Table 1 continued

	n	Frequency (%)	*n*	Frequency (%)	
Corrected calcium level					
<10 mg/dl	48	96.0	84	90.3	0.224
≥10 mg/dl	2	4.0	9	9.7	
C-reactive protein level					
<0.3 mg/dl	21	42.0	38	40.4	0.855
≥0.3 mg/dl	29	58.0	56	59.6	
1st metastatic sites					
Extra-pulmonary					
No	13	26.0	43	45.7	0.021
Yes	37	74.0	51	54.3	
Lung					
No	23	46.0	29	30.9	0.072
Yes	27	54.0	65	69.1	
Lymph nodes					
No	38	76.0	82	87.2	0.085
Yes	12	24.0	12	12.8	
Bone					
No	33	66.0	74	78.7	0.096
Yes	17	34.0	20	21.3	
Liver					
No	44	88.0	87	92.6	0.364
Yes	6	12.0	7	7.4	
Brain					
No	48	96.0	90	95.7	0.942
Yes	2	4.0	4	4.3	
≥3 sites					
No	41	82.0	87	92.6	0.055
Yes	9	18.0	7	7.4	
Metastasectomy					
Yes	12	24.0	35	37.2	0.107
No	38	76.0	59	62.8	
Radiation to metastatic sites					
Yes	24	48.0	43	45.7	0.796
No	26	52.0	51	54.3	

* *t* test for follow-up period and age, and Pearson's chi-squared test for others

(4 %) were treated with a single mTORI. Of 123 patients using cytokine agents, all but 2 patients (98 %) had a treatment history of IFN-α use.

Prognostic factors affecting OS of patients with mRCC

To investigate whether the use of molecular-targeted therapy has any effect on OS, we performed univariate and multivariate analyses using Cox proportional hazards model (Table 3). In the entire study cohort, univariate analysis identified 16 variables as factors significantly affecting OS; however, neither the date at diagnosis of the first metastatic disease nor the mode of systemic therapy had a beneficial effect on OS. Multivariate analysis of these 16 variables identified 5 prognostic factors that were independently associated with worse OS: tumors of histological type other than clear cell [hazard ratio (HR) = 1.89, 95 % confidence interval (CI) 1.09–3.16, $P = 0.024$], decreased blood hemoglobin level (HR = 2.01, 95 % CI: 1.07–3.62, $P = 0.030$), elevated serum lactate dehydrogenase (LDH) level (HR = 3.24, 95 % CI 1.10–7.67, $P = 0.035$), elevated serum C-reactive protein (CRP) level (HR = 2.76, 95 % CI 1.60–4.89, $P = 0.0002$), and metastases at ≥ 3 sites (HR = 4.85, 95 % CI 2.35–9.45, $P < 0.0001$).

Table 2 Contents of immunotherapy and molecular-targeted therapy

	Type of immunotherapy	*n*	Type of molecular-targeted therapy	*n*
Immunotherapy alone	IFNα monotherapy	66		
	IFNγ monotherapy	1		
	IL2 monotherapy	1		
	IFNα + IFNγ	15		
	IFNα + IL2	9		
	IFNα + IFNγ + IL2	1		
Molecular-targeted therapy with prior immunotherapy	IFNα monotherapy	22	2 TKIs and 2 mTORIs	1
	IFNγ monotherapy	0	2 TKIs and 1 mTORI	1
	IL2 monotherapy	0	1 TKI and 1 mTORI	4
	IFNα + IFNγ	0	2 TKIs	7
	IFNα + IL2	6	1 TKI	16
	IFNα + IFNγ + IL2	1		
Molecular-targeted therapy alone			2 TKIs and 2 mTORIs	1
			2 TKIs and 1 mTORI	3
			1 TKI and 1 mTORI	7
			2 TKIs	3
			1 TKI	5
			1 mTORI	2

Comparison of OS between patients treated with molecular-targeted therapy and those treated with immunotherapy

We compared OS between the MT and IT groups using the Kaplan–Meier method. The median OS in the MT group was 45 months compared with 32 months in the IT group (Fig. 1a). In the MT group, the median OS of the 29 patients who received molecular-targeted therapy following prior immunotherapy was 45 months compared with 57 months in the 21 patients who received molecular-targeted therapy without prior immunotherapy (Fig. 1b). To compare the net effect of molecular-targeted therapy on OS, we calculated HR after adjusting for five confounding factors identified as independently prognostic using multivariate analysis. The adjusted HR of the MT group compared with that of the IT group was 0.68 (95 % CI 0.39–1.15, $P = 0.155$), whereas the adjusted HRs of patients treated with molecular-targeted therapy alone and following prior immunotherapy compared with that of the IT group were 0.47 (95 % CI 0.17–1.09, $P = 0.079$) and 0.79 (95 % CI 0.43–1.42, $P = 0.441$).

