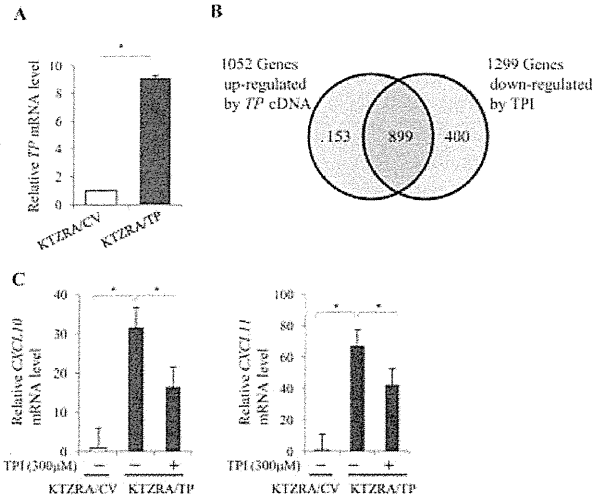

Figure 2. Genes inducible by thymidine phosphorylase (TP) in fibroblast-like synoviocytes. **A**, *TP* (TYMP) mRNA levels in KTZRA cells transfected with a control vector (KTZRA/CV) or *TYMP* complementary DNA (KTZRA/TP), treated with or without thymidine phosphorylase inhibitor (TPI), and incubated for 48 hours, as determined by real-time polymerase chain reaction (PCR). **B**, Microarray analysis using KTZRA/CV cells, KTZRA/TP cells, and KTZRA/TP cells treated with TPI. **C**, Relative expression of *CXCL10* and *CXCL11* mRNA, as determined by real-time PCR. Values in **A** and **C** are the mean ± SD of triplicate experiments. * = $P < 0.01$; ** = $P < 0.05$.

TP-induced genes using microarray analysis. TP has been reported to increase the expression of IL-6, IL-8, vascular endothelial growth factor (VEGF), matrix metalloproteinase 1 (MMP-1), and MMP-3 in cancer cells (3,7). We examined whether TNFα-induced TP enhanced the expression of these genes in FLS. The expression of *TYMP, IL6, IL8, MMP1*, and *MMP3,* but not *VEGF*, was induced by TNFα in KTZRA cells (data not shown). However, both an enzymatic inhibitor of TP at a dose of 300 μ*M* and *TYMP* siRNA did not markedly suppress the expression of these TNFα-induced genes in KTZRA cells or IORA cells (data not shown). TPI at a dose of 300 μ*M* was not cytotoxic to KTZRA cells (data not shown).

We speculated that a large part of the enhanced expression of these genes was caused directly by TNFα. Therefore, in order to explore the TP-induced genes in FLS, we performed microarray analysis of KTZRA cells transfected with a control vector (KTZRA/CV), KTZRA cells transfected with *TYMP* cDNA (KTZRA/TP), and KTZRA/TP cells treated with TPI for 48 hours. We confirmed the expression of *TYMP* mRNA in KTZRA/TP cells (Figure 2A).

Among ~30,000 genes tested using an Agilent Whole-Genome microarray, 1,052 genes were up-regulated more than 2-fold above control in KTZRA/TP cells. When KTZRA/TP cells were treated with TPI, 1,299 genes were down-regulated. Among the genes that were up-regulated by TP, 899 genes were down-regulated by TPI (Figure 2B). Among these 899 genes, we observed 19 that were reportedly related to RA (Table 1). We confirmed the expression of these and other genes by real-time PCR (data not shown). In particular, the expression of *CXCL10* and *CXCL11* mRNA in KTZRA/TP cells was more than 30-fold higher than that in KTZRA/CV cells (Figure 2C). Both CXCL10 and CXCL11 bind to CXCR3, which enhances the migration of Th1 cells and contributes to the Th1 phenotype of RA (11). Moreover, CXCL10 regulates FLS invasion (14) and bone destruction (20) in RA. We thus focused our study on CXCL10.

