Clinicopathological and prognostic significance of interleukin-8 expression and its relationship to *KRAS* mutation in lung adenocarcinoma

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Background: On the basis of our recent findings of oncogenic KRAS-induced interleukin-8 (IL-8) overexpression in non-small cell lung cancer, we assessed the clinicopathological and prognostic significances of *IL-8* expression and its relationship to *KRAS* mutations in lung adenocarcinomas.

Methods: *IL-8* expression was examined by quantitative RT–PCR using 136 of surgical specimens from lung adenocarcinoma patients. The association between *IL-8* expression, clinicopathological features, *KRAS* or *EGFR* mutation status and survival was analysed.

Results: *IL-8* was highly expressed in tumours from elderly patients or smokers and in tumours with pleural involvement or vascular invasion. In a non-smokers' subgroup, *IL-8* level positively correlated with age. *IL-8* was highly expressed in tumours with *KRAS* mutations compared with those with *EGFR* mutations or wild-type *EGFR/KRAS*. Lung adenocarcinoma patients with high *IL-8* showed significantly shorter disease-free survival (DFS) and overall survival (OS) than those with low *IL8*. DFS and OS were significantly shorter in the patients with mutant *KRAS*/high *IL-8* than in those with wild-type *KRAS*/low *IL-8*. Cox regression analyses demonstrated that elevated *IL-8* expression correlated with unfavourable prognosis.

Conclusions: Our findings suggest that *IL-8* expression is associated with certain clinicopathological features including age and is a potent prognostic marker in lung adenocarcinoma, especially in oncogenic *KRAS*-driven adenocarcinoma.

Lung cancer is the major cause of cancer-related death worldwide (Jemal *et al*, 2011). It is histologically divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC represents ∼85% of all lung cancers and adenocarcinoma is the most common subtype of NSCLC (Travis *et al*, 2011). Recent advances in molecular biology have identified several driver mutations, such as *EGFR* mutations, *EML4-ALK* fusions and *ROS1* fusions, which are used or being tested as biomarkers for

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personalised therapy for lung adenocarcinoma (Chin *et al*, 2012; Pao and Hutchinson, 2012). *KRAS* mutations are the most common driver mutations in lung adenocarcinoma (Pao and Hutchinson, 2012). *KRAS* encodes a small GTP-binding protein that has an essential role in the regulation of many cellular processes, including proliferation, differentiation and apoptosis (Suda *et al*, 2010). Despite the high incidence of *KRAS* mutation and its correlation with unfavourable prognosis in lung adenocarcinoma (Mascaux *et al*, 2005), no effective therapeutic strategies have been established to target this subtype of tumour. Therefore, further study is warranted to develop new therapeutic modalities to improve the prognosis for patients with oncogenic *KRAS*-driven lung adenocarcinoma.

The CXC chemokine interleukin-8 (IL-8), which was originally identified as a chemoattractant for neutrophils, has been shown to be a transcriptional target of RAS signalling in human cancer cells (Sparmann and Bar-Sagi, 2004). Subsequently, we reported that IL-8 expression is transcriptionally upregulated by oncogenic KRAS mutations in NSCLC (Sunaga *et al*, 2012). IL-8 is an essential proinflammatory mediator that is involved in cancer development and acts as an angiogenic growth factor that promotes cell proliferation and angiogenesis, thus contributing to the progression and metastasis of NSCLC (Smith *et al*, 1994; Arenberg *et al*, 1996; Wang *et al*, 1996; Yatsunami *et al*, 1997; Zhu *et al*, 2004; Boldrini *et al*, 2005; Luppi *et al*, 2007). IL-8 has also been implicated in the epithelial–mesenchymal transition and cancer cell stemness during tumour progression (Palena *et al*, 2012). Importantly, IL-8 overexpression has been associated with unfavourable prognosis in NSCLC (Bellocq *et al*, 1998; Yuan *et al*, 2000; Chen *et al*, 2003; Seike *et al*, 2007). However, the clinicopathological and prognostic roles of IL-8 and its relationship to *KRAS* mutation status in lung adenocarcinoma are still unclear. To address these issues, we conducted the present study using tumour specimens from lung adenocarcinoma patients.

