Diagnostic and prognostic significance of the alternatively spliced ACTN4 variant in high-grade neuroendocrine pulmonary tumours

A. Miyanaga¹,², K. Honda¹, K. Tsuta³, M. Masuda¹, U. Yamaguchi¹, G. Fuji⁴, A. Miyamoto⁵, S. Shinagawa⁵, N. Miura¹, H. Tsuda³, T. Sakuma⁶, H. Asamura⁷, A. Gemma² & T. Yamada¹*¹

¹Division of Chemotherapy and Clinical Research, National Cancer Center Research Institute, Tokyo; ²Department of Internal Medicine, Division of Pulmonary Medicine, Infection and Oncology, Nippon Medical School, Tokyo; ³Division of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo; ⁴Division of Cancer Prevention Research, National Cancer Center Research Institute, Tokyo; ⁵Kobe Research Center, TransGenic Inc., Kumamoto; ⁶BioBusiness Group, Miitsu Knowledge Industry, Tokyo; ⁷Division of Thoracic Surgery, National Cancer Center Hospital, Tokyo, Japan

Received 2 April 2012; revised 30 May 2012; accepted 31 May 2012

Background: High-grade neuroendocrine tumours (HGNTs) of the lung manifest a wide spectrum of clinical behaviour, but no method for predicting their outcome has been established.

Materials and methods: We newly established a monoclonal antibody specifically recognizing the product of the alternatively spliced ACTN4 transcript (namely, variant actinin-4), and used it to examine the expression of variant actinin-4 immunohistochemically in a total of 609 surgical specimens of various histological subtypes of lung cancer.

Results: Variant actinin-4 was expressed in 55% (96/176) of HGNTs, but in only 0.8% (3/378) of non-neuroendocrine (NE) lung cancers. The expression of variant actinin-4 was significantly associated with poorer overall survival in HGNT patients (P = 0.00021, log-rank test). Multivariate analysis using the Cox proportional hazards model showed that the expression of variant actinin-4 was the most significant independent negative predictor of survival in HGNT patients (hazard ratio (HR), 2.15; P = 0.00113) after the presence of lymph node metastasis (HR, 2.25; P = 0.00023).

Conclusions: The expression of variant actinin-4 is an independent prognostic factor for patients with HGNTs. This protein has a high affinity for filamentous actin polymers and likely promotes aggressive behaviour of cancer cells. The present clinical findings clearly support this notion.

Key words: actinin-4, alternative splice variant, diagnostic marker, high-grade neuroendocrine tumour, pulmonary neoplasm, prognosis

Introduction

Neuroendocrine (NE) tumours comprise 20%–25% of all human lung malignancies and are classified into four histological subtypes: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) [1, 2]. TC and AC are tumours with low- to intermediate-grade malignancy, whereas LCNEC and SCLC are highly aggressive and collectively referred to as high-grade neuroendocrine tumours (HGNTs) [3–6].

LCNEC appeared in the World Health Organization (WHO) Histological Typing of Lung and Pleural Tumours (third version, 1999) as a new entity of large cell carcinoma [2]. LCNEC shows aggressive behaviour distinct from other non-small cell lung cancers (NSCLCs) [7], and thus its accurate discrimination is essential for the management of lung cancer patients. TC, AC and SCLC can be readily diagnosed on the basis of their histological characteristics, but the diagnosis of LCNEC is more complicated. The morphological features of NE differentiation are often inconspicuous, especially in small biopsy or cytology specimens [2].

Three NE markers are used routinely for immunohistochemical assessment of NE differentiation: neural cell adhesion molecule (CD56), chromogranin A (CGA) and synaptophysin (SYN). However, a significant proportion of LCNECs are negative for any of these NE markers [7], and more problematically, some non-NE lung cancers also show equivocal immunoreactivity for these markers [7, 8]. Therefore, it is necessary to develop a new diagnostic biomarker with higher specificity.

Actinin-4 is an actin-binding protein that we originally identified as being associated with enhanced cell motility and cancer invasion [9]. The actinin-4 (ACTN4) gene has a unique structure (supplementary Figure S1, available at Annals of Oncology...
Annals of Oncology online), possessing two exons 8 (8\% of the same size, the mutually exclusive use of which leads to the production of two kinds of mRNA. The transcript obtained with exon 8 is expressed ubiquitously (namely, the ubiquitous form or ACTN4-Ub), whereas the expression of the variant transcript obtained with exon 8\% (the variant form or ACTN4-Va) is undetectable in normal tissues except for testis and brain [10, 11]. We previously found that the variant transcript is frequently expressed in SCLCs and that the gene product can be categorized as a so-called cancer-testis antigen [10]. However, the clinical significance of variant actinin-4 has remained undetermined due to lack of a specific probe.

