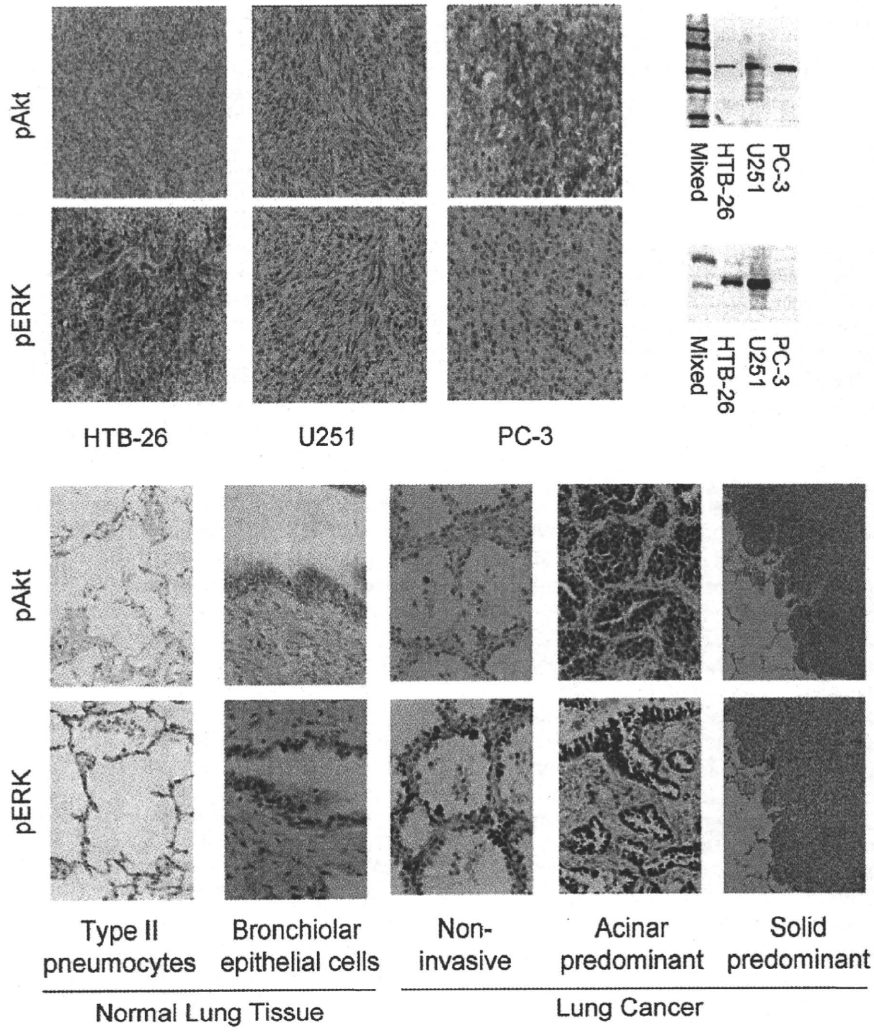

Fig. 1. Immunohistochemical staining of xenografts with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). (a) HTB-26; xenograft of breast cancer cell negative for pAkt and positive for pERK1/2; (b) U251; xenograft of glioma cell moderately positive for both pAkt and pERK1/2; (c) PC-3; xenograft of prostate cancer cell positive for pAkt and negative for pERK1/2. Almost all the cells with mitotic figure were scattered and were positive for pERK1/2. Immunohistochemical staining of resected lung specimen with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). Normal lung tissues (left side) and lung adenocarcinoma of noninvasive, acinar predominant and solid predominant histology (the right side). See that pAkt is almost homogeneously stained compared to that of pERK1/2.

immunohistochemical parameter were calculated and p-values for the statistical significance were given by a two-tails test checking a null hypothesis about zero Pearson's correlation coefficient between two variables.

Two-sided p-value below 0.05 was designated statistically significant.

3. Results

Patient characteristics of the 193 cases and differences in survival according to each clinicopathological factor or protein expression were shown in Table 1.

3.1. Patients and pathological review

The gender distribution was equal, the median age was 63 years and the median follow-up period was 2066 days (5.66 years) (ranged 133–3292 days). Pathological review revealed that more than 80% of the cases were classified as the adenocarcinoma with mixed subtype according to the current WHO classification. Therefore results based on the predominance classification together with invasive/noninvasive dichotomy were presented here. 22.8% ($n=44$) of the cases were classified as pre- and minimally invasive adenocarcinomas. The rest (77.2%; $n=149$) were invasive adenocarcinoma in which papillary (including micropapillary), acinar, solid patterns or patterns of other variants are predominantly recognized. The rates were 61.1% ($n=118$), 9.9% ($n=19$) and 6.2% ($n=12$), respectively.

3.2. Immunohistochemical study and the EGFR/KRAS mutation status

We observed rather homogeneous and tumor-specific staining patterns for pAkt although the intensity was low, 37.8% (73/193) of surgically excised lung adenocarcinomas being positive. The positive rates for pGSK3B, pmTOR, pS6K, pFKHR and TTF-1 were 30.6% ($n=59$), 34.7% ($n=68$), 52.3% ($n=101$), 40.4% ($n=78$) and 79.8% ($n=154$), respectively. The pERK staining pattern was characteristically heterogeneous. The rate of positive tumor cells ranging from 5% to 100% and the staining was not tumor-cell specific but rather

Table 1
Characteristics of the patients ($n = 193$) and differences* in survival rate according to each factor.

	N	5-Year survival (%)	95% CI	p-Value *
Patient and tumor characteristics				
Gender				
Male	96	71.6	0.61–0.80	0.027
Female	97	85.3	0.76–0.91	
Age				
<60	74	81.4	0.70–0.89	0.274
60 or older	119	76.5	0.67–0.83	
Smoking habit				
Never	89	83.2	0.73–0.90	0.151
Ever	104	74.3	0.64–0.82	
Stage				
I	120	94.8	0.89–0.98	<0.001
II–IV	73	48.7	0.36–0.61	
Adenocarcinoma classification				
Pre + minimally invasive**	44	96.5	0.83–0.99	<0.001
Invasive**	149	72.9	0.65–0.80	
Expression status (IHC study)				
pAkt				
–	120	85.3	0.77–0.91	0.007
+	73	68.2	0.56–0.79	
pERK				
–	124	73.8	0.65–0.81	0.058
+	69	86.4	0.76–0.93	
pGSK3B				
–	134	76.7	0.68–0.83	0.289
+	59	82.0	0.69–0.90	
pmTOR				
–	125	75.7	0.77–0.83	0.214
+	68	83.1	0.71–0.90	
pS6K				
–	92	74.1	0.63–0.82	0.260
+	101	82.3	0.73–0.89	
pFKHR				
–	115	85.6	0.77–0.91	0.006
+	78	68.1	0.56–0.77	
TTF-1				
–	39	49.4	0.32–0.65	<0.001
+	154	85.5	0.79–0.90	
Mutation status ($n = 93$)				
EGFR mutation				
–	39	69.2	0.52–0.81	0.175
+	54	84.8	0.72–0.92	
KRAS mutation				
–	86	78.7	0.68–0.86	0.774
+	7	71.4	0.26–0.92	

* Log-rank test (p-value); CI: confidence interval; N: lymph node metastasis.
** Pre + minimally invasive: adenocarcinoma in situ + lepidic pattern predominant adenocarcinoma with minimal invasion (<10% or ≤5 mm invasion); Invasive: adenocarcinoma of papillary (including micropapillary), acinar or solid pattern predominant and other variants; IHC: immunohistochemistry; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

ubiquitous. The contrast between positive and negative cells was excellent. With the 10% cutoff, 35.8% (69/193) of the tumors were positive for pERK (Fig. 1).

The results of mutation analysis of exons 18–21 of *EGFR* and codons 12, 13 and 61 of *KRAS* are detailed in Suppl. Table 2. *EGFR* mutations were detected in 54 cases (58.1%), among which 90% were in exons 19 and 21. Mutations of *KRAS* were seen in seven cases (7/93, 7.5%), all of which were at codon 12. The *EGFR* and *KRAS* mutations were mutually exclusive except in one case. Of note, both pAkt and pERK were strongly stained in this case with double mutation of *EGFR* and *KRAS*. Types of *EGFR* mutation did not appear to affect the pattern of pathway activation. All but one of the cases with *KRAS* mutation were strongly positive for pERK ($p = 0.009$, Table 4), whereas the *EGFR* mutation did not correlate to pERK ($p = 0.294$) nor pAkt ($p = 0.409$) expression.