Identification of patient subgroups benefitting the most from the modern strategy including molecular-targeted therapy

Analysis of the entire study cohort suggested that molecular-targeted therapy may be marginally more effective at improving OS of patients with mRCC than immunotherapy. Therefore, we conducted subgroup analysis using Cox proportional hazards model to identify the subgroups of patients that benefitted the most from treatment with molecular-targeted therapy rather than immunotherapy (Fig. 2). The subgroup effect was examined by the interaction test adjusting for five confounding factors identified in the multivariate analysis. The presence of brain metastases was excluded from this analysis because the number of patients with brain metastases ($n = 6$; 2 in the MT group and 4 in the IT group) was too small for statistical comparison.

Subgroup analysis revealed that the strategy including molecular-targeted therapy was most effective at improving OS of female patients (P value for interaction test = 0.011) compared with male patients, and those classified as MSKCC intermediate risk (P value for interaction test = 0.028) compared with those classified as good or poor risks. It was also suggested that patients who developed metastatic disease less than 1 year after the initial diagnosis of RCC may benefit more from molecular-targeted therapy than from immunotherapy (P value for interaction test = 0.081) compared with those who developed metastases at 1 year or more after the initial diagnosis of RCC.

Discussion

A paradigm shift in treatment strategies for mRCC has occurred worldwide after the introduction of molecular-targeted agents into clinical practice as a result of pivotal RCTs, which have demonstrated beneficial effects of these agents on OS and/or PFS. Moreover, molecular-targeted therapy has almost entirely replaced immunotherapy in the treatment guidelines in Europe and the USA [22, 23]. Although these RCTs have a significant impact on the management of patients with mRCC and almost all mRCC patients are treated with molecular-targeted therapy as an initial treatment or following prior immunotherapy in clinical practice nowadays, these results have not always been reproducible in clinical practice, possibly because a substantial number of patients do not conform to the strict eligibility and ineligibility criteria utilized for the selection

Table 3 Univariate and multivariate analyses for overall survival in 144 patients

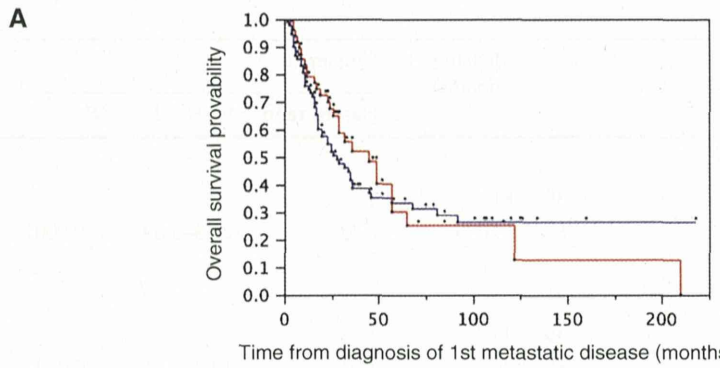
Variable and category	*n*	Median OS (months)	Univariate			Multivariate		
			Hazard ratio	95 % CI	*P**	Hazard ratio	95 % CI	*P**
Era at diagnosis of 1st metastatic disease								
Era II (2002–2011)	100	32	1					
Era I (1992–2001)	44	34	1.05	0.66–1.65	0.820			
Age								
<65 years	83	35	1					
≥65 years	61	26	1.37	0.87–2.13	0.172			
Sex								
Male	112	35	1					
Female	32	23	1.40	0.81–2.31	0.214			
Prior nephrectomy								
Yes	128	35	1					
No	16	11	2.63	1.34–4.75	0.0065			
Tumor histological type								
Clear-cell	97	36	1			1		
Other	30	19	1.76	1.02–2.91	0.041	1.89	1.09–3.16	0.024
Initial diagnosis to 1st metastatic disease								
≥1 year	51	92	1					
<1 year	93	23	2.45	1.51–4.15	0.0002			
Symptom due to metastasis								
Absent	62	57	1					
Present	82	23	2.12	1.35–3.37	0.001			
Karnofsky performance status								
≥80	108	36	1					
≤70	36	16	2.23	1.40–3.49	0.001			
MSKCC risk classification								
Favorable	36	210	1					
Intermediate	95	25	3.81	2.04–7.92	<0.0001			
Poor	13	7	7.93	3.12–19.90	<0.0001			
Laboratory blood test								
Hemoglobin level								
≥Lower limit of normal	113	36	1			1		
<Lower limit of normal	31	16	2.71	1.58–4.48	0.0005	2.01	1.07–3.62	0.030
Neutrophil (%)								
≤Upper limit of normal	50	49	1					
>Upper limit of normal	90	25	1.94	1.21–3.18	0.0053			
Platelet count								
≤Upper limit of normal	123	35	1					
>Upper limit of normal	21	25	1.60	0.80–2.90	0.176			
Lactate dehydrogenase level								
≤Upper limit of normal × 1.5	135	35	1			1		
>Upper limit of normal × 1.5	9	9	4.58	1.87–9.63	0.0019	3.24	1.10–7.67	0.035
Albumin level								
≥Lower limit of normal	106	45	1					
<Lower limit of normal	38	18	2.25	1.39–3.57	0.0012			
Corrected serum calcium level								
<10 mg/dl	132	34	1					
≥10 mg/dl	11	17	1.54	0.64–3.12	0.307			