MATERIALS AND METHODS

Tumour specimens from lung adenocarcinoma patients. Tumour specimens were obtained from 136 patients with primary lung adenocarcinoma who underwent surgery consecutively between July 2003 and September 2009 at the Gunma University School of Medicine Hospital and National Nishigunma Hospital in Gunma, Japan (Supplementary Table 1). At the data cut-off point (end December 2013), 29 patients had developed recurrence and 20 had died due to lung adenocarcinoma. Thirty patients received adjuvant chemotherapy with UFT ($N = 12$) or adjuvant platinum-based chemotherapy ($N = 18$). Among the patients with recurrent disease, 25 patients received EGFR–tyrosine kinase inhibitor (EGFR–TKI) therapy ($N = 17$) or platinum-based chemotherapy ($N = 8$). All tumours were histologically diagnosed as adenocarcinomas according to the criteria of the World Health Organization. Postsurgical pathological stages were assigned according to the tumour-node-metastasis classification. Non-cancerous lung specimens ($N = 10$) that were obtained from 10 patients were used as normal controls. *KRAS* mutation at codon 12 and *EGFR* mutations in exons 19 and 21 were analysed using the Smart Amplification Process version 2 assay (DNAFORM, Kanagawa, Japan) followed by direct sequencing to confirm the presence of these mutations as previously described (Mitani *et al*, 2007; Miyamae *et al*, 2010; Sunaga *et al*, 2013). Disease-free survival (DFS) was defined as the time between surgery and the first evidence of recurrent disease, and overall survival (OS) was defined as the time between surgery and death. For the survival analysis, 17 patients who had received EGFR–TKI therapy were excluded to avoid selection bias. The study protocol was approved by the institutional review board of Gunma University Graduate School of Medicine and National Nishigunma Hospital. The specimens were immediately frozen after collection and stored at $-80\,^\circ$C until mRNA extraction was performed.

Quantitative real-time RT–PCR. IL-8 mRNA expression was analysed by quantitative real-time reverse transcriptase (RT)–PCR (Sunaga *et al*, 2012). Briefly, total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesised from 2 µg of total RNA with the SuperScript II First-Strand Synthesis system using the oligo(dT) primer (Invitrogen, Carlsbad, CA, USA). Primers and probes for IL-8 and GAPDH were purchased from Applied Biosystems (Tokyo, Japan; assay ID: Hs00174103_m1 for *IL-8* and Hs99999905_m1 for *GAPDH*). Real-time PCR was performed using a Lightcycler 480 system (Roche Diagnostics, Tokyo, Japan). Quantitative analysis was performed using the *GAPDH* gene as an internal reference to normalise the input cDNA. The comparative Ct method was used to compute relative expression values. As for IL-8 mRNA expression analysis, we had validated that IL-8 mRNA levels were strongly correlated with IL-8 protein levels in the cultured medium in NSCLC cell lines (Supplementary Figure 1; $r = 0.818$, $P < 0.001$) as evaluated by enzyme-linked immunosorbent assay (Sunaga *et al*, 2012).

Immunohistochemical staining. Immunohistochemical staining for IL-8 and CD34 was performed according to the procedure as previously described (Kaira *et al*, 2012). A rabbit polyclonal antibody against IL-8 (Abcam, Cambridge, MA, USA) and a mouse monoclonal antibody against CD34 (Nichirei, Tokyo, Japan) were used. The intensity of IL-8 staining was scored as follows: 1, <10% of tumour area stained; 2, 10–25% stained; 3, 26–50% stained; and 4, >50% stained. The tumours that had a score of more than 3 were defined as having high expression. The number of CD34-positive vessels was counted in four selected hot spots in a $\times 400$ field (0.26-mm^2 field area). The mean value of two independent readings of the same specimen was calculated.