3.3. Patient survival

By univariate analyses using log-rank test, gender ($p = 0.027$), stage ($p < 0.001$), adenocarcinoma classification (invasive or not) ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and cytoplasmic staining of pAkt ($p = 0.007$) and pFKHR ($p = 0.006$) were significantly related

Table 2
Multivariate analysis for factors predicting poor prognostic outcome ($n = 193$).

Variable	Relative risk	95% CI	p-Value
Cox regression analysis			
Patient and tumor characteristics			
Female	0.48911	0.25–0.93	0.030
Age (60 or older)	1.43993	0.75–2.78	0.268
Ex or current smoker	1.58799	0.84–3.00	0.147
Stages II–IV	10.96302	5.02–23.92	<0.001
Adenocarcinoma classification (invasive*)	1.81110	1.05–2.73	0.030
Expression status (IHC study)			
TTF-1+	0.24379	0.13–0.45	<0.001
pAkt+	2.28909	1.22–4.29	0.009
pERK+	0.50813	0.25–1.04	0.051
pGSK3B+	0.68778	0.34–1.40	0.289
pmTOR+	0.64731	0.32–1.29	0.204
pS6K+	0.70277	0.38–1.30	0.260
pFKHR+	2.34981	1.26–4.38	0.007
Mutation status ($n = 93$)			
EGFR mutation	0.56279	0.24–1.31	0.180
KRAS mutation	1.23578	0.29–5.29	0.780
Cox regression analysis with step wise selection			
Female	0.54	0.27–1.06	0.074
Stages II–IV	10.542	4.61–24.1	<0.001
pAkt+	2.268	1.17–4.38	0.015
pFKHR+	1.812	0.95–3.48	0.073
TTF-1+	0.282	0.15–0.54	<0.001

* CI: confidence interval; invasive: adenocarcinoma with a frankly invasive region; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

to survival (Table 1). Multivariate analysis using the Cox's proportional hazard model revealed that stage ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and pAkt ($p = 0.015$) are statistically significant factors for prognosis independent of any other conditions (Table 2).

3.4. Clinicopathological backgrounds of adenocarcinomas with Akt activation or TTF-1 expression

Since pAkt and TTF-1 expression was revealed to be independent prognostic factors, relationships between their positive status and other factors including clinicopathological characteristics and expression of other phosphorylated proteins are summarized in Table 3. pAkt expression was significantly associated with advanced stage (stage II-IV, $p = 0.021$) and lymph node metastasis ($p = 0.002$) but not with expression of any other signal proteins or TTF-1. TTF-1 expression, on the contrary, was significantly associated with never-smoker status ($p = 0.013$) and pre- or minimally invasive nature ($p < 0.001$) as well as with expression of pERK ($p = 0.039$) and pmTOR ($p = 0.014$). Also, TTF-1 expression was related to *EGFR* mutation ($p = 0.017$). Regarding invasiveness of tumor, the pAkt activation frequency tended to be lower in the pre- and minimally invasive adenocarcinomas (18.2%; 8/44) as compared with invasive types (43.6%; 65/149) and this tendency was highly and statistically significant ($p = 0.004$). In contrast, TTF-1 staining was more frequently seen among the pre- and minimally invasive adenocarcinomas ($p < 0.001$).

Correlation coefficients between each factor were shown in Table 4. The general tendency was largely in concert with results of survival analysis (Tables 1 and 2) and expression of Akt and TTF-1. Interestingly, activation of signal proteins of ERK had similar and statistically (or marginally) significant correlation to non-smoking ($p = 0.035$), stage I ($p = 0.029$) and noninvasive status ($p = 0.001$). GSK3B expression was related significantly to activation of ERK ($p = 0.001$), mTOR ($p < 0.001$) and S6K ($p = 0.018$). *EGFR* mutations were significantly associated with female gender ($p = 0.031$), non-smokers ($p = 0.016$), TTF-1 expression ($p = 0.008$) and pS6K expression ($p = 0.008$).

4. Discussion

By IHC applied to primary adenocarcinoma tissues and mouse xenografts of cell lines used as controls, we succeeded in demonstrating Akt activation to be an independent marker of poor prognosis. In addition, TTF-1, which is known to be a marker of type II cell differentiation, was also proved to be a significant favorable prognostic marker in correlation with activation of ERK and mTOR or *EGFR* mutation regardless of tumor stages. The expression of TTF-1 is important in outcome estimation of lung carcinoma at any tumor stage whereas Akt activation is abnormally affected according to the aggressiveness of the tumors regardless of their cell origin. While *EGFR* mutations had no correlation to activation of Akt or ERK pathways, six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors. Finally, FKHR expression was established to be a marker for poor prognosis.

This study demonstrated that activated Akt was associated independently and significantly with poor prognosis ($p = 0.015$), which was in agreement with previous studies [14–19]. In fact, pAkt expression correlated with factors such as stages II-IV, positive lymph node metastasis and the invasive histology (Table 3), suggesting that Akt activation is an acquired characteristic according to tumor aggressiveness. To cast light on actual regulators and substrates of Akt that might mediate regulatory mechanisms in primary lung adenocarcinoma, we compared the activation status of Akt with upper and downstream components of the signal transduction. However our results somewhat differed from those obtained earlier with cell lines. For example, *EGFR* mutations in NSCLC cell lines were reported to selectively activate the Akt pathway [3], but in our primary tumors, this correlation between Akt and constitutively activated RTK was not significant ($p = 0.409$). Moreover, in our study, expression of pAkt did not correlate with activation of any of the downstream signal proteins such as GSK3B, mTOR, S6K and FKHR in the burgeoning list of Akt substrates implicated in oncogenesis [20,21]. Linkage of Akt with those activated downstream signal proteins has been confirmed in vitro, but the results in clinical samples have been inconsistent [18,22–24]. Certainly, Akt activation is an important factor for development and proliferation of cancer cells and perhaps a marker for targeted therapies, the exploration of this area especially in real tumors has definitely been inadequate and further study is needed.

Our multivariate analysis revealed that TTF-1 expression was also a statistically significant and independent prognostic factor ($p < 0.001$). TTF-1 is a regulator of normal lung development or maintenance of type II pneumocytes [25] and was expressed in 79.8% (154/193) of the cases. Recently, a model of lineage specific dependency on TTF-1 in a subset of adenocarcinoma, that is, the terminal respiratory unit (TRU) type adenocarcinoma, has been proposed [26]. In our study, TTF-1 staining was correlated with never-smoker ($p = 0.013$) and *EGFR* mutation status ($p = 0.039$) independently of tumor stage, which is consistent with the concept of lineage specific tumorigenesis in this subset of lung adenocarcinoma. These insights suggest the importance of the cell lineage that tumors were derived from in outcome estimation of lung carcinoma.

ERK was activated in 35.8% (69/193) of the cases in our study and was associated with pathologically early stages, the pre or minimally invasiveness and TTF-1 expression ($p = 0.039$). Normal tissues, such as type II pneumocytes or interstitial fibroblasts, were also positive with varying intensity (Fig. 1). Generally, ERK activation is known not only as the result of oncogenic dysregulation

Table 3
Backgrounds of patients with expression of a significant prognostic factors; pAkt and TTF-1 ($n = 193$).

	No. of cases with expression of pAkt and TTF-1 (%)				
	Total	pAkt	p-Value*	TTF-1	p-Value*
	193	73 (37.8)		154 (79.8)	
Patient and tumor characteristics					
Gender					
Male	96	33 (35.1)	0.371	73 (76.0)	0.214
Female	97	40 (42.1)		81 (83.5)	
Age					
<60	74	30 (40.5)	0.760	62 (83.8)	0.357
60 or over	119	43 (37.4)		92 (77.3)	
Smoking habit					
Never	89	37 (42.0)	0.374	78 (87.6)	0.013
Ever	104	36 (35.6)		76 (73.1)	
Stage					
I	120	37 (31.9)	0.021	99 (82.5)	0.269
II–IV	73	36 (49.3)		55 (75.3)	
N					
–	135	41 (31.3)	0.002	112 (83.0)	0.118
+	57	32 (56.1)		42 (73.7)	
Adenocarcinoma classification**					
Pre + minimally invasive	44	8 (18.2)	0.004***	44 (100.0)	<0.001
Invasive	149	65 (43.6)		110 (73.8)	
Pap-pred	118	52 (44.1)		91 (77.1)	
Acinar-pred	19	9 (47.4)		14 (73.7)	
Solid-pred and other variants	12	4 (33.3)		5 (41.7)	
Other IHC results					
pAkt					
–	120			94 (78.3)	0.851
+	73			58 (79.5)	
pERK					
–	124	51 (41.8)	0.275	93 (75.0)	0.039
+	69	22 (32.8)		61 (88.4)	
pGSK3B					
–	134	48 (36.6)	0.421	103 (76.9)	0.331
+	59	25 (43.1)		50 (84.7)	
pmTOR					
–	125	49 (39.2)	0.754	93 (74.4)	0.014
+	68	24 (35.3)		61 (89.7)	
pS6K					
–	92	39 (42.4)	0.370	73 (79.3)	1.000
+	101	34 (33.7)		81 (80.2)	
pFKHR					
–	115	39 (33.9)	0.879	87 (75.7)	0.101
+	78	34 (43.6)		67 (85.9)	
TTF-1					
–	39	15 (38.4)	0.851		
+	154	58 (37.7)			
Mutation status ($n = 93$)					
EGFR mutation					
–	39	16 (41.0)	0.409	26 (66.7)	0.017
+	54	27 (50.0)		48 (88.9)	
KRAS mutation					
–	86	41 (47.7)	0.445	70 (81.4)	0.148
+	7	2 (28.6)		4 (57.1)	

* Results of Fisher's exact test; pAkt: phosphorylated Akt; TTF-1: thyroid transcription factor-1; N; lymph node metastasis.