Table 3 continued

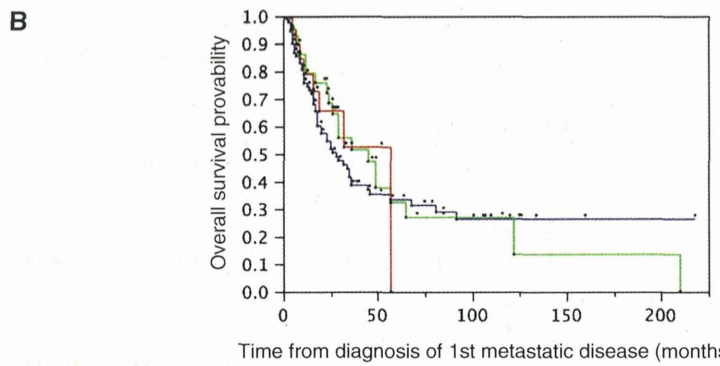
Variable and category	*n*	Median OS (months)	Univariate			Multivariate		
			Hazard ratio	95 % CI	*P**	Hazard ratio	95 % CI	*P**
C-reactive protein level								
<0.3 mg/dl	59	81	1			1		
≥0.3 mg/dl	85	19	3.43	2.13–5.69	<0.0001	2.76	1.60–4.89	0.0002
1st metastatic sites								
Extra-pulmonary								
No	56	49	1					
Yes	88	26	1.61	1.02–2.61	0.042			
Lung								
No	92	35	1					
Yes	52	29	1.12	0.70–1.74	0.639			
Lymph nodes								
No	120	35	1					
Yes	24	26	1.59	0.90–2.67	0.108			
Bone								
No	107	36	1					
Yes	37	20	1.94	1.18–3.10	0.0094			
Liver								
No	131	35	1					
Yes	13	11	3.30	1.49–6.52	0.0047			
Brain								
No	138	35	1					
Yes	6	12.5	4.78	1.81–10.54	0.0032			
≥3 sites								
No	128	36	1			1		
Yes	16	11.5	3.80	2.00–6.78	0.0001	4.85	2.35–9.45	<0.0001
Therapy against metastatic disease								
Systemic therapy								
Immunotherapy alone	94	28	1					
Molecular-targeted therapy	50	45	0.87	0.55–1.37	0.564			
Immunotherapy alone	94	28	1					
1st line molecular-targeted therapy	21	57	0.81	0.35–1.61	0.568			
2nd line molecular-targeted therapy	29	45	0.90	0.53–1.49	0.699			
Metastasectomy								
Yes	47	36	1					
No	97	26	1.50	0.95–2.41	0.081			
Radiation								
No	77	45	1					
Yes	67	28	1.31	0.85–2.05	0.221			

* Wald test based on Cox proportional-hazards model

of participants in RCTs. In addition, several studies have reported a somewhat better prognosis for Japanese mRCC patients in the cytokine era [9–11]. Therefore, it is important to know whether the modern treatment strategy including molecular-targeted therapy improves the prognosis for patients with mRCC in clinical practice. However, to date, limited studies have examined the beneficial effects of molecular-targeted therapy compared with those of immunotherapy in clinical practice on survival of patients with mRCC [19–21]. Our primary aim was to