Figure 1. IL-8 mRNA expression in 136 surgical specimens from lung adenocarcinoma patients. (**A**) Comparisons of *IL-8* expression between patients who were ≥70 years old and those who were <70 years old; between males and females; between smokers and non-smokers; and between patients with different pathological tumour stages. (**B**) Comparisons of *IL-8* expression between patients who were ≥70 years old and those who were <70 years old in the smoker subgroup and the non-smoker subgroup. (**C**) Correlations between *IL-8* expression and age in smokers and non-smokers. (**D**) Comparisons of *IL-8* expression between tumours with or without pleural involvement; tumours with or without lymphatic permeation; and tumours with or without vascular invasion. (**E**) Differential *IL-8* expression among the three groups that were classified according to *KRAS* and *EGFR* mutation status. (**F**) Comparisons of *IL-8* expression between ≥70-year-old patients and <70-year-old patients in the *KRAS*-mutants, *EGFR*-mutants and wild-type *EGFR/KRAS* subgroups. (**G**) Correlations between *IL-8* expression and age in the *KRAS*-mutant, *EGFR*-mutant and wild-type *EGFR/KRAS* subgroups. (**H**) Significant correlations between *IL-8* expression and age in non-smokers with *KRAS* mutations and non-smokers with *EGFR* mutations. (**I**) Comparisons of *IL-8* expression between smokers and non-smokers in the *KRAS*-mutant, *EGFR*-mutant and wild-type *EGFR/KRAS* subgroups. *IL-8* expression levels were determined by quantitative RT–PCR analysis and normalised to the mean values (= 1 a.u.) from 10 non-cancerous lung tissue samples. The points represent the mean *IL-8* levels obtained from four independent experiments. Differences between two groups were tested with the Mann–Whitney test, and differences between three or more groups were tested with the Kruskal–Wallis test.

Statistical analysis. The data were statistically analysed with GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA, USA). Differences between two groups were analysed by Mann–Whitney test and differences between more than two groups were analysed by Kruskal–Wallis test. The correlation between different variables was analysed using the non-parametric Spearman's rank test. The Kaplan–Meier method was used to estimate survival as a function of time, and survival differences
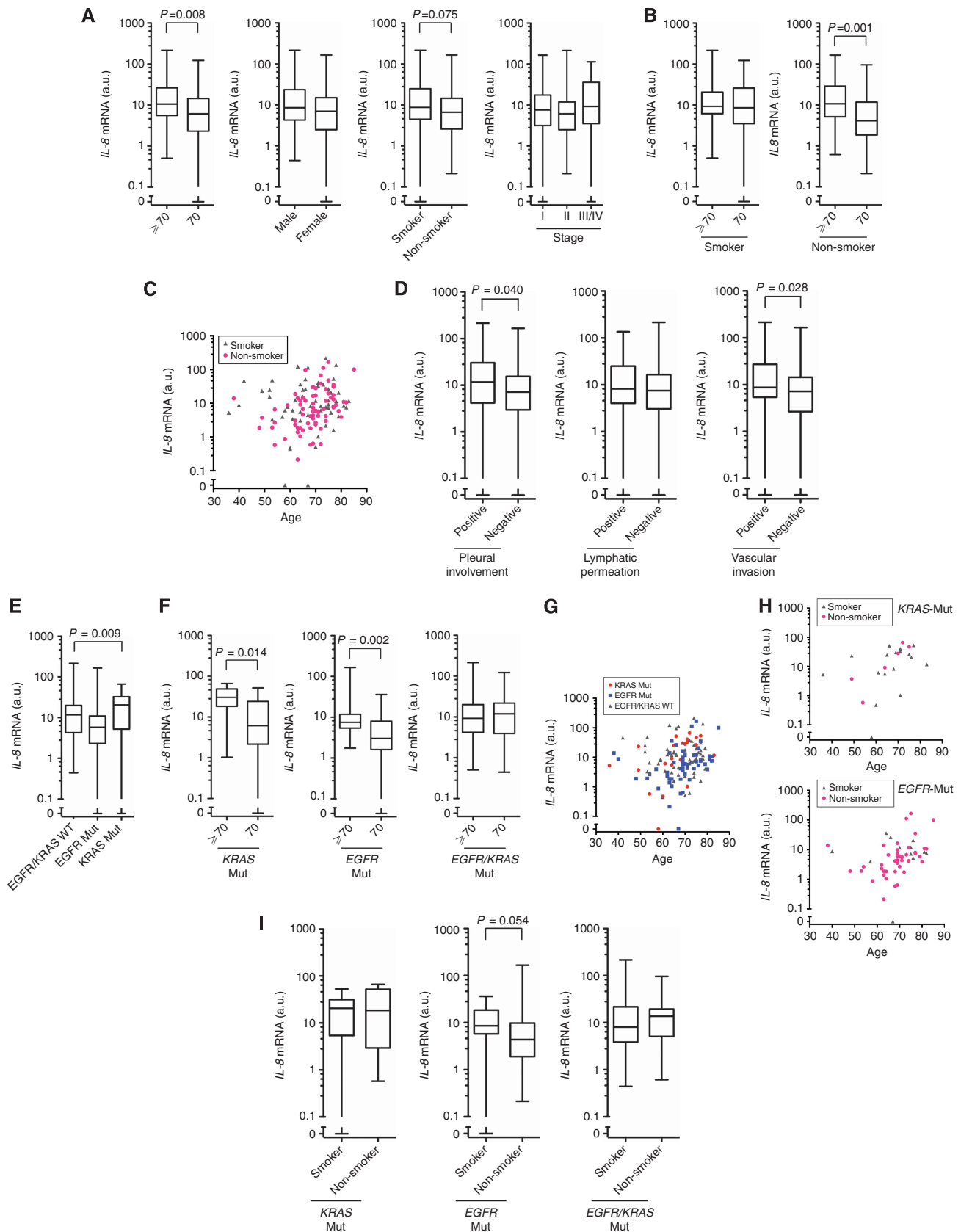
were analysed by the log-rank test. Multivariate analyses were performed with StatView version 5.0 software (SAS Institute Inc., Cary, NC, USA) using a stepwise Cox proportional hazards model to identify independent prognostic factors. $P < 0.05$ was considered significant and $0.1 > P \geqslant 0.05$ was considered borderline significant.