The alternatively spliced actinin-4 variant transcript is predicted to encode a polypeptide differing in only three amino acids. To produce an antibody that can detect this small difference, we used the GANP (germline centre-associated nuclear protein) technology [12]. GANP transgenic mice generate a highly diverse spectrum of antibodies and have been used to produce high-affinity antibodies against various difficult antigens, such as those with small protein modifications [13]. Here, we report the establishment of a highly specific antibody recognizing variant actinin-4 protein and the potential utility of variant actinin-4 not only as a new diagnostic biomarker but also as a highly potent prognostic biomarker for HGNTs.

materials and methods

cell lines and sequencing

Total RNA was extracted from 91 human cancer cell lines (31 lung cancers, 23 colorectal cancers, 7 stomach cancers, 5 hepatocellular carcinomas, 6 pancreatic cancers, 4 choriocarcinomas, 4 ovary cancers, 4 oral cancers, 3 prostate cancers, 2 breast cancers, 1 bladder cancer and 1 cervical cancer) using TRIzol reagent (Life Technologies, Grand Island, NY) (supplementary Table S1, available at Annals of Oncology online).

First-strand complementary DNA (cDNA) was synthesized in the presence of random primers using the high-capacity cDNA reverse transcription kit (Life Technologies) in accordance with the manufacturer's instructions. The entire coding region of ACTN4 was amplified and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Life Technologies).

production of monoclonal antibodies

GANP transgenic mice were immunized with a synthetic peptide (NQSYQGSPSSAGNGAG) to produce a monoclonal antibody (namely 13G9) reactive with all the known forms (ubiquitous and variant) of actinin-4. Another monoclonal antibody specific to variant actinin-4 (namely 15H2) was raised against a synthetic peptide (DIVGTLRPEDEKAIMTYVSC). The reactivity and titre of antibodies against the various peptides were assessed by the antibody capture assay as described previously [13].

western blot analysis

The PCR-amplified fragments encoding the ubiquitous and variant forms of actinin-4 (amino acids 28–911) were cloned into the EcoRI and KpnI sites of the pEFGP-C1 vector (Takara Bio, Otsu, Japan) to express the ubiquitous and variant actinin-4 proteins fused with green fluorescent protein (GFP) at the N-termini (namely pEFGP-ACTN4-Ub and pEFGP-ACTN4-Va, respectively). The nucleotide sequences of all the PCR-amplified fragments were verified by sequencing. HEK293 cells (Health Science Research Resources Bank, Osaka, Japan) were transfected with each plasmid using Lipofectamine 2000 reagent (Life Technologies).

Western blotting was carried out following standard procedures, as described previously [14, 15]. Cells were extracted with lysis buffer (10 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 1% NP40 and 1 mg/ml NaN3) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) on ice for 30 min. Ten micrograms of cell lysate were reduced, denatured at 70°C for 10 min and fractionated through NuPAGE 4%-12% Bis-Tris gel (Life Technologies). The fractionated proteins were then blotted onto PVDF membranes (Life Technologies). After incubation with the primary antibody at 4°C overnight, the blots were incubated with appropriate horse-radish peroxidase-linked secondary antibodies and then detected by enhanced chemiluminescence (Western Lightning ECL Pro; Perkin-Elmer, Waltham, MA).

patients and tissue samples

Thirty-one tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded tissue blocks of 609 primary lung tumours that had been surgically resected at the National Cancer Center Hospital (Tokyo, Japan) from 1982 to 2010 using a tissue-arraying instrument (KIN-1; Azumaya, Tokyo, Japan). Based on the Pathology and genetics of Tumours of the Lung, Pleura, Thymus and Heart [IARC (International Agency for Research on Cancer) WHO Classification of Tumours] (2004) [16], the 609 tumours were classified into carcinoid (51 cases), SCLC (70 cases), LCNEC (106 cases), adenocarcinoma (164 cases), squamous cell carcinoma (166 cases) and other NSCLCs (52 cases). To reduce sampling bias due to tissue heterogeneity, we took duplicate core samples measuring 2.0 mm in diameter from two different areas of every tumour.