No. of patients at risk						Adjusted HR*
Immunotherapy alone	94	22	11	3	2	1
Molecular-targeted therapy	50	12	4	2	2	0.68 (95%CI:0.39-1.15, *P*=0.155)



No. of patients at risk						Adjusted HR*
Immunotherapy alone	94	22	11	3	2	1
Molecular-targeted therapy alone	21	3	0	0	0	0.47 (95%CI:0.17-1.09, *P*=0.079)
Molecular-targeted therapy with prior immunotherapy	29	10	3	2	2	0.79 (95%CI:0.43-1.42, *P*=0.441)

Fig. 1 **a** Overall survival curves of patients treated with immunotherapy alone (*blue line*) and with molecular-targeted therapy (*red line*). **b** Overall survival curves of patients treated with immunotherapy alone (*blue line*) and with molecular-targeted therapy alone (*red line*) or with molecular-targeted therapy with prior immunotherapy (*green line*). *Hazard ratios are adjusted for confounding factors (i.e., tumor histological type, blood Hb level, serum LDH level, serum CRP level, metastatic sites ≥ 3 or less)

identify the patients with mRCC who benefitted the most from the modern strategy using molecular-targeted therapy with or without prior immunotherapy in clinical practice.

We identified a marginally positive effect of molecular-targeted therapy compared with that of immunotherapy on OS. This observation is consistent with that of 2 recent population-based studies and an epidemiological study showing statistically significant improvements in the survival of patients treated with TKIs [19, 20] or in patients with distant disease in the post-cytokine era [21]. A possible explanation for the failure of the present study to demonstrate a statistical significance of this observation could be that more patients in the MT group had extra-pulmonary metastases or metastases at ≥ 3 sites compared with those in the IT group whereas the patients in the IT group had undergone tumor nephrectomy more frequently than those in the MT group (Table 1). These biases could have a beneficial effect on OS of patients in the IT group. Moreover, we found that a history of nephrectomy or the presence of extra-pulmonary metastases affected OS and identified the presence of metastases at ≥ 3 sites as a significant prognostic factor. In addition, previous studies have shown that higher rates of nephrectomy and fewer metastases or metastatic sites may improve the prognosis [9, 20, 24].

Subsequent subgroup analysis demonstrated that compared with patients showing favorable or poor risk features, patients with MSKCC intermediate risk features benefitted significantly more from molecular-targeted therapy than from immunotherapy. This observation is partly consistent with that of the population-based studies that demonstrated improved survival in MSKCC intermediate risk patients treated with TKI than in those treated with IFN-α [19, 20]. In contrast, there are inconsistencies between our study and the 2 population-based studies in terms of the beneficial effect of molecular-targeted therapy over immunotherapy for patients showing favorable or poor risk features. In addition, Warren et al. demonstrated a survival benefit for favorable risk patients, whereas Heng et al. demonstrated a survival benefit for poor risk patients; however, these effects were not observed in the other two studies. Because

