD was lower in SCLC than in LCNEC in immunohistochemical analysis. The authors stated that LCNEC and SCLC are different morphologically, phenotypically, and genetically, although there are some overlapping features [15]. Nitadori et al. performed tissue microarray analysis of surgically resected LCNEC and SCLC specimens using 48 antibodies, and demonstrated that significant expression of CK7, CK18, E-cadherin, and β-catenin is more characteristic of LCNEC than of SCLC, suggesting that LCNEC and SCLC have a different biologic phenotype [17]. Ullmann et al. examined comparative genomic hybridization for LCNEC and SCLC, and reported that there were differences in the expression at 3q, 6p, 10q, 16q, and 17p [19]. On the other hand, Jones et al. demonstrated that cDNA microarrays gene expression profiles showed LCNEC was not differently clustered from SCLC, but different from large cell carcinoma or other NSCLC histology [20]. Although the clinicopathological features of LCNEC were similar to those of SCLC, and there is a histological ambiguity with regard to a diagnosis of LCNEC or SCLC, some biological behaviors of LCNEC were different from those of SCLC. Because there is actually the difficulty regarding the use of kinds of imuunohistochemical antibodies in daily practice, we have used the unique diagnostic criteria for HG-pLCNEC developed specifically for biopsy specimens by Igawa et al. [8]. However, lung cancer including LCNEC diagnosed by biopsy materials might not be representative of the whole tumor characteristics, particularly in heterogeneous cancers. Combinations with SCLC do occur, but such tumors are classified as combined variants of SCLC. Therefore, when using only biopsy materials for diagnosis, misdiagnosis may be unavoidable. The HG-pLCNECs examined in the present study might be mostly LCNECs and other related tumors, which included combined subtypes or other histological types, and excluded pure SCLC. This is one of the potential limitations of the present study.

To the best of our knowledge, there are few retrospective studies on the therapeutic efficacy of chemotherapy and/or radiation therapy for LCNEC [8,21,22], and this is the first study to examine

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Table 3
Treatments and clinical response.

(a) Initial therapy and chemotherapy regimens

	HGpL	SCLC
No. of patients	25	180
Initial therapy		
Chemotherapy (%)	18 (72)	101 (56)
CDDP + CPT-11	8	21
CDDP + VNR	4	0
CBDCA + PTX	2	0
CBDCA + ETP	1	42
CDDP + DTX	1	0
CDDP + AMR	1	2
CPT-11	1	0
CDDP + ETP	0	23
Others	0	13
Chemoradiotherapy (%)	7 (28)	79 (44)
CDDP + VNR	3	0
CBDCA + ETP	2	5
CDDP + ETP	2	72
Others	0	2

(b) Clinical response after initial therapy

	HGpL (n = 25)		SCLC (n = 180)	
Initial therapy	Chemotherapy only	CRT	Chemotherapy only	CRT
No. of patients	18	7	101	79
CR	1	0	2	21
PR	10	6	62	56
SD	5	1	19	2
PD	2	0	12	0
NE	0	0	6	0
Response rate (%)	11/18 (61)	6/7 (86)	64/101 (63)	77/79 (98)

(c) Second-line chemotherapy regimens

	HGpL	SCLC
No. of patients	12	102
AMR	4	18
DTX	3	0
CDDP + CPT-11	3	34
CBDCA + ETP	1	12
CDDP + ETP + CPT-11	1	11
CDDP + ETP	0	10
Others	0	17

(d) Clinical response after second-line chemotherapy

Response	HGpL (n = 12)	SCLC (n = 102)	p-Value
CR	0	4	
PR	2	41	
SD	4	16	
PD	6	42	
NE	0	1	
Response rate (%)	2/12 (17)	45/102 (43)	0.12

HGpL, high-grade neuroendocrine carcinoma probable large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; CDDP, cisplatin; CPT-11, irinotecan; VNR, vinorelbine; CBDCA, carboplatin; PTX, paclitaxel; ETP, etoposide; DTX, docetaxel; AMR, amrubicin; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; CRT, chemoradiotherapy.

second-line chemotherapeutic efficacy. In the current study, the majority of patients with HG-pLCNEC were predominantly men, smokers, with elevated NSE and ProGRP values. These resemble the clinical features of those of SCLC, similarly to several previous reports regarding the clinicopathological characteristics of LCNEC [11,12]. We obtained a response rate to initial chemotherapy of 61% and that to chemoradiotherapy of 86% in patients with HG-pLCNEC, which was similar to those of SCLC. The survival of patients with stage IV HG-pLCNEC was also similar to that of ED-SCLC patients. Considered together, these results suggest that there was no statistically significant difference in the initial treatment efficacy between the HG-pLCNEC and SCLC groups. Some authors have reported no statistically significant difference in survival outcome between LCNEC and SCLC [11,13], whereas the survival of patients with surgically resected LCNEC is reported to be intermediate between that of atypical carcinoid and SCLC [23]. Many authors reported that survival in LCNEC was poorer than that in stage-matched NSCLC, and adjuvant therapy might be effective in cases of early stage LCNEC [24–27].