The 176 patients with HGNTs included 143 men and 33 women with a mean age of 66 years (19–84 years). One hundred and sixty-nine (96%) patients had a history of habitual cigarette smoking. The follow-up periods ranged from 1 to 286 months (median follow-up, 46 months). Patients were staged postsurgically into IA (52 cases), IB (25 cases), IIA (33 cases), IIB (16 cases), IIIA (37 cases), IIIB (7 cases) and IV (6 cases) according to the International Union against Cancer (UICC) TNM classification of malignant tumours (7th edition, 2010) [17]. This study was conducted with approval from the Institutional Review Board of the NCC.

immunohistochemistry

TMA blocks were then cut into 4-mm thick sections and subjected to immunohistochemistry (IHC). Immunostaining of actinin-4 proteins was carried out using the Ventana DABMap detection kit and an automated slide stainer (Discovery XT, Ventana Medical Systems, Tucson, AZ) [18, 19]. Immunoreactivity was classified as positive when ≥10% of cancer cells showed cytoplasmic or membrane staining detectable at a magnification of ×40 [20, 21].

Deparaffinized TMA slides were incubated with anti-CD56 mouse monoclonal (1B6, Novocastra, Newcastle upon Tyne, UK), anti-CGA rabbit polyclonal (Dako, Glostrup, Denmark) or anti-SYN mouse monoclonal (27G12, Nichirei Biosciences, Tokyo, Japan) antibody for 1 h at room temperature. Immunoreactivity was detected with the EnVision plus kit (Dako) using 3, 3' diaminobenzidine as the chromogen. For diagnosis of LCNEC, positive staining for at least one of the three NE markers was stipulated.

The results of IHC were judged by two investigators (AM and KT) who were blinded to any clinical-pathological information, and any discrepancy in judgement was discussed.
statistical analysis

Overall survival was measured as the period from surgery to the date of death or last follow-up. Progression-free survival was defined as the length of time from surgery to the first detection of new lesions or death. Overall and progression-free survival was estimated by the Kaplan–Meier method using the StatFlex statistical software package (version 5.0, Ariteck, Osaka, Japan). Differences between the survival curves were assessed with the log-rank test. Univariate and multivariate analyses were carried out using the Cox regression model. Other statistical tests were carried out using tools available in the R statistical package (version 2.12.0; http://www.r-project.org/). Differences were considered to be statistically significant at \( P < 0.05 \).

results

expression of the ACTN4 splice variant in cancer cell lines

We sequenced the entire coding region of the ACTN4 transcript in 91 cell lines derived from human cancers of various origins (supplementary Table S1, available at *Annals of Oncology* online). No non-synonymous nucleotide substitution was detected except for those registered in the single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/snp), indicating that somatic mutation of the ACTN4 gene is infrequent. However, we detected overlap of two sequences appearing in nucleotides 793–872 of the ACTN4 transcript (NM_004924.2) (supplementary Figure S2A, available at *Annals of Oncology* online) in 25% (23/91) of the cell lines. These two transcripts were separately cloned, and their nucleotide sequences were confirmed to be identical to the ubiquitous and variant transcripts of ACTN4 that we had described previously [10] (supplementary Figure S2B, available at *Annals of Oncology* online).

The ubiquitous form of the ACTN4 transcript (namely ACTN4-Ub) was expressed in all of the 91 human cancer cell lines examined, but the variant form (namely ACTN4-Va) was detected in 90% (18/20) of the SCLC cell lines examined (SBC-3, SBC-5, MS-1-L, Lu-135, Lu-143, STC-1, Lu-138, Lu-140, DMS153, DMS53, H1688, Wa-hT, H69AR, RERF-LCMS, Lu165, H69, Lu134-AH, Lu134-B, Lu-141 and Lu-139) and 25% (1/4) of the cell lines derived from pulmonary carcinoid tumour (NCI-H727, NCI-H835, UMC-11 and NCI-H720) (supplementary Table S1, available at *Annals of Oncology* online). In addition, the variant ACTN4 mRNA was detected in 33% (11/33) of prostate cancers and 75% (3/4) of choriocarcinoma cell lines. However, none of the seven non-SCLC-derived cell lines examined (A549, PC9, LCD, LCKJ, JK-2, Lu-65 and Lu-99) expressed the variant transcript.

detection of antibody to variant actinin-4 protein

Although there was a difference of only three amino acid residues between the ubiquitous and variant actinin-4 protein sequences deduced from their cDNA sequences (supplementary Figure S2C, available at *Annals of Oncology* online), we were able to produce a monoclonal antibody (15H2) that reacted specifically with a peptide for which the amino acid sequence was derived from the variant actinin-4 protein (DIVNTARPDEKAIMTYVSS) (underlining indicates the amino acids that differed) (Figure 1A).