RESULTS

IL-8 mRNA expression was examined in 136 surgical specimens from primary lung adenocarcinoma patients, and the association between *IL-8* expression and clinicopathological parameters was analysed. *IL-8* expression was significatly higher in tumours from patients who were $\geqslant 70$ years old than in those from patients who were < 70 years old ($P = 0.008$) and was higher in tumours from smokers than in those from non-smokers with borderline significance ($P = 0.075$), whereas there were no significant differences in *IL-8* expression on the basis of gender or pathological stage (Figure 1A). When the patient population was classified into four groups according to age and smoking history, a significant difference in *IL-8* expression levels was observed only in the non-smoker subgroup (Figure 1B; $P = 0.001$). There was a significant correlation between age and *IL-8* expression among the non-smokers but not among the smokers (Figure 1C; $r = 0.436$, $P < 0.001$), indicating that *IL-8* expression levels might increase with age when patients have no history of smoking. In regard to pathological factors, *IL-8* expression was significantly higher in tumours with pleural involvement (PI; $P = 0.040$) or vascular invasion (VI; $P = 0.028$) than in those without such characteristics (Figure 1D). We also validated that IL-8 protein was highly expressed in tumour cells of lung adenocarcinoma specimens with high *IL-8* mRNA levels (Supplementary Figure 2) and that *IL-8* mRNA expression was significantly correlated with IL-8 protein score ($r = 0.526$, $P < 0.001$), indicating that the results obtained from the *IL-8* mRNA expression analysis are also valid at the protein level.