** Adenocarcinoma classification; see text for details; -pred: predominant; Pap: papillary pattern including micropapillary pattern; Acinar: acinar pattern; Solid: solid with mucin formation pattern.

*** Comparison between "Preinvasive + minimally invasive" vs other "invasive" carcinoma; IHC: immunohistochemistry; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

Table 4
Correlation analysis of clinicopathological data, expression of phosphoryated proteins and mutation status.

N=193	Gender [Female]	Age [60≦]	Smoking habit [Ever]	Stage [II-IV]	Adenocarcinoma Classification [Invasive]	TTF-1 [+]	pAkt [+]	pERK [+]	pGSK3B [+]	pmTOR [+]	pS6K [+]	pFKHR [+]	*EGFR* mutation
Age [60≦]	-0.049	1											
Smoking habit [Ever]	-0.630 (<0.001)	0.011	1										
Stage [II-IV]	-0.063	-0.028	0.079	1									
Adenocarcinoma Classification [Invasive]	-0.021	0.058	-0.191	0.252 (<0.001)	1								
TTF-1 [+]	0.110	-0.058	-0.191 (0.009)	-0.087	-0.153 (0.039)	1							
pAkt [+]	0.083	-0.048	-0.069	0.185 (0.012)	0.107	-0.041	1						
pERK [+]	0.107	0.138	-0.156 (0.035)	-0.161 (0.029)	-0.235 (0.001)	0.178 (0.016)	-0.006	1					
pGSK3B [+]	0.098	-0.014	-0.119	-0.083	0.008	0.073	0.064	0.250 (<0.001)	1				
pmTOR [+]	0.164 (0.026)	-0.107	-0.213 (0.004)	-0.011	-0.160 (0.003)	0.203 (0.006)	-0.036	0.246 (<0.001)	0.234 (0.001)	1			
pS6K [+]	0.279 (<0.001)	0.063	-0.257 (<0.001)	-0.086	-0.134 (0.072)	0.054	-0.049	0.175 (0.018)	0.241 (0.001)	0.233 (0.001)	1		
pFKHR [+]	-0.029	-0.048	0.087	0.140	0.261 (<0.001)	-0.099	0.020	-0.138	0.040	-0.036	-0.096	1	
N=93													
EGFR mutation	0.224 (0.031)	0.040	-0.249 (0.016)	0 (1.000)	0.063	0.272 (0.008)	0.089	0.112	0.111	-0.005	0.274 (0.008)	-0.181	1
KRAS mutation	0.032	-0.024	0.125	-0.115	-0.012	-0.159	-0.101	0.268 (0.009)	0.051	0.095	0.044	0.072	-0.253 (0.014)

Correlation coefficients were calculated assuming the conditions described in the parenthesis [ ] to be observed. For abbreviations, see text. Coefficients values highlighted by underline imply statistical significance. Numbers in ( ) are *p*-values. For details of invasiveness, see text. Darkly shadowed cells imply significantly positive correlation and weakly shadowed cells imply significantly (or marginally) negative correlation. TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK; phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

but also as an essential component of epithelial cell development or adaptation to changing circumstances [27–29]. Moreover, ERK regulation depends on very complex mechanisms, involving several intracellular parameters [30], timing or balance of the signals [31–34] and other unknown factors [35]. Considering these arguments, we may conjecture that ERK activation in lung adenocarcinoma mostly reflects the intracellular signal transduction of normal cells, which is still preserved within early-staged adenocarcinoma especially with TTF-1 expression, and that the ERK pathway is gradually switched off as the tumor cells progress to a more malignant phenotype. Also, these results shown here is in line with the recent studies of American cases [36], where the ERK pathway was more activated in earlier stages and the Akt pathway in advanced stages. In further studies, considering significant heterogeneity of ERK activation, we may use more detailed judgment criteria for immunoreactivity and whole sections of tumor, rather than tissue microarrays.

Six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors, consistent with previous reports [37,38]. It is notable that the clinical impact of such *KRAS* mutation-induced ERK activation is enormous since it has already been shown in mice models that tumors with ERK activation due to *KRAS* or *BRAF* mutations can be successfully treated by an inhibitor of MEK, a signal protein upstream of ERK [39]. Our results imply so far the presence of at least two causes for ERK activation in lung adenocarcinoma, one is the vestige of normal intracellular signal and the other is the impact of *KRAS* mutation.

Two other supplementary implications were obtained from this study. Among downstream proteins of the Akt pathway, only S6K was significantly expressed in cases with the *EGFR* mutation ($p = 0.008$) and may potentially be an alternative marker for *EGFR* mutation. S6K is known to regulate ribosomal biogenesis and to play an important role in progression of G1 phase of the cell cycle [41,42]. This correlation between *EGFR* and S6K suggests again a cross talk between the Akt and ERK pathways and similar result was previously described by Conde et al. [40]. Our results also indicated cytoplasmic localization without intranuclear accumulation of pFKHR protein to be an adverse prognostic factor. FKHR is a member of a transcription factor family and represents a mammalian counterpart of DAF16, first identified at chromosomal breakpoints in human tumors [20]. Subcellular localization of FKHR is known to play an important role by regulating cell cycle and apoptosis in normal cell, which is consistent with our result. Further accumulation of cases will be needed to confirm those possibilities.

As a reference for IHC evaluation we here used mouse xenografts selected from a cell line panel repeatedly used in drug research [12,43]. This resulted in more accurate and reproducible evaluation of protein expression, implying the usefulness of tumor xenografts for clinical researches.

Conflict of interest statement

Kengo Takeuchi is a consultant for DAKO.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.01.001.

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Correspondence

EML4-ALK Fusion in Lung

To the Editor-in-Chief:

The recent article by Martelli et al[1] reports (i) the detection of *EML4-ALK* fusion cDNA[2] not only in non-small cell lung cancer (NSCLC) specimens but in non-tumor lung tissues, (ii) a very low proportion of FISH-positive cells for *ALK* rearrangements among *EML4-ALK*–positive specimens, and (iii) the failure to detect EML4-ALK protein by immunohistochemistry (IHC) and Western blotting. Based on these lines of observation, the authors questioned the clinical relevance of *EML4-ALK* in the carcinogenesis of NSCLC.

Although detection of fusion kinases in normal tissues is a potentially interesting observation, caution is warranted in the interpretation of their results.[1,3] They replicated thrice the reverse transcription-polymerase chain reaction (RT-PCR) for *EML4-ALK* and noted that "In half of the (positive) cases, one replicate experiment did not confirm the fusion transcript was present." They then suggested that the fusion gene was "expressed at very low level." It is, however, also quite possible that such unstable PCR results may simply represent contaminated experiments. If this is the case, a discussion on FISH and protein analyses would become irrelevant. In their report, the presence of the *EML4-ALK* fusion gene was only evidenced by unstable RT-PCR results and a small proportion of FISH-positive cells among specimens.

In this regard, it was surprising that the authors had not tried genomic PCR to exclude the possibility of PCR contamination.[1,3] In most of their fusion-positive cases, they found the *EML4-ALK* variant 1 cDNA, in which exon 13 of *EML4* cDNA is connected to exon 20 of *ALK* cDNA. Because the length of intron 14 of *EML4* gene and intron 19 of *ALK* gene is 5724 bp and 1932 bp, respectively, the maximum size of the genomic PCR to detect the gene fusion should be ≈7.7 kbp, which is within the scope of current long-range PCR systems. Indeed, we have been able to detect genomic PCR products among >50% of the fusion cDNA-positive cases. Interestingly, the break/fusion points in the genome vary substantially among NSCLC specimens,[2,4,5] and we have not obtained, to date, any pairs of NSCLC specimens carrying identical break/fusion points in their genome (even among those positive for the same *EML4-ALK* variants).