Enhanced expression of *TYMP*-induced *CXCL10* by the combination of TNFα and IFNγ. We next examined the expression of TP and CXCL10 in KTZRA cells

Table 1. Genes shown to be related to thymidine phosphorylase (TP) by microarray analysis

	Gene	GenBank accession no.	Fold change: KTZRA/TP to KTZRA/CV*	Fold change: KTZRA/TP + TPI to KTZRA/TP†
1	*CXCL11*	NM_005409	48.16	0.78
2	*CXCL10*	NM_001565	30.34	0.83
3	*HSPA12B*	NM_001197327	11.67	0.06
4	*CD40*	NM_001250	7.06	0.84
5	*CCL3*	NM_002983	6.34	0.73
6	*IL32*	NM_001012631	5.34	0.93
7	*EGR4*	NM_001965	4.19	0.14
8	*SAA1*	NM_000331	3.34	0.77
9	*ERBB4*	NM_001042599	3.22	0.42
10	*IL24*	NM_001185156	3.13	0.61
11	*CCL7*	NM_006273	3.03	0.95
12	*ANGPTL4*	NM_001039667	2.82	0.84
13	*CCL5*	NM_002985	2.72	0.81
14	*TNFSF13B*	NM_006573	2.48	0.63
15	*IL5*	NM_000879	2.47	0.69
16	*PIK3CG*	NM_002649	2.43	0.35
17	*PIK3AP1*	NM_152309	2.32	0.70
18	*HLA-DQA1*	NM_002122	2.31	0.43
19	*ADAMTS4*	NM_005099	2.06	0.87

* Messenger RNA level in KTZRA cells transfected with *TP* (*TYMP*) cDNA (KTZRA/TP) divided by that in KTZRA cells transfected with a control vector (KTZRA/CV). Values >1 indicate that the gene is induced by TP.

† Messenger RNA level in KTZRA/TP cells treated with 300 μ*M* TP inhibitor (TPI) divided by that in KTZRA/TP cells not treated with TPI. Values <1 indicate that the TP-induced gene is suppressed by TPI.

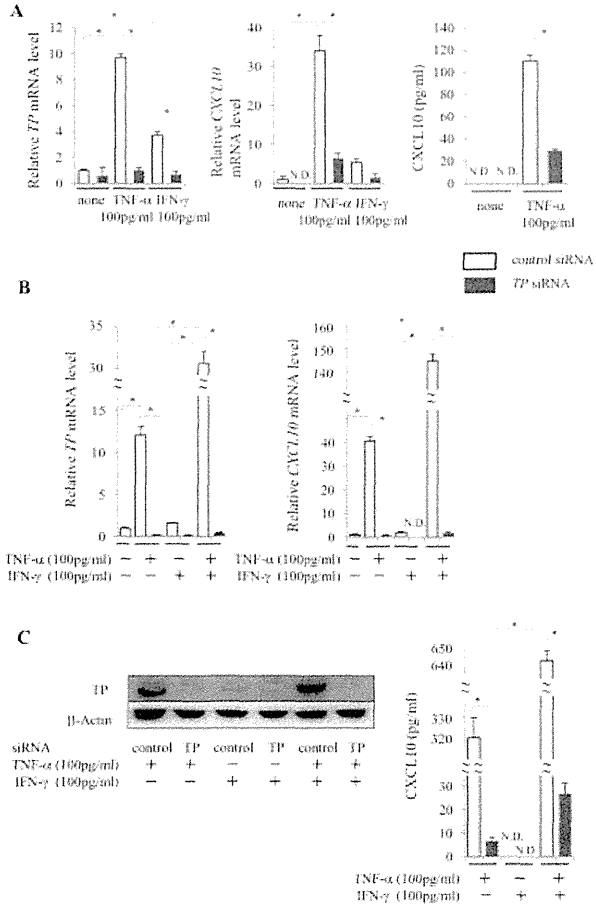

Figure 3. Synergistic effect of TNFα and IFNγ on thymidine phosphorylase (TP) and CXCL10 expression. **A**, Left and middle, Expression of *TP* (*TYMP*) (left) and *CXCL10* (middle) mRNA in KTZRA cells transfected with control small interfering RNA (siRNA) or *TYMP* siRNA and incubated with or without TNFα or IFNγ for 48 hours, as determined by real-time polymerase chain reaction (PCR). Right, CXCL10 protein secretion in the culture medium of the transfected KTZRA cells in the absence or presence of TNFα, as determined by enzyme-linked immunosorbent assay (ELISA). **B**, Expression of *TYMP* (left) and *CXCL10* (right) mRNA levels in KTZRA cells treated with or without TNFα alone, IFNγ alone, or the combination of TNFα and IFNγ, as determined by real-time PCR. **C**, Expression of TP protein in cell lysates and of CXCL10 protein in culture supernatants, as determined by immunoblotting (left) and ELISA (right). Values are the mean ± SD of triplicate experiments. * = $P < 0.01$. N.D. = not determined (see Figure 1 for other definitions).