The present study showed that the ORRs of second-line chemotherapy were 17% and 45% for patients with LCNEC and SCLC, respectively. In patients with SCLC, the prognosis at relapse is poor, and response to second-line chemotherapy correlates with response to first-line therapy and also to the interval between first-line chemotherapy and disease progression. Second-line chemotherapy has been recognized to be well-tolerated and effective, with an ORR of 15–88% in patients with chemotherapy-sensitive SCLC [3–7]. The present study suggested that chemotherapeutic efficacy in patients with HG-pLCNEC might be lower than in those with SCLC, even though the chemotherapeutic regimens were heterogeneous. The number of patients with HG-pLCNEC in this study was too small to draw any definite conclusion in terms of differing benefits of chemotherapy regimens for NSCLC and SCLC, or a possible difference in second-line chemotherapeutic sensitivity between LCNEC and SCLC. However, although LCNEC is categorized as a NSCLC, molecular findings in SCLC and LCNEC showed some differences but much overlap, and overall clinicopathological features and the initial treatment response of LCNEC in our study or several published articles suggest that these tumors would be better classified as a high-grade neuroendocrine tumor comparable with SCLC, suggesting that chemotherapies using an SCLC-based standard protocol might be effective and significantly improves the survival of patients with LCNEC compared with those using a NSCLC-based protocol [12,24,26].

In conclusion, these results, although limited, that the clinical efficacy of initial chemotherapy and/or radiation therapy for patients with HG-pLCNEC is similar to that of SCLC, and there might be a different sensitivity to second-line chemotherapy between HG-pLCNEC and SCLC. Improved diagnostic criteria, specifically developed for biopsy specimens, are needed to analyze the biological behavior of LCNEC. Moreover, prospective additional studies in a larger series are clearly mandatory to confirm our data, and the role of a therapy strategy with SCLC-based regimens deserves sensitivity to chemotherapeutic agents and the optimal treatment protocol.

Conflict of interest statement

The authors declare no potential conflicts of interest regarding this study.

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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

See commentary by Lauring and Park, p. 7508

Quantitative Detection of *EGFR* Mutations in Circulating Tumor DNA Derived from Lung Adenocarcinomas

Kazuya Taniguchi[1], Junji Uchida[2], Kazumi Nishino[2], Toru Kumagai[2], Takako Okuyama[2], Jiro Okami[3], Masahiko Higashiyama[3], Ken Kodama[3], Fumio Imamura[2], and Kikuya Kato[1]

Abstract

Purpose: Examination of somatic epidermal growth factor receptor (*EGFR*) mutations is now a diagnostic routine for treatment of cancer using EGFR tyrosine kinase inhibitors (EGFR-TKI). Circulating tumor DNA is a promising target for noninvasive diagnostics. We evaluated its utility by quantitatively detecting activating and resistant mutations, which were measured with BEAMing (beads, emulsion, amplification, and magnetics).

Experimental Design: Twenty-three patients with lung cancer with progressive disease after EGFR-TKI treatment and 21 patients who had never been treated with EGFR-TKIs were studied. Their primary tumors were confirmed to have activating mutations. In the plasma DNA of each patient, the activating mutation found in the corresponding primary tumor and the T790M resistance mutation were quantified by BEAMing.

Results: In 32 of 44 patients, activating mutations were detected in the plasma DNA [72.7%; 95% confidence interval (CI), 58.0%–83.6%]. The T790M mutation was detected in 10 of 23 patients in the first group (43.5%; 95% CI, 25.6%–53.4%). The ratio of T790M to activating mutations ranged from 13.3% to 94.0%. The peak of the distribution of the mutation allele fraction in the plasma DNA was in the 0.1% to 1% range.

Conclusions: The major advantage of BEAMing is its ability to calculate the fraction of T790M-positive alleles from the alleles with activating mutations. This feature enables the detection of increases and decreases in the number of T790M mutations in cancer cells, regardless of normal cell DNA contamination, which may be useful for monitoring disease progression. Circulating tumor DNA could potentially be used as an alternative method for *EGFR* mutation detection. *Clin Cancer Res; 17(24); 7808–15. ©2011 AACR.*

Introduction

The strong effects of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKI; i.e., gefitinib and erlotinib) on non-small cell lung cancer (NSCLC) are correlated with activating somatic mutations in the epidermal growth factor receptor (*EGFR*; refs. 1–3). Patients subjected to these drugs are currently selected on the basis of the presence of these activating mutations. In addition, a mutation known as T790M has been identified as a cause of gefitinib resistance (4, 5). The T790M mutation appears in about half of the cases of acquired resistance to EGFR-TKIs. Detection of T790M may have prognostic value in the patients with acquired resistance to EGFR-TKIs, because the presence of T790M defines a clinical subset with a relatively favorable prognosis and more indolent progression (6).

Detecting *EGFR* mutations using tumor tissues obtained via a biopsy or surgical resection is now routinely used to diagnose NSCLC. Because a biopsy is an invasive procedure, it is desirable to replace it with a noninvasive procedure. In particular, noninvasive tests allow the frequent monitoring of disease progression in patients with the T790M mutation (7).

For some time, circulating nucleic acids in the plasma or serum have been considered to be candidates for noninvasive cancer diagnostics (8, 9). In particular, circulating tumor DNA (ctDNA) has been explored to detect somatic mutations derived from malignant tumors. For example, in 2 studies, somatic mutations in ctDNA were used to monitor disease status with the appearance of target mutations (10, 11). One major problem is that detecting rare mutant alleles is technically difficult. Diehl and colleagues used their proprietary technique called BEAMing (beads, emulsion, amplification and magnetics; ref. 12) to measure somatic mutations in ctDNA and monitor the tumor burden during the course of the disease. In BEAMing, PCR

Authors' Affiliations: [1]Research Institute, Departments of [2]Thoracic Oncology and [3]Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka, Japan

Corresponding Author: Kikuya Kato, Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. Phone: 81-6-6973-1209; Fax: 81-6-6973-1209; E-mail: katou-ki@mc.pref.osaka.jp

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