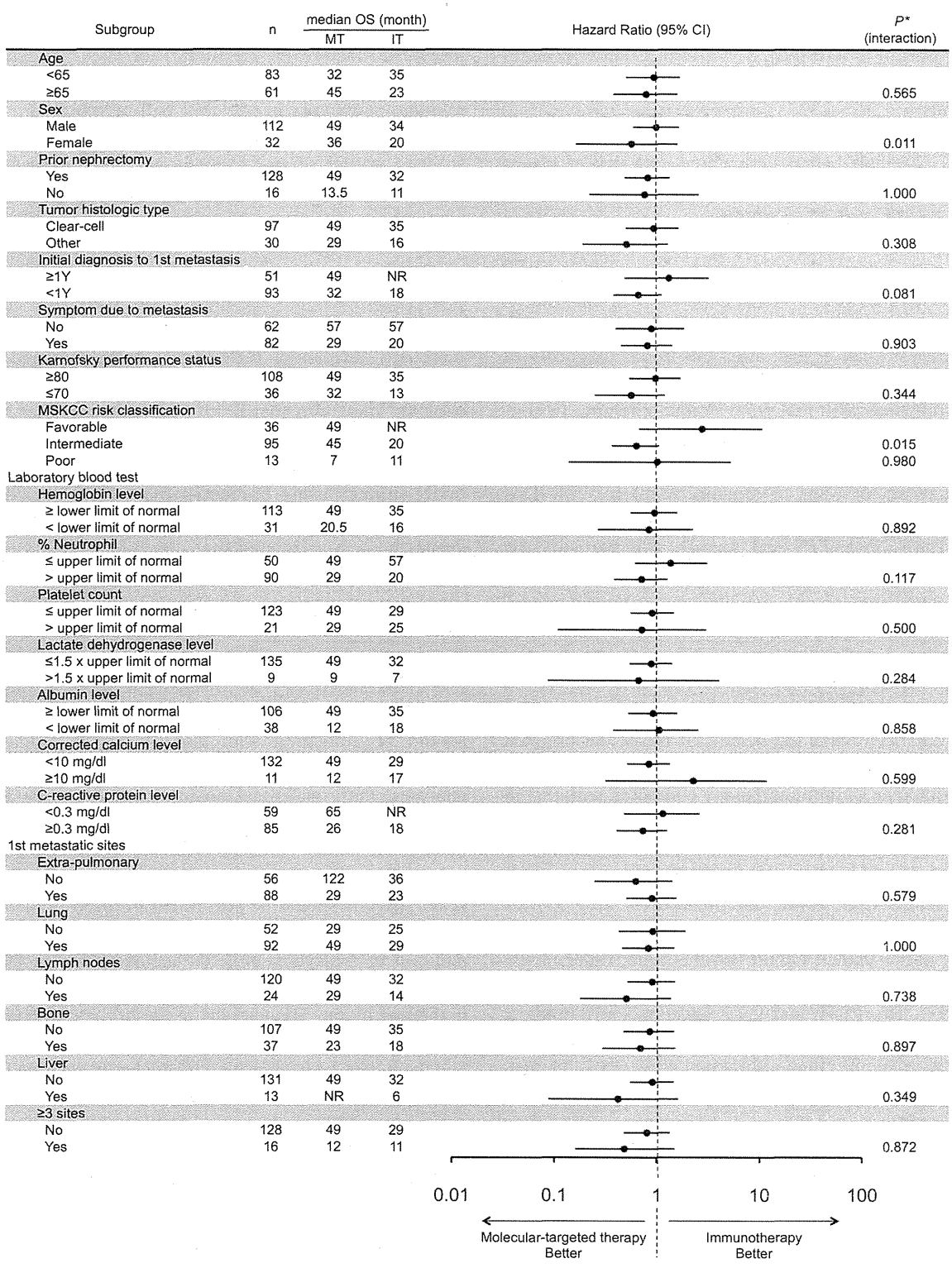


Fig. 2 Hazard ratios for overall survival among subgroups of patients. Hazard ratios (indicated by *circles*) with 95 % confidence intervals (indicated by *horizontal lines*) are shown for subgroups of patients treated with immunotherapy alone or molecular-targeted therapy. *P values for interaction are calculated by Wald test based on Cox proportional-hazards model after adjustment for confounding factors (i.e., tumor histological type, blood Hb level, serum LDH level, serum CRP level, metastatic sites ≥ 3 or less)

each study had a relatively small number of patients ($n = 200$ [19]; $n = 275$ [20]; $n = 144$ [this study]), large-scale studies are required in order to draw definitive conclusions.

Subgroup analysis identified a significant improvement in the OS of female patients compared with that of males, which is the first observation identifying an association between gender and susceptibility to molecular-targeted agents in patients with mRCC to date. However, a definite conclusion should not be drawn on this matter because of the small sample size of female patients in this study. Furthermore, it was suggested that patients who developed metastases less than 1 year after the initial diagnosis of RCC may derive the most benefit from molecular-targeted therapy compared with that from immunotherapy. In contrast, RCTs found that both sunitinib and temsirolimus were beneficial regardless of the length of time from the initial diagnosis of RCC to randomization [6, 25]. This discrepancy may result from inherent differences in patient selection for RCTs and clinical practice. Alternatively, this discrepancy may be specific to Japanese patients, whose treatment outcomes in the cytokine era were reportedly superior to those of Western patients. Similarly to our study results, Naito et al. [9] reported that Japanese patients who developed metastases more than 1 year after the initial diagnosis of RCC had a good prognosis even during the cytokine era. Therefore, the benefit of molecular-targeted therapy in these patients may be relatively small because of the good outcome of immunotherapy compared with that in patients who developed metastases within 1 year.