To further confirm that the 15H2 monoclonal antibody reacted specifically with the variant form of actinin-4 protein, HEK 293 cells were transfected with a plasmid encoding the ubiquitous (pEGFP-ACTN4-Ub) or variant (pEGFP-ACTN4-Va) form of actinin-4. We found that the 15H2 antibody reacted only with a lysate prepared from HEK 293 cells transfected with pEGFP-ACTN4-Va, but not with those prepared from cells transfected with pEGFP-ACTN4-Ub or the parental pEGFP-C1 vector (ACTN4-Va, Figure 1B). On the other hand, the monoclonal antibody 13G9 reacted with both the ubiquitous and variant actinin-4 proteins (pan-ACTN4, Figure 1B).

![Figure 1](image-url)}
The expression of endogenous variant actinin-4 was detected in all three SCLC cell lines examined (SBC-3, Lu-135 and Lu-165), but in none of the three non-SCLC cell lines (LCD, LK-2 and EBC-1) (Figure 1C), being consistent with the results of cDNA sequencing described above.

**significance of variant actinin-4 expression in the diagnosis of HGNT**

We next investigated immunohistochemically the expression of both actinin-4 proteins and classical NE markers (CGA, SYN and CD56) in 609 primary lung tumours. Representative results of immunohistochemical staining are shown in Figure 2, and the positivity rates for each histological subtype are summarized in Table 1. CGA was expressed in 88% (59/67) of SCLCs and 56% (59/105) of LCNECs. SYN was expressed in 88% (58/66) of SCLCs and 56% (59/105) of LCNECs. CD56 was expressed in 96% (64/67) of SCLCs and 72% (76/105) of LCNECs. The three NE markers were also expressed in 76%–100% of pulmonary carcinoid tumours (Table 1). The expression of variant actinin-4 protein was detected in 55% (96/176) of HGNTs [60% (42/70) of SCLCs and 51% (54/106) of LCNECs], but in only 10% (5/51) of carcinoid tumours. The difference in the frequency of variant actinin-4 expression between HGNTs and carcinoid tumours was statistically significant ($P = 6.0 \times 10^{-6}$, Fisher’s exact test).

**Figure 2.** Expression of the variant actinin-4 protein in high-grade neuroendocrine tumour (HGNT). Representative cases of small cell lung carcinoma (SCLC) (A–C) and large cell neuroendocrine carcinoma (LCNEC) (D–F) showing positive (A, B, D and E) and negative (C and F) immunoreactivity with the 15H2 monoclonal antibody. Original magnification: A, C; D and F, ×20; B and E, ×400.

CGA, SYN and CD56 were found to show variable expression in non-NE lung tumours (2%–11% of adenocarcinomas, 2%–14% of squamous cell carcinomas and 6%–17% of other NSCLCs). However, variant actinin-4 protein was expressed in only 0.8% (3/382) of non-NE NSCLCs. These results indicated that variant actinin-4 was highly specific to HGNTs.

**prognostic significance of variant actinin-4 expression**

There was no significant difference between patients with HGNTs that were positive ($n = 96$) and negative ($n = 80$) for variant actinin-4 protein expression with respect to gender, age, smoking status, histological subtype (LCNEC versus SCLC), pathological stage, tumour size or frequency of lymph node or distant metastasis (Table 2). However, the frequency of relapse after surgery was much higher in stage I to III HGNT cases that were positive for variant actinin-4 protein expression [67% (53/79)] than in cases that were negative [43% (39/91)] ($P = 0.0020$, Fisher’s exact test) (Table 2). The sites of first recurrence in patients with HGNTs that were positive for variant actinin-4 expression included the brain (16 cases), lymph nodes (16 cases), lung (9 cases), liver (6 cases), and bone (5 cases), but the site distribution did not differ significantly from that in negative cases.