We speculate, therefore, that (i) if none of the fusion cDNA-positive cases reported by Martelli et al[1,3] produce specific genomic PCR products, then the fusion cDNA products likely arose from cDNA-contamination, (ii) if the fusion cDNA–positive cases yield identical genomic PCR products, then the fusion cDNAs likely arose from specimen-contamination, and (iii) if the fusion cDNA-positive cases display distinct genomic fusion points, then each specimen was truly positive for the *EML4-ALK* fusion gene. Without such careful examination, we have to conclude that their claims in the article have not as yet been clearly demonstrated.

As described previously,[6] immunohistochemical detection of the EML4-ALK protein is highly difficult, probably owing to the weak activity of the *EML4* promoter that drives the expression of *EML4-ALK* messages. We have thus examined the suitability of commercially available antibodies to ALK for IHC and successfully developed the intercalated antibody–enhanced polymer (iAEP) method, which enables reliable detection of EML4-ALK among formalin-fixed and paraffin-embedded specimens.[6] The same specimen positive for *EML4-ALK* RT-PCR can be, for instance, readily stained to be positive with iAEP, but negative with conventional IHC methods (see Supplemental Figure S1 in Ref. 6). We thus agree with Martelli et al that screening of NSCLC specimens with conventional IHC methods will not detect EML4-ALK protein, but strongly argue that such failure does not simply indicate the absence of EML4-ALK. For such screening, we recommend iAEP or other sensitive techniques.[7]

It should be further noted that, in both our[6] and other researchers' IHC analyses,[7] almost all tumor cells in a given *EML4-ALK*–positive specimen were positively immunostained with anti-ALK antibodies, suggesting a homogenous presence of *EML4-ALK* within a tumor. Such observation is, however, in contrast to the FISH data by Martelli et al, which show that the *ALK* rearrangement was only positive in ≈2% of tumor cells in a given *EML4-ALK*–positive specimen. On the contrary, FISH analyses of our *EML4-ALK*–positive samples clearly demonstrate that most of the tumor cells harbor rearranged *ALK* alleles, implying that the generation of the *EML4-ALK* fusion gene is an early event in NSCLC carcinogenesis. The homogenous presence of EML4-ALK in our fusion-positive tumors, as demonstrated by both FISH and IHC, further raises a concern about the "EML4-ALK–positive tumors" as defined by Martelli et al.

Specific inhibitors to ALK enzymatic activity are already in clinical trial, as reported at the 2009 annual meeting of America Society of Clinical Oncology and the European Cancer Organization and Congress of the European Society for Medical Oncology.[8] Such reports reveal only

modest and transient side effects (nausea, vomiting, and diarrhea) with their ALK inhibitor, but without severe damage in hematopoiesis or renal function. On the other hand, the marked therapeutic efficacy of their compound against EML4-ALK–positive NSCLC makes it one of the rare, highly successful molecular targeted therapies against human cancer, in line with imatinib mesylate and gefitinib/erlotinib. These data further reinforce the essential role of EML4-ALK in the carcinogenesis of NSCLC, and question the validity of the conclusions led by Martelli et al.[1,3]

Hiroyuki Mano

The University of Tokyo
Tokyo, Japan

Kengo Takeuchi

The Cancer Institute
Tokyo, Japan

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Authors' reply:

In their letter, Mano and Takeuchi claim that our unstable PCR results in normal and cancerous lung tissues could be attribtuable to contamination. However, as clearly illustrated in our article,[1] serial dilution experiments in the H2228 cell line demonstrate the specificity and sensitivity of our RT-PCR assay. Furthermore, the identification in our EML4-ALK fusion positive tissues of alternative isoforms of variant 3, rather than the described two isoforms coexpressed in the H2228 cell line, is indicative of exclusive events in tumors, making contamination unlikely. Lastly, our experiments were confirmed independently in two laboratories (Milan and Barcelona) and always contained appropriate negative PCR controls.

We disagree with Mano et al's claim that the results of genomic PCR could be used to prove a possible RT-PCR contamination in our samples, which can only be excluded by the use of appropriate controls and procedures, as outlined above. However, we used genomic PCR to amplify the sequence flanking the *EML4-ALK* variant 1 breakpoint in four positive NSCLC samples. Even though a strong amplification product had been obtained from the same DNA templates using primer sets amplifying a control genomic locus of similar size to that of the cases so far reported in literature, no amplification of the *EML4-ALK* variant 1 fusion product was identified, suggesting only a minority of cells carried the *EML4-ALK* gene. These findings concur with Maes et al[2] who reported that, in lymphoid tissues, high level detection of *NPM-ALK* and *ATIC-ALK* fusion transcripts coincided with *ALK* gene rearrangements (as detected by cytogenetics and FISH), whereas low-level detection was not supported by genomic evidence of rearrangements.

In our article,[1] we clearly stated that, unlike observations in ALK+ lymphomas, tumor cells from NSCLC specimens expressed such a low amount of the EML4-ALK fusion protein that immunoprecipitation and immunohistochemistry performed with the commercially available antibodies are unable to detect it. This is in keeping with the observation that the EML4-ALK fusion protein is detectable only using highly sensitive methods, such as mass spectrometry[3] or the intercalated antibody-enhanced polymer (iAEP) method[4] which, unfortunately, are not available in all pathology laboratories and are difficult to standardize. Therefore, the question of how best to detect the EML4-ALK fusion protein remains unanswered.

Issues concerning the frequency, heterogeneity, and tissue specificity of the *EML4-ALK* rearrangement must also be addressed carefully.

Frequency

We recently extended our FISH analysis to 173 surgically resected lung cancer specimens (mainly adenocarcinoma) from an unselected group of Caucasian patients. The incidence of truly positive cases (>50% FISH positive, fusion transcript, and protein positive) was only 0.6% (1/173 cases), which reinforces the results in our artile and is in keeping with Rodig et al's[5] recent report of 1/227 (0.45%) ALK rearranged case in a series of surgically treated Western adenocarcinoma.

Heterogeneity

The heterogeneity of the *EML4-ALK* rearrangement we detected by FISH was confirmed by others in primary tumors and cell lines[6,7] and is supported by functional studies showing that the magnitude of growth inhibition by siRNA-

mediated silencing did not correlate with the number of cells harboring the rearrangement and the lack of growth inhibition in 50% of *EML4-ALK*–positive cell lines. These observations suggest that additional signaling mechanisms independent of ALK may regulate growth and cell proliferation.

Specificity

Claims from Mano's group that the *EML4-ALK* product is specific for NSCLC is contradicted by our findings in normal tissues[1,8] and by a recent study from Lin E. et al,[6] who found *EML4-ALK* fusions in breast (2.4%) and colorectal (2.4%) cancer, in addition to NSCLC.

Finally, we wonder whether it is really appropriate to compare treatments such as ALK inhibitors in NSCLC with imatinib mesylate and gefinitinib/erlotinib in other human neoplasms. In fact: i) the role of EML4-ALK in NSCLC is not as well established as that of BCR/ABL in chronic myeloid leukemia (CML); ii) NSCLC responses to ALK inhibitors[9] are not as remarkable as the CML response to imatinib mesylate; and iii) patients with NSCLC were treated with a multikinase, c-MET and ALK, inhibitor.[9] Considering that about 20% of NSCLC have MET amplification and overexpression and that MET rearrangements are homogeneous in lung cancer,[10] it may be possible that responses to the multikinase inhibitor may be related to other coexisting oncogenic events, independently of ALK.

In conclusion, although we fully acknowledge the importance of Soda et al's discovery,[11] we believe that additional studies are required to elucidate the concurrent genetic events and cellular settings necessary for *EML4-ALK* to exert an oncogenic function and to better define the role of *EML4-ALK* in diagnosis and targeted therapy of NSCLC.