We found that tumor histological types other than clear cell, decreased blood Hb level, elevated serum LDH level, elevated serum CRP level, and metastases at ≥ 3 sites were independent prognostic factors associated with worse OS in the entire patient cohort. Furthermore, other investigators and several studies supported the theory that each variable, i.e., Hb, LDH, and CRP, independently predicts poor outcome in mRCC patients treated with molecular-targeted therapy or immunotherapy [18, 20, 26–29]. MSKCC risk classification itself did not remain as an independent prognostic factor in the present multivariate analysis. Probably, Hb and LDH were more powerful prognostic factors at least in the present study cohort, and it is partly because MSKCC risk classification is based on the analyses using mRCC patients in the cytokine era [11, 28]. In a large-scale epidemiological study investigating trends in RCC in the cytokine and post-cytokine era, Shek et al. [21] demonstrated that non-clear cell tumors are associated with worse OS. The number of metastatic sites is also an independent prognostic factor in a population-based study comparing the effect of TKI and IFN-α [20]. Collectively, these findings suggest that the existing treatment modalities, including molecular-targeted therapy, are not sufficient for the management of patients with these prognostic factors. In the future, for such patients, novel treatment options, including next-generation TKI and immunomodulatory agents [30] will be required.

Our study had weaknesses and limitations. The retrospective nature of our analysis and the relatively small sample size may have compromised the quality of analyses. Our cohort consists of heterogeneous groups of patients. When the background characteristics are compared among patients treated with molecular-targeted therapy alone ($n = 21$), molecular-targeted therapy with prior immunotherapy ($n = 29$), and immunotherapy alone ($n = 94$), there are substantial differences, partly because of the small number of patients in each MT subgroup (data not shown). Since our goal is to investigate whether the new treatment strategy with addition of molecular-targeted agents improves the prognosis compared with the traditional strategy using immunotherapy alone, we compared the MT subgroups together as an MT group with the IT group. In such a circumstance, the differences were minimized as shown in Table 1. The content of molecular-targeted therapy was also heterogeneous in the MT group as shown Table 2. Thus, we could not mention the usefulness of individual molecular-targeted agents. In addition, to prove the superiority of molecular-targeted therapy over immunotherapy, the outcome of the MT alone group should be compared with that of the IT alone group. Although we did such analyses along with the presented analyses, these statistical analyses could not be successfully performed (data not shown) because of the small number of patients treated with MT alone ($n = 21$). To draw a definitive conclusion on this matter, a further large-scale analysis should be performed in future. For now, we can only mention whether the new treatment strategy with addition of molecular-targeted agents improves the prognosis compared with the traditional strategy using immunotherapy alone. The patients in the MT group had started their treatment more recently than those in the IT group and there may be background biases based on the treatment era. Although we did not identify any effect of the treatment era on OS, we cannot exclude an unexpected bias on the outcome of each group.

In conclusion, our results suggest that the modern strategy including molecular-targeted therapy may have the potential to improve OS of patients with mRCC and MSKCC intermediate risk features in clinical practice, compared with those with other risk features. However, our results also suggest that the prognosis for patients with tumors of histological type other than clear cell, decreased blood Hb level, elevated serum LDH or CRP levels, or metastases at ≥ 3 sites remains poor even in the modern molecular-targeted era. Further development of novel treatment strategies is needed to improve the prognosis in such patients.

Conflict of interest The authors declare that they have no conflict of interest.

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Non-terminal respiratory unit type lung adenocarcinoma has three distinct subtypes and is associated with poor prognosis