We next examined the association between *IL-8* expression and the mutation status of *KRAS* and *EGFR*. *IL-8* was differentially expressed among groups in which the mutation status of *KRAS* and *EGFR* differed (Figure 1E; $P = 0.009$); *IL-8* was more highly expressed in tumours with *KRAS* mutations than in those with wild-type *KRAS/EGFR* or with *EGFR* mutations. We further examined whether *IL-8* expression levels differed according to age or smoking history in these three subgroups. In both *KRAS*-mutant and *EGFR*-mutant subgroups, *IL-8* expression was significantly higher in tumours from patients who were $\geqslant 70$ years old (Figure 1F; *KRAS*-mutants: $P = 0.014$; *EGFR*-mutants: $P = 0.002$). There was a significant correlation between age and *IL-8* expression (Figure 1G; *KRAS*-mutants: $r = 0.628$, $P = 0.001$; *EGFR*-mutants: $r = 0.452$, $P < 0.001$). When these subgroups were further classified by smoking history, the significant correlation between age and *IL-8* expression was limited to the non-smoker subgroups (Figure 1H; *KRAS*-mutants: $r = 0.886$, $P = 0.033$; *EGFR*-mutants: $r = 0.549$, $P < 0.001$). In addition, *IL-8* expression was higher in the tumours of smokers than in those of non-smokers with borderline significance only in the *EGFR*-mutant subgroup (Figure 1I; $P = 0.054$).

As IL-8 has been well described as an angiogenic factor, we evaluated expression of CD34, an angiogenic marker, in the tumour specimens from lung adenocarcinoma patients. There is a significant correlation between CD34 scores and *IL-8* mRNA levels (Figure 2A; $r = 0.221$, $P = 0.011$). When the tumour specimens were classified according to *EGFR* and *KRAS* mutation status, the correlation between CD34 and *IL-8* expression appears to be stronger in *KRAS*-mutated tumours ($r = 0.352$, $P = 0.108$) compared with those with *EGFR*-mutant ($r = 0.096$, $P = 0.495$) or those with wild-type *EGFR/KRAS* ($r = 0.133$, $P = 0.329$), although there were no significant correlations in each group. Furthermore, when
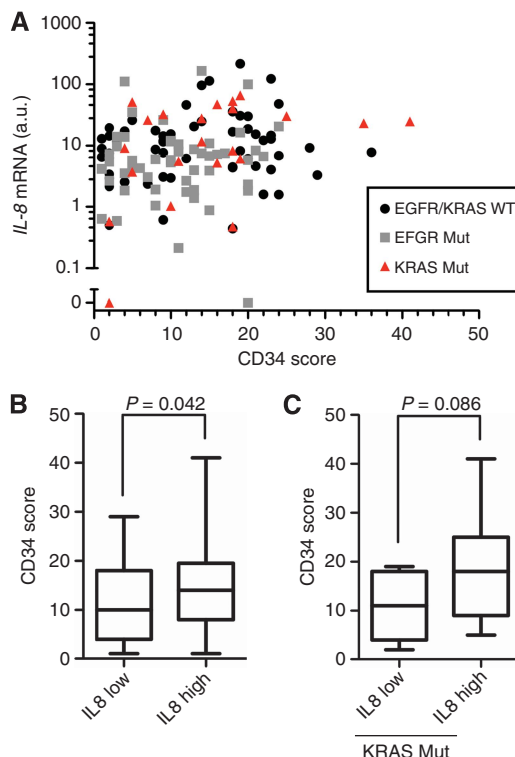

Figure 2. (**A**) Correlations between *IL-8* expression and CD34 score in lung adenocarcinomas. Grey squares: *EGFR*-mutants; red triangles: *KRAS*-mutants; black circles: tumours with wild-type *EGFR*/KRAS. Comparisons of CD34 score between lung adenocarcinomas with high *IL-8* expression and those with low *IL-8* expression (cut-off point: the median *IL-8* level) in (**B**) the whole group or (**C**) the *KRAS*-mutants subgroup.

the tumours were divided into two groups according to *IL-8* mRNA expression levels, CD34 levels were significantly higher in tumours with high *IL-8* than in those with low *IL-8* (Figure 2B; $P = 0.042$). In the *KRAS*-mutated tumours, CD34 levels tended to be higher in tumours with high *IL-8* levels than in those with low *IL-8* levels (Figure 2C; $P = 0.086$). These findings suggest that IL-8 could have an important role in tumour progression by participating tumour angiogenesis, especially in *KRAS*-mutated lung adenocarcinomas.