(Figure 3A) and IORA cells (data not shown). In this experiment, we used clinically relevant doses of TNFα or IFNγ in the synovial fluid of patients with RA (21–23). The expression of *TYMP* mRNA was increased by both TNFα and IFNγ, and this increase was suppressed by *TYMP* siRNA (Figure 3A). CXCL10 was also induced by TNFα at a dose of 100 pg/ml, and this induction was suppressed by *TYMP* siRNA (Figure 3A). TPI reduced the expression of *CXCL10* mRNA in KTZRA cells treated with TNFα (data not shown). These results suggested that the enzymatic activity of TPI is essential for the induction of CXCL10. The expression of *CXCL10* mRNA was not induced by a clinically relevant dose of IFNγ (100 pg/ml) (Figure 3A), but a higher concentration of IFNγ (1 ng/ml) significantly augmented the expression of *CXCL10* (data not shown).

The combination of TNFα and IFNγ synergistically enhanced the expression of TP and CXCL10 (Figures 3B and C) in KTZRA cells. Although TNFα alone up-regulated the expression of TP and CXCL10, IFNγ alone did not significantly augment the expression of CXCL10. However, IFNγ potentiated the effect of TNFα on the expression of TP and CXCL10 in KTZRA cells. The induction of CXCL10 by both cytokines was inhibited by *TYMP* siRNA (Figure 3C). These findings indicated that TNFα and IFNγ synergistically induced the expression of TP, which increased CXCL10 expression. Meanwhile, the expression of *CXCL10* mRNA was

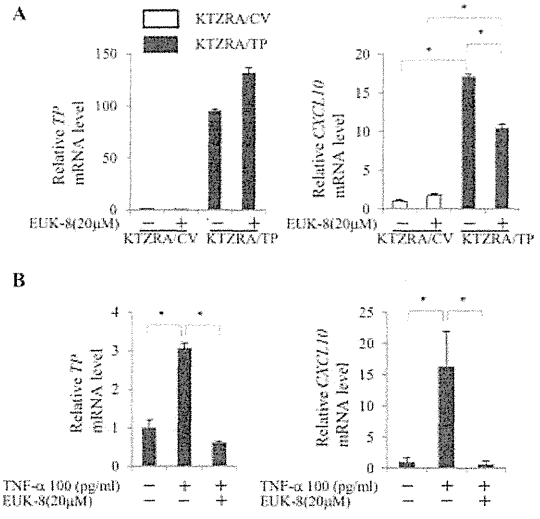

Figure 4. Effect of EUK-8 on *TP* (*TYMP*) and *CXCL10* expression. **A**, Expression of TP (left) and *CXCL10* (right) mRNA in KTZRA cells transfected with a control vector (KTZRA/CV) or thymidine phosphorylase complementary DNA (KTZRA/TP), treated with or without EUK-8, and incubated for 48 hours, as determined by real-time polymerase chain reaction (PCR). **B**, Expression of *TYMP* (left) and *CXCL10* (right) mRNA in KTZRA cells incubated in the presence or absence of TNFα with or without EUK-8 for 48 hours, as determined by real-time PCR. Values are the mean ± SD of triplicate experiments. * = $P < 0.01$. See Figure 1 for other definitions.