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ABSTRACT

Objectives: The characteristics of non-terminal respiratory unit (TRU) type lung adenocarcinoma are still unclear. The aim of the present study was to characterize non-TRU type lung adenocarcinoma.
Materials and methods: We analyzed the expression of mucins MUC5B and MUC5AC, as well as thyroid transcription factor-1 (TTF-1), using a tissue microarray comprising lung adenocarcinoma specimens from 244 consecutive patients. The presence of mutations in *EGFR* and *KRAS* were also determined.
Results: TTF-1, MUC5B, and MUC5AC were detected in 219 (89.8%), 75 (30.7%), and 33 cases (13.5%), respectively. Cluster analysis of protein expression profiles and *EGFR* and *KRAS* mutations yielded five groups of tumors as follows: TRU1-type [TTF-1(+), MUC5B(−), MUC5AC(−), *EGFR* mutations(−)]; TRU2-type [TTF-1(+), MUC5B(−), MUC5AC(−), *EGFR* mutations(+)]; Combined-type [TTF-1(+), MUC5B(+), and/or MUC5AC(+)]; Bronchiolar-type [TTF-1(−), MUC5B(+) and/or MUC5AC(+)]; and Null-type [TTF-1(−), MUC5B(−), MUC5AC(−), *EGFR* mutations(−), *KRAS* mutations(−)]. TRU-type tumors, which include TRU1- and TRU2-type tumors, were significantly associated with TRU morphology, whereas Bronchiolar-type tumors were associated with non-TRU morphology. Combined-type cases exhibited intermediate morphologies between TRU-type and Bronchiolar-type cases. TRU-type was associated with significantly better prognosis, followed by Combined-type, Bronchiolar-type, and Null-type (disease-free survival [DFS] $P = 0.017$; overall survival [OS], $P = 0.002$). Multivariate analyses indicated that non-TRU type tumors, which include Bronchiolar-, Combined-, Null-type tumors, were significantly correlated with poorer prognoses for DFS (hazard ratio = 1.785; 95% CI, 1.041–3.063; $P = 0.035$) and OS (hazard ratio = 1.928; 95% CI, 1.084–3.421; $P = 0.025$).
Conclusion: This study revealed three distinct subtypes of non-TRU type adenocarcinomas. Additionally, non-TRU type tumors were associated with worse prognoses than TRU type tumors. The results presented here may be useful for select patients should appropriate therapies become available.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1], and adenocarcinoma is the most common histologic subtype of primary lung cancer [2,3]. Yatabe et al. [4–7] proposed the existence of a distinct subset of lung adenocarcinomas arising from a terminal respiratory unit (TRU), which develops in periphery of the lung parenchyma is similar in cell morphology to type II pneumocytes or Clara cells, is positive for the expression of thyroid transcription factor-1 (TTF-1), and harbors mutations in the gene encoding the epidermal growth factor receptor (*EGFR*). The clinicopathological characteristics of these tumors are generally well defined.

In contrast, few studies have focused on non-TRU type adenocarcinomas. For example, Yatabe et al. [4] reported that non-TRU type adenocarcinomas originate centrally, are TTF-1 negative, are solid in morphology and poorly differentiated, and are often necrotic. Other researchers have reported that a relatively high proportion of mucinous-type lung adenocarcinomas, particularly mucinous adenocarcinoma in situ (AIS), can be classified as non-TRU type adenocarcinomas, which do not express TTF-1 [8,9]. Mucinous AIS is characterized by the presence of mucous columnar cells, which

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are similar to mucinous cells of the bronchi-bronchiolar epithelium, and evidence suggests that it is a precursor of non-TRU type adenocarcinoma. The mucin core proteins MUC5B and MUC5AC are expressed in goblet-type epithelial cells in normal airways and serve as specific markers for these cells [10–12]. Therefore, we reasoned that MUC5B and MUC5AC may be candidate markers for non-TRU type adenocarcinoma.

In the present study, we analyzed the expression of MUC5B, MUC5AC, and TTF-1 in resected lung adenocarcinomas using immunohistochemistry to better define non-TRU type adenocarcinoma. The presence of *EGFR* and *KRAS* mutations was also determined.

2. Patients and methods

2.1. Patient selection and histologic evaluation

Between January 2001 and December 2007, 337 consecutive patients with lung adenocarcinomas underwent pulmonary resection at Kyoto University Hospital. Patients were excluded if they had multiple primary lung cancers, underwent chemo- or radiotherapy before surgery, underwent incomplete resection, or lacked complete follow-up data retrieved from the Thoracic Surgical Database. Tumor staging was performed according to the 7th Edition of the TNM classification of the International Union Against Cancer [13].

All resected specimens were formalin-fixed, sectioned, and stained with hematoxylin and eosin (H&E) in the conventional manner. Periodic acid Schiff (PAS) and Alcian-blue stains were performed to detect mucins. Elastic stains were also performed to detect invasion of the pleura or vessels. Slides were reviewed by two pathologists (AY, SS), who were blinded to patient outcomes. First, we attempted to divide the lung adenocarcinomas into TRU type and non-TRU type according to previous studies [4–7]. Because some of the adenocarcinomas exhibited a mixture of cytologic subtypes, we categorized the tumor as non-TRU type when morphologic resemblance to mucinous columnar cells of bronchi-bronchiolar epithelium and/or bronchial glandular cells was seen. All cases were classified according to IASLC/ATS/ERS criteria [14]. Findings of significant prognostic factors for lung adenocarcinomas prompted further analyses of lymphatic invasion, vascular invasion, pleural invasion, and/or tumor grade, which were assessed according to the IASLC/ATS/ERS criteria [14].