Brunangelo Falini
Maria Paola Martelli

University of Perugia
Perugia, Italy

Stefano A. Pileri

University of Bologna
Bologna, Italy

Gabriella Sozzi
Patrizia Gasparini

Istituto Nazionale dei Tumori
Milan, Italy

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ORIGINAL ARTICLE

SUVmax in FDG-PET at the biopsy site correlates with the proliferation potential of tumor cells in non-Hodgkin lymphoma

REINA WATANABE[1], NAOTO TOMITA[1], KENGO TAKEUCHI[2], SEIJI SAKATA[2], UKIHIDE TATEISHI[3], MASATSUGU TANAKA[1], HIROYUKI FUJITA[1], YOSHIAKI INAYAMA[4], & YOSHIAKI ISHIGATSUBO[1]

[1]*Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, Yokohama, Japan,* [2]*Department of Pathology, The Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan, Departments of Radiology*[3] *and* [4]*Pathology, Yokohama City University Graduate School of Medicine, Yokohama, Japan*

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Abstract

The maximum standard uptake value (SUVmax) of the whole body on 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) reflects the tumor aggressiveness in non-Hodgkin lymphoma (NHL). To clarify the correlation between SUVmax at the biopsy site and the proliferation potential of tumor cells, we studied 36 patients with untreated NHL and five with untreated Hodgkin lymphoma (HL) by measuring the Ki-67 proliferation index (MIB-1 labeling index) in biopsy specimens. The measured MIB-1 labeling index was categorized into seven levels: nearly 0%, 5–20%, 21–40%, 41–60%, 61–80%, 81–95%, and nearly 100%. Twenty-four lymph nodes (LNs) and 17 extranodal (EN) sites were biopsied. The reviewed diagnosis was eight indolent lymphomas, two mantle-cell lymphomas, 26 aggressive lymphomas, and five HLs. A positive correlation was observed between the SUVmax at the biopsy site and the MIB-1 labeling index in the 36 patients with NHL ($r = 0.69$, $P < 0.001$). The correlations were also observed in LN group ($r = 0.60$, $P = 0.006$) and EN group ($r = 0.87$, $P < 0.001$), respectively. In the five patients with HL, the MIB-1 labeling index was uniformly categorized in nearly 100%. The SUVmax correlates with the proliferation potential in the case of NHL.

Keywords: *MIB-1 labeling index, non-Hodgkin lymphoma, positron emission tomography, proliferation potential, SUVmax*

Introduction

In recent years, positron emission tomography (PET), particularly with [18F] fluorodeoxyglucose (FDG), has emerged an alternative to computed tomography (CT) not only in treatment evaluation [1,2] but also in the staging of malignant lymphomas [3–6] even in cases of bone marrow involvement [7]. It enables the assessment of the extent of lymphoma with a higher sensitivity than that of CT [3–5,7].

In non-Hodgkin lymphoma (NHL), the Ki-67 proliferating index (MIB-1 labeling index) indicates the proliferation potential of tumor cells, which often affects the prognosis [8–10]. Although the correlation between the standardized uptake value (SUV) on PET and the proliferation potential of tumor cells has been reported in several tumors such as brain tumors [11], head and neck cancer [12], lung cancer [13], and bone and soft tissue tumors [14], it has barely been elucidated in the case of malignant lymphoma, which is one of the tumors that is most sensitive to chemotherapy/radiotherapy. Previous reports have described methods of distinguishing between indolent histology NHL and aggressive histology NHL [15–19]. However, it is important to know the proliferation potential and, in turn, determine the speed of tumor growth to decide the appropriate regimen of initial chemotherapy. In this study, we attempted to clarify the correlation between maximum SUV (SUVmax) at the biopsy site

Correspondence: Naoto Tomita, MD, Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Tel: +81-45-787-2800. Fax: +81-45-786-3444. E-mail: cavalier@ch-yamate.dlenet.com

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and the tumor proliferation potential using the MIB-1 labeling index in the cases of NHL, along with the cases of Hodgkin lymphoma (HL).

Materials and methods

Patients

Of 200 consecutive patients with malignant lymphoma in Yokohama City University Hospital during the period February 2001 through October 2007, 53 patients were staged by PET or PET/CT. In seven of them, pathologically evaluable specimens could not be prepared, and in other five cases, the pathological diagnosis was composite lymphoma. The remaining 41 patients (36 NHL and 5 HL) were the subjects of this study. The SUVmax on PET or PET/CT at the biopsy site was evaluated. The decision of the biopsy site was dependent on each attending physician. PET or PET/CT was not necessarily performed before the biopsy. The biopsy was not always performed at the site showing the maximum SUVmax in the whole body.

The biopsied specimens were reviewed by a hematopathologist (KT) according to the WHO classification [20], and the proliferation potential of lymphoma cells was evaluated by two pathologists (SS and KT) by measuring the MIB-1 labeling index in tumor cells. After evaluating the approximate proportion and distribution of lymphoma cells on the preparation stained by hematoxylin and eosin or immunostaining such as CD20, the MIB-1 labeling index was measured by Ki-67 immunostaining on the seriate section. The MIB-1 labeling index was measured by semiquantitative analysis and categorized into seven levels on the basis of the MIB-1 labeling grade (MG); MG-1 (nearly 0%), MG-2 (5–20%), MG-3 (21–40%), MG-4 (41–60%), MG-5 (61–80%), MG-6 (81–95%), and MG-7 (nearly 100%), respectively. The correlation between SUVmax at the biopsy site and the MIB-1 labeling index was analyzed in NHL. It was also compared between the cases biopsied from lymph nodes (LNs) and the cases done from extranodal (EN) sites. Furthermore, correlation was also analyzed for the subgroup of DLBCL. This study was approved by the ethical committee of Yokohama City University Hospital.

PET study

Studies were performed with the dedicated full ring PET (SET2400, Shimadzu, Kyoto, Japan) or LSO-based whole-body PET/CT scanner (Aquiduo, Toshiba Medical Systems, Tokyo, Japan). PET images were acquired with the following condition: field of view (FOV), 59.5 cm; body axis length, 20 cm; slice thickness, 3.125 mm; central resolution, 4.2 mm; and half width, 5.0 mm. The CT component of the PET/CT scanner was same as Aquillion 16, which has 16-rows detector. The PET component of the PET/CT scanner has a transaxial FOV of 68.3 cm and an axial FOV of 16.2 cm without septa and rotating rod source. The scanner was used in a three-dimensional mode with image resolution of 4.0 mm in full width at half maximum (FWHM). Before the PET/CT study, the patients fasted for at least 6 h. CT was performed from the head to the midthigh according to a standardized protocol with the following setting: axial 2.0-mm collimation × 16 modes; 120 kVp; Auto-Exposure Control (SD10); and a 0.5-s tube rotation, a table speed of 11.0 mm/s. Patients maintained normal shallow respiration during the acquisition of CT scans. No iodinated contrast material was administered. Emission scans from the head to the midthigh were obtained starting approximately 60 min adjusted for time decay until start of scan after the intravenous administration of 296–414 MBq of [18]F-FDG. The acquisition time for each PET was 2 min per table position. Images were reconstructed with attenuation-corrected, ordered-subset expectation maximization with two iterations and two subsets for dedicated PET or four iterations and 14 subsets using emission scans and CT data for PET/CT.

The initial review of the attenuation-corrected PET images was performed using transverse, coronal, and sagittal planes. The images were reviewed, and a diagnostic consensus was reached by two board-certified radiologists who were unaware of any clinical or radiologic information using a multimodality computer platform. Focal FDG uptake was considered to be abnormal when it was substantially greater than that of the surrounding normal tissue. A pixel region of interest (ROI) was outlined in the peak activity within regions of increased FDG uptake and measured on each slice. For quantitative interpretations, SUVmax was determined according to the standard formula, with activity in the ROI given in Bq per milliliter/injected dose in Bq per weight (kg). However, time decay correction for whole-body image acquisition was not conducted.

Statistical analysis

Mann–Whitney's test was used to calculate the differences between groups. Pearson's product moment correlation coefficient was used to calculate the correlations between SUVmax in the biopsy sites and the MIB-1 labeling index. The regression line has been depicted.

Results

There were eight indolent lymphoma cases, 26 aggressive lymphoma cases, two mantle-cell lymphoma cases, and five HL cases. The indolent lymphoma cases included three cases of follicular lymphoma (grade 1), and five cases of EN marginal zone lymphoma of mucosa-associated lymphoid tissue. Aggressive lymphoma cases included one case of T lymphoblastic lymphoma; 16 diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS); one anaplastic lymphoma kinase (ALK)-positive large B-cell lymphoma; one plasmablastic lymphoma; one Burkitt lymphoma; one NK/T cell lymphoma, nasal type; one peripheral T-cell lymphoma, NOS; two angioimmunoblastic T-cell lymphoma; one anaplastic large-cell lymphoma, ALK positive; and one anaplastic large-cell lymphoma, ALK negative. All of the five cases with HL were nodular sclerosis subtype. HIV status was negative in all the 30 tested cases, including one case of plasmablastic lymphoma.