The overall survival of patients with variant actinin-4-positive HGNT, SCLC and LCNEC was significantly worse than that of patients whose tumours were negative [$P = 0.0021$ (HGNT, Figure 3A), 0.0283 (SCLC, supplementary Figure S3A, available at *Annals of Oncology* online) and 0.0022 (LCNEC, supplementary Figure S3B, available at *Annals of Oncology* online), log-rank test]. Furthermore, progression-free survival also differed significantly between patients whose tumours were positive and negative for variant actinin-4 expression [$P = 0.0021$ (HGNT, Figure 3B), 0.018 (SCLC, supplementary Figure S3C, available at *Annals of Oncology* online) and 0.048 (LCNEC, supplementary Figure S3D, available at *Annals of Oncology* online), log-rank test]. The 5-year survival rates of patients with variant actinin-4-negative HGNT, SCLC and LCNEC were 62%, 62% and 62%, respectively, whereas those of

**Table 1.** Expression of variant actinin-4 and three NE markers in various histological subtypes of lung cancer

<table>
<thead>
<tr>
<th>Histological subtype</th>
<th>Variant actinin-4</th>
<th>CGA (%)</th>
<th>SYN (%)</th>
<th>CD56 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td>70</td>
<td>42 (60%)</td>
<td>59 (88%)</td>
<td>58 (88%)</td>
</tr>
<tr>
<td>LCNEC</td>
<td>106</td>
<td>54 (51%)</td>
<td>59 (56%)</td>
<td>59 (56%)</td>
</tr>
<tr>
<td>Carcinoid</td>
<td>51</td>
<td>5 (10%)</td>
<td>49 (99%)</td>
<td>49 (100%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>164</td>
<td>1 (1%)</td>
<td>16 (11%)</td>
<td>6 (4%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>166</td>
<td>2 (1%)</td>
<td>23 (14%)</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>Others</td>
<td>52</td>
<td>0 (0%)</td>
<td>9 (17%)</td>
<td>5 (10%)</td>
</tr>
</tbody>
</table>

SCLC, small cell lung carcinoma; LCNEC, large cell neuroendocrine carcinoma; NE, neuroendocrine; CGA, chromogranin A; SYN, synaptophysin; IARC, International Agency for Research on Cancer; WHO, World Health Organization.

*B based on the Pathology and Genetics of Tumours of the Lung, Thymus and Heart (IARC WHO Classification of tumours) (third version, 2004).
patients whose tumours were positive were 42%, 32% and 49%, respectively. The prognostic significance of variant actinin-4 protein expression was reproducibly observed in subgroup analyses of patients with stages I and II and stages III and IV HGNT ($P = 0.0089$ and $P = 0.049$, respectively) (Figure 3C and D), whereas the expression of total actinin-4 proteins (detected by 13G9 monoclonal antibody) (supplementary Figure S4, available at *Annals of Oncology* online) or the three conventional NE markers (CGA, SYN and CD56) (supplementary Figure S5, available at *Annals of Oncology* online) did not show such prognostic significance.

Univariate analysis with the Cox proportional hazards model (Table 3) revealed that lymph node metastasis ($P = 0.000082$) and immunoreactivity for the actinin-4 splice variant ($P = 0.000467$) were significantly correlated with the outcome of the 173 patients with HGNTs. Multivariate analysis indicated that the expression of the actinin-4 splice variant was the most significant independent predictor of unfavourable outcome ($P = 0.00113$; hazard ratio (HR), 2.15; 95% confidence interval (CI) 1.36–3.40) after the presence of lymph node metastasis ($P = 0.00023$; HR, 2.25; 95% CI 1.46–3.47). The variant actinin-4 protein expression was also a significant independent predictor of outcome for SCLCs (supplementary Table S2, available at *Annals of Oncology* online) ($P = 0.050$; HR, 2.16; 95% CI 1.00–4.68) and LCNECs (supplementary Table S3, available at *Annals of Oncology* online) ($P = 0.0038$; HR, 2.37; 95% CI 1.32–4.26).

**discussion**

In this study, we first determined the distribution of expression of the alternatively spliced *ACTN4* transcript in a large panel of human cancer cell lines derived from various tissues. We confirmed the frequent expression of *ACTN*-Va in SCLCs and the absence of its expression in NSCLCs. The variant transcript was also expressed frequently in choriocarcinoma, but its relationship with NE differentiation as well as its clinical significance remained undetermined. We then investigated the expression of the variant actinin-4 protein in a variety of histological subtypes of lung cancer using a newly established monoclonal antibody (Figure 1). We found that the expression pattern of the variant protein was apparently different from any of the conventional NE markers. CGA, SYN and CD56 (supplementary Figure S4, available at *Annals of Oncology* online) did not show such prognostic significance.