2.2. Tissue microarray (TMA)

A portion of the present cohort was described in our previous report [15]. Briefly, after case selection described above, paraffin-embedded tumor blocks with sufficient tissue were selected to prepare a TMA. The most representative region of the tumor was selected based on the morphology of the H&E-stained slide. Tissue cores measuring 2 mm in diameter were punched out from each donor tumor block using thin-walled stainless steel needles (Azumaya Medical Instruments Inc., Tokyo, Japan), and core were arrayed in a recipient paraffin block. Non-neoplastic lung tissue cores from selected patients were also arrayed in the same block.

2.3. Immunohistochemistry (IHC)

A standard two-step technique was implemented, using polymeric conjugates as secondary antibodies for MUC5B and MUC5AC [16], and the standard avidin–biotin–peroxidase complex technique was used to detect TTF-1. Primary anti-mucin antibodies were as follows: anti-MUC5B (H-300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-MUC5AC (CLH2, Novocastra, New Castle Upon Tyne, UK). We performed immunohistochemistry using the SPT24 antibody clone (Novocastra), which was recently reported to be one of the most sensitive antibodies against TTF-1 [17–19]. IHC was performed using an auto-immunostainer (Benchmark, Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's instructions. We confirmed that TTF-1 was expressed in the alveolar epithelial cells, bronchial epithelial cells, and focal cells of the bronchial submucosal glands in normal lung tissue. We also confirmed that MUC5B and MUC5AC were expressed in the goblet cells of the bronchial epithelium and bronchial submucosal glands in normal lung tissue.

Scoring was based on the distribution and intensity of staining according to previous study [20]. The sums of the distribution and intensity scores were expressed as total scores. Here, a total score of 0 was regarded as a negative result. Alveolar epithelial, bronchiolar epithelial, and bronchial gland cells in the same TMA section were used as internal controls. Immunostaining was scored independently by two investigators (AY, SS), and when the scores differed, a consensus decision was made by viewing the specimen with a multiheaded microscope.

In general, compared to TTF-1 expression, mucin expression could be heterogeneous within individual tumors. To avoid false-negative results (cases showing TTF-1(+), MUC5B(−), and MUC5AC(−) in TMA sections, but TTF-1(+), MUC5B(+), and/or MUC5AC(+) in whole-slide sections), we performed IHC using anti-mucin antibodies with whole-slide sections for such cases.

To detect co-expression of TTF-1 and MUC5B or MUC5AC in the same cells, double immunostaining was carried out. Briefly, sections were immunostained with mouse monoclonal anti-TTF-1 antibodies using a standard avidin–biotin–peroxidase complex technique with horseradish peroxidase (HRP) and DAB on an automated stainer. Sections were then incubated with rabbit monoclonal anti-MUC5B or anti-MUC5AC antibodies and visualized with alkaline phosphatase and a fuchsin substrate system on the automated stainer.

2.4. Somatic EGFR and KRAS mutations

EGFR and *KRAS* mutations were detected using published methods [21,22]. Briefly, a section from each tumor was frozen immediately, and a part of the section was observed microscopically to confirm that the sample included sufficient numbers of tumor cells. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was then employed to detect mutations within exons 18, 19, 20, and 21 of *EGFR* [21,22]. For detecting *KRAS* mutations, the mutagenic PCR-restriction enzyme fragment length polymorphism method was used according to a published method [23]. Because *KRAS* mutations were previously detected in codon 12, but not codon 13 [23,24], we only assayed for codon 12 mutations [21,22].

2.5. Statistics

Chi-square and Fisher's exact tests were used to analyze categorical data. Hierarchical cluster analysis was conducted using the Ward's minimum variance method. Tissue samples were clustered based on protein expression profiles and *EGFR* and *KRAS* mutations. The factors evaluated by univariate and multivariate analyses to assess their impact on overall survival (OS) and disease-free survival (DFS) rates were as follows: sex, age, smoking status, tumor size, stage, tumor grade, lymphatic invasion, vascular invasion, pleural invasion, *EGFR* status, and *KRAS* status. The survival rates were calculated using the Kaplan–Meier method, and the differences were analyzed using the log rank test. Multivariate analysis was performed using Cox's proportional hazards model. All statistical tests were two-sided at a 5% level of significance. Data analysis and summary graphs were generated using the JMP statistical software package, version 8 (SAS Institute, Cary, NC, USA).