In NHL, the SUVmax at the biopsy sites ranged from 1.1 to 32.8 (Table I). It was significantly higher in the aggressive lymphoma cases than in the indolent lymphoma cases ($P < 0.001$). All eight cases with indolent lymphoma as well as the mantle-cell lymphoma cases exhibited a SUVmax below eight. On the other hand, only 23% (6 of 26) of aggressive lymphoma cases exhibited a SUVmax below eight. With regard to the MIB-1 labeling index, there were seven MG-1 cases, three MG-2 cases, three MG-3 cases, six MG-4 cases, 11 MG-5 cases, two MG-6 cases, and four MG-7 cases. The MIB-1 labeling index of the eight indolent NHL cases was significantly lower than that of the 26 aggressive NHL cases ($P < 0.001$). A strong positive correlation was observed between SUVmax at the biopsy sites and the MIB-1 labeling index in the 36 patients (Figure 1(a), $r = 0.69$, $P < 0.001$). The mathematical expression of the regression line was as follows: MIB-1 labeling index (%)=17.32 + 2.84 SUVmax ($P < 0.001$, $R^2 = 0.47$).

Table I. SUVmax in biopsy site and MIB-1 index in NHL.

	n	SUVmax	MIB-1 index* (%)
Total	36	9.1 (1.1–32.8)	50 (0–100)
Indolent lymphoma	8	4.0 (1.1–7.1)	0 (0–10)
Aggressive lymphoma	26	11.7 (2.1–32.8)	70 (10–100)
Mantle-cell lymphoma	2	3.5 (2.1–4.9)	20 (10–30)
LN	19	9.1 (1.1–32.8)	50 (0–100)
EN	17	9.2 (1.3–22.5)	50 (0–100)

LN, lymph node; EN, extranodal site.
*Median (range).

All 15 cases with a SUVmax more than 10 exhibited an MG $\geq$ 4. Nineteen LN and 17 EN sites were biopsied. The EN sites were: tonsils–three cases; pharynx–two cases; bone–two cases; lung–two cases; orbita–one case; paranasal sinus–one case; salivary glands–one case; lip–one case; gingival–one case; stomach–one case; small intestine–one case; and prostate–one case. The SUVmax and MIB-1 labeling index were not significantly different between the LN and EN groups. A positive correlation was also observed between SUVmax at the biopsy sites and the MIB-1 labeling index in LN group ($r = 0.60$, $P = 0.006$) and EN group ($r = 0.87$, $P < 0.001$), respectively [Figure 1(b)]. In DLBCL only, a positive correlation was also observed ($r = 0.61$, $P = 0.01$) (data not shown). Samples of three cases with DLBCL, NOS indicating the positive correlation between SUVmax at the biopsy site, and the MIB-1 labeling index are shown in Figure 2. In HL, the SUVmax at the biopsy sites

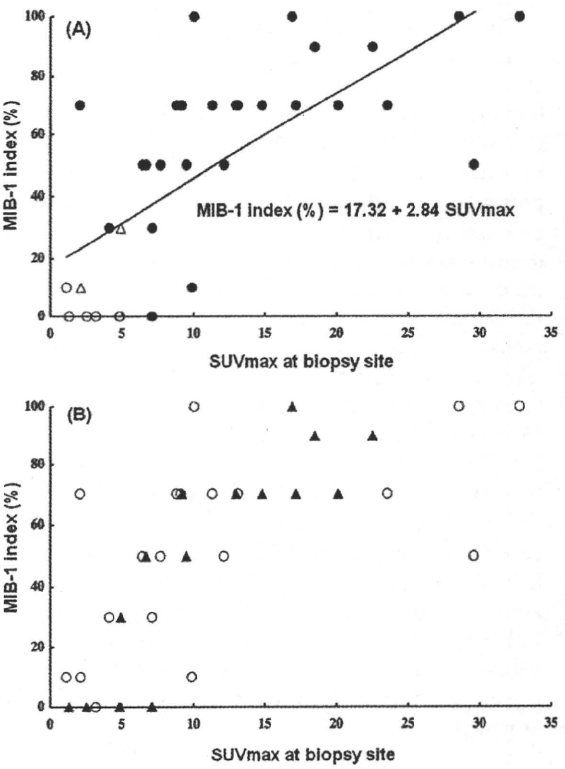

Figure 1. (a) There is a positive correlation between the SUVmax at biopsy site and MIB-1 labeling index in 36 cases of NHL ($r = 0.69$, $P < 0.001$). Regression line is depicted (○, indolent lymphoma; ●, aggressive lymphoma; △, mantle-cell lymphoma); (b) There are positive correlations between the SUVmax at biopsy site and MIB-1 labeling index in LN cases (○; $n = 19$, $r = 0.60$, $P = 0.006$) and in EN cases (▲; $n = 17$, $r = 0.87$, $P < 0.001$), respectively.

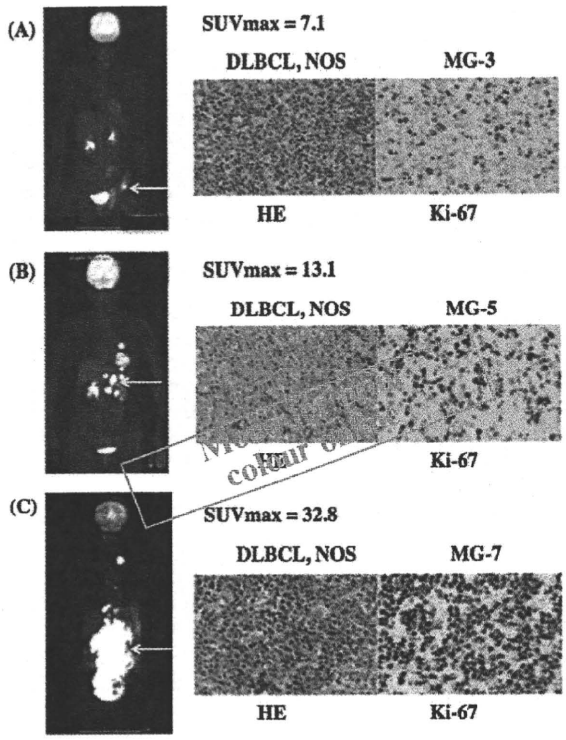

Figure 2. Samples showing the correlation between SUVmax at the biopsy site and the MIB-1 labeling grade (MG). (a) Left inguinal lymph node biopsy (SUVmax 7.1 and MG-3); (b) mesenteric lymph node biopsy (SUVmax 13.1 and MG-5); and (c) retroperitoneal lymph node biopsy (SUVmax 32.8 and MG-7).

ranged from 1.9 to 17.2 (median, 10.1). Almost all of the tumor cells were positive for Ki-67 staining and all of them were categorized in MG-7.

Discussion

The result of this study indicates that it is possible to estimate the proliferation potential from SUVmax on FDG-PET. It demonstrates the speed of tumor growth more directly and objectively compared with several previous reports [15–19] that indicate that SUVmax can be used as a predictor to distinguish between indolent histology and aggressive histology NHL cases. Therefore, our results have increased the importance of the SUV value on PET in the case of NHL. We can also estimate the proliferation potential of tumors in cases where the diagnostic biopsy is performed at sites other than the site of maximum SUVmax in the whole body or when PET is possible, but rebiopsy is clinically impossible in relapsed cases. The positive correlation between the SUVmax in biopsied site and the MIB-1 labeling index was stronger in EN group than in LN group, although the reason was uncertain.

In addition, we examined five cases with HL of the nodular sclerosis subtype during this period. Almost all the Hodgkin and Reed-Sternberg (HRS) cells were stained with MIB-1. The high MIB-1 labeling index of HRS cells, however, does not reflect the clinical behavior of the HL. Such a small number limits any meaning analysis. We excluded the HL cases in discussing the correlation between SUVmax and MIB-1 labeling index, all of which were MG-7.

Although our findings suggest an important role of SUVmax on PET in NHL, there are limitations to the use of SUVmax in the management of patients with NHL. In our study, 23% of aggressive lymphomas had SUVmax below eight, whereas all indolent lymphoma had SUVmax less than eight. There was still a considerable overlap between indolent and aggressive lymphoma. It suggests that SUVmax cannot completely distinguish between indolent and aggressive lymphoma. A positive correlation was observed between the SUVmax on FDG-PET at the biopsy site and the MIB-1 labeling index in NHL. The SUVmax is an useful predictor of the proliferation potential in NHL.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. ①

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original article

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Bone metastasis and poor performance status are prognostic factors for survival of carcinoma of unknown primary site in patients treated with systematic chemotherapy

M. Kodaira[1], S. Takahashi[1]*, S. Yamada[1], K. Ueda[1], Y. Mishima[1], K. Takeuchi[2], N. Yamamoto[2], Y. Ishikawa[2], M. Yokoyama[1], T. Saotome[1], Y. Terui[1] & K. Hatake[1]

[1]*Department of Medical Oncology, Cancer Institute Hospital and* [2]*Division of Pathology, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan*

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Background: Cancer of unknown primary site (CUP) generally has a poor prognosis, and there is no established standard therapy. There have been no reports of a prognostic model for CUP patients treated with a single regimen of systemic chemotherapy.