We next assessed the prognostic significance of *IL-8* expression in 119 patients with lung adenocarcinomas who had not received EGFR–TKI therapy. The patients with high *IL-8* expression had significantly shorter DFS (Figure 3A; $P = 0.006$) and OS (Figure 3B; $P = 0.005$) compared with those with low *IL-8* expression. Considering that oncogenic *KRAS*-induced IL-8 over-expression contributes to aggressive phenotypes of NSCLC (Sunaga *et al*, 2012), we hypothesised that *KRAS* mutation would negatively affect the survival of lung adenocarcinoma patients who also have high *IL-8* expression. When the patient population was classified into four groups according to *IL-8* expression level and *KRAS* mutation status, the patients with *IL-8*-high and *KRAS*-mutant tumours had significantly shorter DFS (Figure 3C; $P = 0.001$) and OS (Figure 3D; $P = 0.007$) than those with *IL-8*-low and *KRAS* wild-type tumours. Furthermore, univariate Cox regression analysis revealed that smoking history, advanced pathological stage, presence of PI, lymphatic permeation (LP) or VI, presence of *KRAS* mutation, absence of *EGFR* mutation and elevated *IL-8* expression were all unfavourable prognostic factors for DFS (Table 1). Similarly, smoking history, advanced pathological stage, presence of PI, LP or VI, presence of *KRAS* mutation and elevated *IL-8* expression were unfavourable prognostic factors for OS
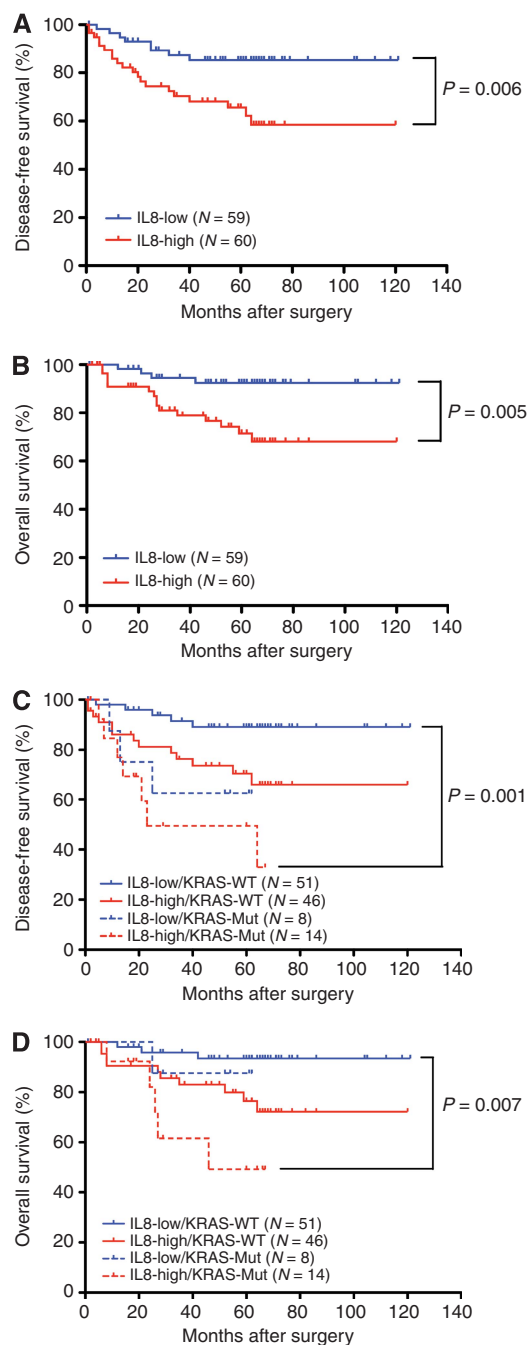
Figure 3. Kaplan–Meier analysis of (A) disease-free survival (DFS) and (B) overall survival (OS) in 119 lung adenocarcinoma patients who had not received EGFR–TKI therapy. The patients were classified into two groups with low *IL-8* expression and those with high *IL-8* expression according to the median *IL-8* level. Differences in DFS and OS were analysed with the log-rank test. Comparison of (C) DFS and (D) OS in four subgroups according to *IL-8* expression levels and *KRAS* mutation status. KRAS-WT, *KRAS* wild-type; KRAS-Mut, *KRAS*-mutant. Differences in DFS and OS between IL-8-High/KRAS-Mut and IL-8-Low/KRAS-WT were analysed with the log-rank test with Bonferroni's correction for multiple comparisons.