Univariate analysis with the Cox proportional hazards model (Table 3) revealed that lymph node metastasis ($P = 0.000082$) and immunoreactivity for the actinin-4 splice variant ($P = 0.000467$) were significantly correlated with the outcome of the 173 patients with HGNTs. Multivariate analysis indicated that the expression of the actinin-4 splice variant was the most significant independent predictor of unfavourable outcome ($P = 0.00113$; hazard ratio (HR), 2.15; 95% confidence interval (CI) 1.36–3.40) after the presence of lymph node metastasis ($P = 0.00023$; HR, 2.25; 95% CI 1.46–3.47). The variant actinin-4 protein expression was also a significant independent predictor of outcome for SCLCs (supplementary Table S2, available at *Annals of Oncology* online) ($P = 0.050$; HR, 2.16; 95% CI 1.00–4.68) and LCNECs (supplementary Table S3, available at *Annals of Oncology* online) ($P = 0.0038$; HR, 2.37; 95% CI 1.32–4.26).
Three patients with LCNEC, for whom no data regarding adjuvant therapy were available, were excluded from the analysis.

Variant actinin-4 protein expression is not a simple marker for NE differentiation, but seems to be associated with the malignant progression of NE tumours.

We originally identified actinin-4 as an actin-bundling protein associated with enhanced cell motility and cancer invasion [9]. Actinin-4 directly regulates cell motility through remodelling of the actin cytoskeleton [9, 22]. Increased expression of actinin-4 protein is closely associated with a poor outcome in patients with breast cancer [9], colorectal cancer [22], pancreatic cancer [18], ovarian cancer [19] and NSCLC [23]. However, the expression of total actinin-4 protein (i.e. the ubiquitous and variant actinin-4 proteins together) detected by the monoclonal antibody 13G9 was not significantly correlated with the outcome of HGNT patients (supplementary Figure S4, available at Annals of Oncology online). The amino acid sequence encoded by exon 8 is crucial for the function of the ACTN4 gene. In fact, a germ-line missense mutation in exon 8 is responsible for a hereditary renal disease, familial focal segmental glomerulosclerosis [24]. The splice variant as well as mutated actinin-4 proteins have a higher affinity for actin polymers [10]. SCLC shows an abnormal actin cytoskeleton structure [10]. Existing experimental data as well as the current clinical observations suggest that variant actinin-4 very likely plays a functional role in the aggressive behaviour of NE lung tumours. Because of its limited expression in normal tissues, variant actinin-4 may also serve as a therapeutic target.

If a biomarker capable of defining a subset of HGNT patients whose prognosis is likely to be unfavourable were to be identified, allowing them to be selected for intense adjuvant chemotherapy, then their survival might be improved. Recently Klotho was newly characterized as a biomarker predictive of a favourable outcome in patients with LCNEC [25] and limited-disease SCLC [26], although the number of cases examined was relatively small. The expression of CD117 was also reported to show marginally significant correlation with recurrence of LCNEC (P = 0.046) [27]. It is expected that combination of these emerging prognostic biomarkers with variant actinin-4 would further improve the accuracy of HNGT prognostication. Future investigation to select and validate an optimal biomarker set is anticipated.

**funding**

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences conducted by the National Institute of Biomedical Innovation of Japan, Research on Biological Markers for New Drug Development conducted by the Ministry of Health, Labor and Welfare of Japan and the National Cancer Center Research and Development Fund.

**disclosure**

The authors have declared no conflicts of interest.

**references**

Phase Ib safety and pharmacokinetic study of volociximab, an anti-α5β1 integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer


*Correspondence to: Dr B. Besse, Department of Oncology Medicine/Thoracic Unit, Institut Gustave Roussy, Villejuif Cedex 94805, France. Tel: +33-1-42114322; Fax: +33-1-42-11-52-19; E-mail: benjamin.besse@igr.fr

© The Author 2012. Published by Oxford University Press on behalf of the European Society for Medical Oncology. All rights reserved. For permissions, please email: journals.permissions@oup.com.