Methods: Univariate and multivariate prognostic factor analysis for overall survival (OS) were conducted retrospectively in 58 consecutive CUP patients treated with carboplatin plus paclitaxel (Taxol) therapy as a first-line treatment.

Results: Univariate prognostic factor analysis revealed baseline performance status (PS) of two or more, low serum albumin level, pleural effusion, bone metastasis, and liver metastasis as adverse prognostic factors. Cox proportional hazards analysis showed that poor PS and bone metastasis had the most powerful adverse impact on survival. We developed a prognostic model using those two variables—a good-risk group (PS 0–1 without bone metastasis) and a poor-risk group (PS ≥2 or bone metastasis). The poor-risk group showed significantly poorer OS than the good-risk group (1 year OS 36.8% versus 67.1%, $P = 0.0003$).

Conclusions: Poor PS and bone metastasis were identified as independent adverse prognostic factors in CUP. A simple prognostic model was developed and seems useful for decision making as to whether chemotherapy is indicated for CUP patients.

Key words: cancer of unknown primary site, carboplatin plus paclitaxel, bone metastasis

introduction

Cancer of unknown primary site (CUP) is pathologically diagnosed metastatic carcinoma in which no obvious primary site is identified with a conventional work-up. It is not a rare clinical entity, accounting for 3%–5% of all solid malignancies [1, 2]. The prognosis of CUP is generally considered poor, with median survival ~6–12 months [3]. Briasoulis et al. [4] reported encouraging results from phase II data of carboplatin and paclitaxel combination therapy for patients with CUP. In this study, the overall response rate by an intention-to-treat analysis was 38.7%, and median overall survival (OS) was 13 months at median follow-up time of 28 months. Platinum and taxane combination therapy is now widely used in clinical practice [4–8], but recent multiple-treatment meta-analysis showed that no type of chemotherapy has been proven to prolong survival in patients with CUP [9]. CUP consists of heterogeneous neoplasms with variable biological features, making it difficult to identify clinically useful prognostic survival factors. But several subsets have been identified requiring a specific treatment and having a better prognosis. Women with peritoneal carcinomatosis of serous adenocarcinoma [10], women with adenocarcinoma of axillary lymph nodes [11] or cervical lymph node metastasis of squamous cell carcinoma [12], young adults with poorly differentiated carcinoma of midline distribution [13], and undifferentiated carcinoma with neuroendocrine features [14] are CUP subgroups known to have a better prognosis. But the majority of CUPs have a poor prognosis, as mentioned above. In this article, we report the results of a prognostic factor analysis conducted in a population of 58 patients of CUP treated with carboplatin and paclitaxel as a first-line systemic chemotherapy. We retrospectively investigated baseline characteristics as prognostic factors for survival to identify a subset of patients who would benefit from chemotherapy.

Correspondence to: Dr S. Takahashi, Department of Medical Oncology, Cancer Institute Hospital, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan. Tel: +81-3-3520-0111; Fax: +81-3-3570-0343; E-mail: stakahas@jfcr.or.jp

methods

patient characteristics

The medical and pathological records of 58 consecutive newly diagnosed patients with CUP who received carboplatin and paclitaxel (Taxol, Bristol-Myers Squibb, Tokyo, Japan) combination therapy as first-line therapy at the Cancer Institute Hospital, Japanese Foundation for Cancer Research, from March 2004 to January 2008 were retrospectively reviewed. Patients had pathologically confirmed metastatic cancer and were surveyed for detailed medical history, complete physical examination, blood counts, chemistry profile, chest radiograph, computed tomography (CT) scan of chest and abdomen, and further radiological survey or endoscopy of suspected areas. Serum prostate-specific antigen (PSA) was measured in male patients, and CA 125 was measured in female patients. Women with adenocarcinoma of axillary lymph nodes also received mammography and breast ultrasound. Young adults with poorly differentiated adenocarcinoma involving the mediastinal region were surveyed with α-fetoprotein and β-human chorionic gonadotropin. The gastrointestinal tracts of male and female patients with adenocarcinoma involving abdominal and pelvic lesion were surveyed by upper gastrointestinal endoscopy and colonoscopy. Gynecologic examination was carried out in female patients with abdominal and pelvic disease. Patients with squamous cell carcinoma of cervical lymph nodes also underwent laryngeal endoscopy and upper gastrointestinal endoscopy. Bone metastases were assessed by the combination of bone scintigraphy or positron emission tomography with chest X-ray, CT, or magnetic resonance imaging. Histopathological review including immunohistochemistry (IHC) was carried out to detect primary sites and to exclude other malignancies. Low-molecular cytokeratins (CKs) 7 and 20 were routinely stained for all patients with CUP, and thyroid transcription factor 1, caudal type homeobox transcription factor 2, and PSA were stained for patients with adenocarcinoma of CUP. When a specific origin was suspected by morphological examination and clinical history, distinctive IHC was carried out (chromogranin, synaptophysin, and CD56 for neuroendocrine cell carcinoma; D2-40, placental alkaline phosphatase, human chorionic gonadotropin, and CD30 for germ-cell tumor; and D2-40 and calretinin for mesothelioma). In the case of difficulty in diagnosing epithelial carcinoma, several IHC of S100, vimentin, leukocyte common antigen, and CKs are used for distinguishing melanoma, sarcoma, and lymphoma from the anaplastic cell type of carcinoma.

We excluded patients in favorable subsets that have specific treatments other than carboplatin and paclitaxel—such as women with adenocarcinoma of axillary lymph nodes or cervical lymph node metastasis of squamous cell carcinoma, young adults with poorly differentiated carcinomas of midline distribution, and patients with undifferentiated carcinomas of neuroendocrine features. However, women with peritoneal carcinomatosis of adenocarcinoma who were treated with carboplatin and paclitaxel as first-line treatment were included in this study.

treatment

Carboplatin was administered by a 2-h i.v. infusion, dosed with 6 mg/ml/min target area under the free carboplatin plasma concentration versus time curve and was followed by paclitaxel 200 mg/m^2 in 500 ml of normal saline administered over 3 h. The Calvert formula was used for carboplatin dosing, on the basis of a glomerular filtration rate calculated by the Cockcroft–Gault equation using serum creatinine, body surface area, and age. Chemotherapy cycles were repeated every 3 weeks and responding patients continued the chemotherapy until disease progression or intolerable toxicity. Response to chemotherapy was assessed by Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0). Progression-free survival (PFS) and OS were calculated from day 1 of the first cycle of chemotherapy.

statistical analysis

Survival curves were estimated using the Kaplan and Meier method, compared using the log-rank test, and prognostic factors were identified by univariate analysis. Then the forward stepwise Cox proportional hazards analysis was carried out to identify independent prognostic factors. Statistical analyses were carried out using SPSS software (version 17.0; SPSS Inc., Chicago, IL).

results

patient characteristics

Patient characteristics are shown in Table 1. Fifty-eight CUP patients treated with at least one cycle of carboplatin and paclitaxel combination therapy were retrospectively analyzed. Twenty-eight (48.3%) patients were male, and the median age was 64 years (range 28–79 years). Forty-nine patients (84.5%) had a good performance status (PS) of zero to one. Twenty-six (44.8%) patients had well-differentiated adenocarcinoma, 21 (36.2%) patients had anaplastic or poorly differentiated carcinoma, and 5 patients (8.6%) had squamous cell carcinoma. Another six (10.3%) patients had clear-cell carcinoma, transitional cell carcinoma, or adenosquamous cell carcinoma. Metastatic sites are listed in Table 1. Lymph nodes, lung, bone, and liver were frequently involved sites and cervical, mediastinum, and retroperitonium were common sites for lymph node metastasis.

PSA was measured in 20 male patients (median PSA level 2.04 ng/ml, range 0.34–4.04 ng/ml), and CA 125 was obtained in 26 female patients (median CA 125 level 462 U/ml, range 4.8–50000 U/ml). Five of six male patients with bone metastasis showed a PSA level <4.0 ng/ml, and the PSA value before treatment of one young male patient was not available.

outcome of chemotherapy

A total of 315 cycles were administered, and patients received a median of five cycles of treatment (range 1–21 cycles).