(Table 1). Multivariate analysis showed that elevated *IL-8* expression remained an unfavourable prognostic factor for both DFS and OS even after adjustment for pathological stage (Table 1). These results indicate that elevated *IL-8* expression is a biomarker for unfavourable prognosis in lung adenocarcinoma patients, especially in those with *KRAS* mutations.

DISCUSSION

We recently reported the association between *IL-8* expression, *KRAS* mutations and certain clinicopathological parameters of NSCLC and the potential role of IL-8 as a therapeutic target (Sunaga *et al*, 2012). Here, we extended our recent study to include 136 primary lung adenocarcinomas, to further elucidate the clinicopathological and prognostic significance of *IL-8* expression in lung adenocarcinomas. We found that *IL-8* expression was higher in lung adenocarcinomas from elderly patients and smokers. A recent study demonstrated that an increased level of circulating IL-8 is associated with a lung cancer risk several years before diagnosis (Pine *et al*, 2011), suggesting that IL-8 has a critical role in lung tumour initiation. In fact, IL-8 was shown to be upregulated in smokers with lung cancer in a microarray study comparing gene expression in normal large-airway epithelial cells between smokers with or without lung cancer (Spira *et al*, 2007). Other studies have implicated tobacco smoking as one of the possible mechanisms for IL-8 upregulation in bronchial epithelial cells (Kuschner *et al*, 1996; Mio *et al*, 1997; Chalmers *et al*, 2001). It is thus likely that IL-8 upregulation in bronchial epithelial cells induced by tobacco smoking is involved in the carcinogenesis of lung adenocarcinomas. On the other hand, *IL-8* expression positively correlated with age in our non-smoker subgroup. Cellular senescence is part of the aging process (Collado *et al*, 2007; Lopez-Otin *et al*, 2013), and is accompanied by a senescence-associated secretory phenotype (SASP) that involves a marked increase in the secretion of proinflammatory cytokines (Coppe *et al*, 2008). SASP is thought to have a role in tumour progression (Davalos *et al*, 2010), and one of the most upregulated cytokines in SASP is IL-8, which is expressed in premalignant epithelial cells (Kuilman *et al*, 2008; Davalos *et al*, 2010). Therefore, age-related IL-8 upregulation in bronchial epithelial cells may be one of the mechanisms that lead to the development of lung adenocarcinomas in non-smokers. Further investigation regarding the *IL-8* levels in normal lung tissues stratified by age are needed to address this issue.

In the present study, *IL-8* was preferentially expressed in lung adenocarcinomas with pleural involvement and vascular invasion, which contribute to aggressive tumour phenotypes. Furthermore, elevated IL-8 expression was associated with unfavourable prognosis in lung adenocarcinoma patients. Our results are consistent with those of previous studies that showed a correlation between high *IL-8* mRNA expression or IL-8 protein expression and poor prognosis in NSCLC patients (Bellocq *et al*, 1998; Yuan *et al*, 2000; Chen *et al*, 2003; Seike *et al*, 2007), although the number of lung adenocarcinoma patients was relatively small in these studies. In addition, we found that the presence of *KRAS* mutation along with high IL-8 expression in tumours was associated with the worst prognosis for patients with lung adenocarcinoma. Given that *KRAS* mutation induces IL-8 over-expression in lung adenocarcinoma cells (Sunaga *et al*, 2012), elevated IL-8 expression is likely to be a biomarker for predicting unfavourable clinical outcome in patients with lung adenocarcinoma who have *KRAS* mutations.