Table 1. Patient characteristics

Number of patients	58
Age, median (range)	64 (28–79)
Sex	
Male	28
Female	30
Performance status	
0–1	49
2–4	9
Pathology	
Adenocarcinoma	26
Squamous cell carcinoma	5
Poorly differentiated/anaplastic carcinoma	21
Other	6
Sites of metastasis	
Lung	15
Bone	13
Liver	11
Pleural effusion	15
Ascites	11
Lymph node	44

The response rates by main histopathological types of adenocarcinoma, squamous cell carcinoma, poorly differentiated carcinoma, or poorly differentiated adenocarcinoma were 42.3%, 60.0%, and 23.8%, respectively (Table 2). For other histology types, one patient with transitional cell carcinoma had partial response. Sixteen patients were treated with second-line chemotherapy. At a median follow-up time of 12 months (range 6–1659 days), median OS and PFS were 16.7 months and 5.9 months, respectively. Six patients had PFS >2 years and one of these patients survived >4 years.

prognostic model of clinical and biological variables

The outcome of univariate analysis of clinical and biological factors is listed in Table 3. Five parameters have prognostic relevance: poor PS (≥2) ($P = 0.01$), low serum albumin level (<3.7 g/dl) ($P = 0.003$), pleural effusion ($P = 0.04$), bone metastasis ($P = 0.02$), and liver metastasis ($P = 0.02$). Multivariate analysis for these five variables was conducted and showed that bone metastasis ($P = 0.002$) and PS of two or more ($P = 0.016$) had significant adverse impact for survival (Table 3). Poor PS was not correlated with presence of bone metastasis.

Table 2. Treatment results

	N	CR (*n*)	PR (*n*)	ORR (%)
Total	58	5	15	34.5
Pathology				
Adenocarcinoma	26	5	6	42.3
Squamous cell carcinoma	6	0	3	50.0
Poorly differentiated anaplastic carcinoma	21	0	5	23.8

CR, complete response; PR, partial response; ORR; overall response rate.

Table 3. Univariate and multivariate analysis of prognostic factors for survival

	Univariate	Multivariate	
	P value	HR (95% CI)	*P* value
PS ≥2	0.01	2.93 (1.22–7.04)	0.016
Age (>65 years)	0.29		
Sex (male)	0.41		
ALP (>UNL)	0.13		
LDH (>UNL)	0.45		
ALB (<3.7 g/dl)	0.003		
Hb (<11.0 g/dl)	0.77		
Pleural effusion	0.04		
Ascites	0.69		
Lung metastasis	0.58		
Bone metastasis	0.02	3.48 (1.56-7.78)	0.002
Liver metastasis	0.02		
Adenocarcinoma	0.81		
Poorly/anaplastic carcinoma	0.32		

HR, hazard ratio; CI, confidence interval; PS, performance status; ALP, alkaline phosphatase; UNL, upper normal limit; LDH, lactate dehydrogenase; ALB, albumin; Hb, hemoglobin.

The incidence of bone metastasis was not significantly different between males and females (6 of 28 males, 5 of 30 females). A prognostic model was developed with those two variables. Nineteen (32.8%) patients were assigned to the good-risk group (defined as PS 0–1 without bone metastasis), and 38 (67.2%) patients were assigned to the poor-risk group (defined as PS ≥2 or bone metastasis). The poor-risk group ($n = 19$) showed significantly poorer OS than good-risk group ($n = 39$) (1 year OS 36.8% versus 67.1%, $P = 0.0003$) (Figure 1).

discussion

To identify a favorable or poor prognostic group of patients with CUP is of great concern when physicians consider whether systemic chemotherapy is indicated. No randomized trial showed better survival with chemotherapy than best supportive care. To our knowledge, the current study is the first that assesses prognostic factors for survival of patients with CUP treated with a single first-line regimen and should give us information as we choose an optimal therapy.

We demonstrated an overall response rate of 34.5% and a median OS of 16.7 months in CUP patients by an intention-to-treat analysis. This result seems similar to the results previously reported by Briasoulis et al. [4] and slightly better than other reports. One reason might be that both studies included female patients with peritoneal carcinomatosis (11 of 58 in ours and 19 of 75 in Briasoulis et al.). In our study, seven women (63.6%) responded to chemotherapy. A second reason might be that our group included a marginally larger number of patients with good PS. Golfinopoulos et al. [9] reported in recent multiple-treatment meta-analysis for CUP that 10 randomized trials assessed in that study included variable rates for patients with poor PS (median 24.5%, interquartile range 12.8%–38.9%). Third, our study included a slightly smaller number of patients

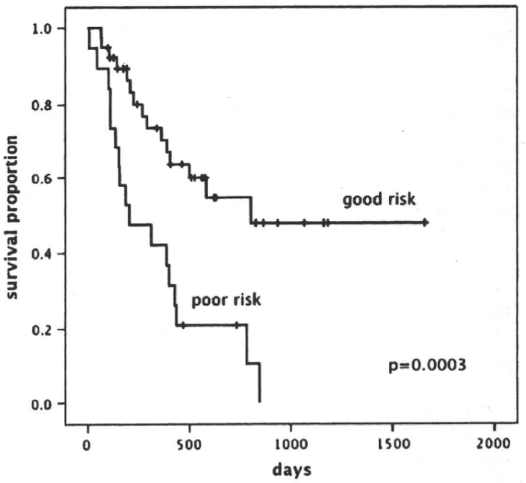

Figure 1. The prognostic model incorporating two variables. The good-risk group ($n = 39$) was defined as performance status (PS) of zero to one without bone metastasis and the poor-risk ($n = 19$) group as PS of two or more, or bone metastasis.

with liver metastasis, which was reported as an independent poor prognostic factor by Seve et al. [15]. But the rates of liver metastases in previous studies are variable from 16% to 76% [4–8, 16, 17]. The patients in the present study were treatable with combination therapy, so most of them maintained good PS and end organ function. No prospective studies or meta-analysis of prognostic factors for CUP have been published. But several retrospective studies have shown a number of independent adverse factors such as age, male gender, poor PS, adenocarcinoma histology, number of metastatic sites, liver metastasis, bone metastasis, lung metastasis, pleural metastasis, brain metastasis, comorbidity scoring of adult comorbidity evaluation-27 (ACE-27), low serum albumin, high serum lactate dehydrogenase (LDH), high serum alkaline phosphatase, lymphopenia, anemia, thrombocytopenia, high serum carcinoembryonic antigen, and high serum CA 125 [15, 18–20]. Abbruzzese et al. [18] reported adverse prognostic variables from a study of 657 cases of CUP at M. D. Anderson Cancer Center, and multivariate analysis identified male gender, a large number of metastatic sites, adenocarcinomatous histological type, and the presence of liver metastasis as unfavorable indicators. Culine et al. [19] proposed a simple prognostic model using PS and serum LDH levels in a population of 150 CUP patients, excluding favorable subsets, at a French cancer center. More recently, Seve et al. conducted a retrospective study assessing the influence of comorbidities, age, PS, and chemotherapy on survival in a population of 389 patients with CUP in Canada. Multivariate analysis showed that patients who had a PS of two or more and a high overall ACE-27 score had a poor prognosis. They concluded that the impact of comorbidity on survival was limited to patients with low PS [20]. The same author showed in another study that low serum albumin level and liver metastasis were the two most powerful adverse prognostic factors. The prognostic significance of those two factors was validated in another set of 124 patients with CUP [15]. In our study, bone metastases and poor PS (≥2) had a powerful adverse impact on survival. In clinical practice, bone metastases could be the cause of declining PS, but in this study, bone metastases and poor PS were not significantly correlated. Poor PS was also an adverse prognostic factor in studies by Culine et al. and by Seve et al. Bone metastases have been identified as an independent poor prognostic factor for the first time in uniformly treated patients with CUP. Prognostic significance of bone metastases in advanced cancer depends on the primary sites. In breast cancer or prostate cancer, the presence of bone metastases or bone-only metastases indicates a better prognosis [21]. On the other hand, the presence of bone metastases indicates a worse prognosis in lung cancer [22], thyroid cancer [23], or renal cell carcinoma [24]. The worse prognosis of patients with bone metastases in our series might be due to the apparent absence of occult breast cancer or prostate cancer in this set of patients.

Although our study might be small for finding independent prognostic factors retrospectively, it is important to identify clinically useful prognostic factors for CUP patients treated with platinum and taxane combination therapy, which are used frequently in daily practice. It has not been proven that systemic chemotherapy would prolong the survival of unfavorable CUP patients, and the best supportive care is a reasonable choice for patients who have little benefit from systemic chemotherapy.

We designed a new prognostic model that incorporated those two factors, poor PS and bone metastasis. The OS of patients with at least one or more prognostic factor was significantly shorter than those with no adverse prognostic factor. This model might be useful for decision making regarding the use of chemotherapy for CUP patients in daily clinical practice. A validation study of our prognostic model is warranted in the near future.

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disclosure

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