Many human cancers, including lung adenocarcinomas, over-express IL-8 (Xie, 2001; Sunaga *et al*, 2012). Secreted IL-8 from tumour cells activates downstream signalling pathways that regulate many cellular processes including cell proliferation, migration, invasion and angiogenesis to promote tumour progression and metastasis (Waugh and Wilson, 2008). In particular, IL-8 is a well-known angiogenic factor and intratumoural IL-8 expression has been shown to be associated with angiogenesis in NSCLC (Yuan *et al*, 2000; Masuya *et al*, 2001; Boldrini *et al*, 2005). Anti-angiogenesis therapy is a key strategy for treating NSCLC. Several anti-angiogenic agents including bevacizumab are currently

Table 1. Cox regression analysis for survival in 119 patients with lung adenocarcinomas

Variable	Hazard ratio	95% Confidence interval	*P*-value
Disease-free survival			
Univariate analysis			
Age (≥70 *vs* <70)	1.194	0.576–2.476	0.634
Gender (male *vs* female)	1.644	0.787–3.434	0.186
Smoking history (smoker *vs* non-smoker)	2.650	1.227–5.723	0.013
Stage (III–IV *vs* I–II)	9.048	4.305–19.018	<0.001
Pleural involvement (positive *vs* negative)	7.116	3.135–16.154	<0.001
Lymphatic permeation (positive *vs* negative)	3.912	1.771–8.639	<0.001
Vascular invasion (positive *vs* negative)	9.514	3.596–25.170	<0.001
KRAS gene (mutation *vs* wild-type)	3.093	1.426–6.709	0.004
EGFR gene (mutation *vs* wild-type)	0.381	0.155–0.939	0.036
IL8 expression (as continuous variable)	1.011	1.006–1.017	<0.001
Multivariate analysis			
Stage (III–IV *vs* I–II)	9.594	4.484–20.530	<0.001
IL-8 expression (as continuous variable)	1.013	1.007–1.020	<0.001
Overall survival			
Univariate analysis			
Age (≥70 *vs* <70)	1.428	0.591–3.450	0.428
Gender (male *vs* female)	2.063	0.839–5.075	0.115
Smoking history (smoker *vs* non-smoker)	2.622	1.041–6.601	0.041
Stage (III–IV *vs* I–II)	10.637	4.313–26.230	<0.001
Pleural involvement (positive *vs* negative)	9.613	3.205–28.839	<0.001
Lymphatic permeation (positive *vs* negative)	6.973	2.319–20.967	<0.001
Vascular invasion (positive *vs* negative)	10.116	2.946–34.735	<0.001
KRAS gene (mutation *vs* wild-type)	2.480	0.944–6.516	0.065
EGFR gene (mutation *vs* wild-type)	0.371	0.124–1.113	0.077
IL-8 expression (as continuous variable)	1.012	1.006–1.018	<0.001
Multivariate analysis			
Stage (III–IV *vs* I–II)	10.700	4.254–26.913	<0.001
IL-8 expression (as continuous variable)	1.014	1.007–1.022	<0.001

used or being tested for the treatment of NSCLC patients (Horn and Sandler, 2009). In addition, several researchers including our group have demonstrated that attenuation of IL-8 leads to inhibition of cell proliferation, migration and angiogenesis in NSCLC (Smith *et al*, 1994; Arenberg *et al*, 1996; Yatsunami *et al*, 1997; Zhu *et al*, 2004; Sunaga *et al*, 2012). Importantly, IL-8 was shown to be upregulated in human cancers including NSCLC harbouring the oncogenic *KRAS* mutation (Sparmann and Bar-Sagi, 2004; Wislez *et al*, 2006; Sunaga *et al*, 2012), which is one of the most common driver mutations in lung adenocarcinoma (Pao and Hutchinson, 2012). Thus, targeting IL-8 may be an attractive therapeutic option to improve the survival of patients with oncogenic KRAS-driven lung adenocarcinoma.

In conclusion, the present study suggests that IL-8 expression is a predictor of unfavourable prognosis for lung adenocarcinoma patients, especially for those with *KRAS* mutation. Although tobacco smoking is likely to be one mechanism that is linked to IL-8 upregulation, aging may be the main cause of IL-8 overexpression in lung adenocarcinomas arising in non-smokers. Our findings also raise the possibility that an anti-IL-8 therapeutic strategy could improve the clinical outcome in patients with oncogenic KRAS-driven lung adenocarcinoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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