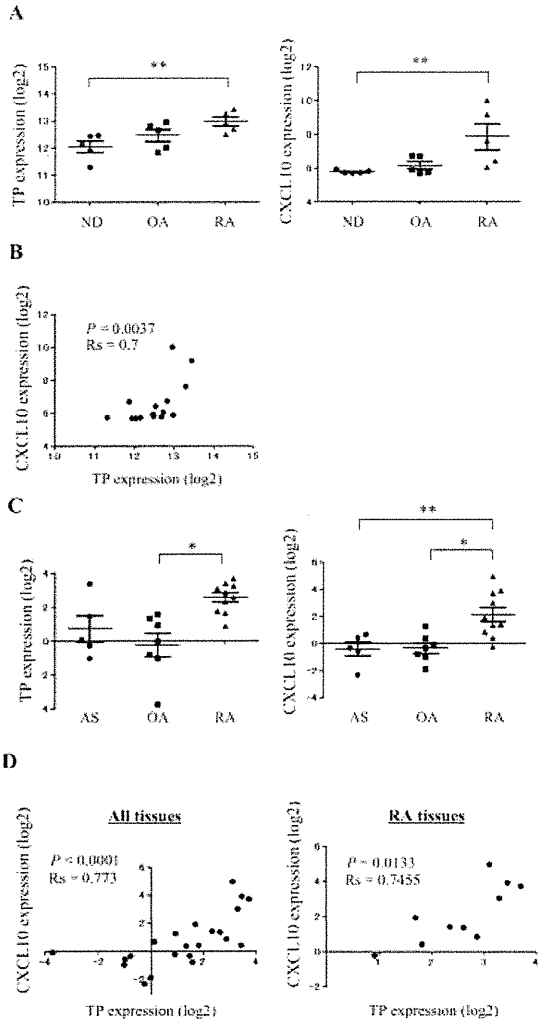

Figure 5. Evaluation of thymidine phosphorylase (TP) and CXCL10 expression in synovial tissue, using the GSE1919 and GSE39340. **A,** Expression of thymidine phosphorylase (TP) and CXCL10 in synovial tissue from normal donors (NDs), patients with osteoarthritis (OA), and patients with rheumatoid arthritis (RA), as determined using the GSE1919. **B,** Correlation between TP levels and CXCL10 levels in all tissue samples, as determined using the GSE1919. **C,** Expression of TP and CXCL10 in synovial tissue from patients with ankylosing spondylitis (AS), patients with OA, and patients with RA, as determined using GSE39340. **D,** Correlation between TP levels and CXCL10 levels in all tissue (left) and in RA tissue only (right) as determined using GSE39340. In **A** and **C,** each symbol represents an individual sample. Bars show the mean ± SD. In **B** and **D,** each symbol represents an individual subject. * = $P < 0.01$; ** = $P < 0.05$.

enhanced with increasing concentrations of IL-1β and LPS but not IL-17 (data not shown). However, IL-1β–induced *CXCL10* expression was not significantly suppressed by TPI (data not shown), indicating that the induction of *CXCL10* by IL-1β may not be regulated by TP.

Effect of antioxidant on the expression of *TYMP*-induced *CXCL10* in FLS. We previously reported that TP enhanced the generation of reactive oxygen species (ROS) in cancer cells, and that the enzymatic activity of TP was required for the generation of ROS (24). To examine whether TP induces CXCL10 expression through ROS generation in FLS, KTZRA/TP cells (Figure 4A) and KTZRA cells treated with TNFα (Figure 4B) were treated with the antioxidant EUK-8. The increased *CXCL10* expression in KTZRA/TP cells was significantly suppressed by EUK-8 (Figure 4A). The increased expression of *CXCL10* induced by TNFα was also significantly attenuated by EUK-8 (Figure 4B). These results suggested that TP augments CXCL10 expression through ROS production. Interestingly, the TNFα-induced *TYMP* expression observed in KTZRA cells was attenuated by EUK-8 (Figure 4B). ROS may also be involved in the increased expression of *TYMP* by TNFα.

Expression of TP and CXCL10 in the synovial tissue of patients with RA. We investigated TP and CXCL10 levels in synovial tissue using the Ungethuem data set (GSE1919) and the Chang data set (GSE39340) of the GEO. The Ungethuem data set included gene expression levels in synovial tissue from 5 normal donors, 5 patients with OA, and 5 patients with RA. The Chang data set included gene expression levels in synovial tissue from 5 patients with ankylosing spondylitis, 7 patients with OA, and 10 patients with RA.

We first used the Ungethuem data set. The expression levels of TP and CXCL10 in RA tissue were higher than those in tissue from normal donors (Figure 5A). The expression levels of TP and CXCL10 in OA tissue were not significantly different from those in normal donors. TP levels in all of these tissues were correlated with CXCL10 expression (Figure 5B). In the Chang data set, the expression of TP and CXCL10 was higher in RA tissue than in OA and AS tissue (Figure 5C). TP levels were significantly correlated with CXCL10 levels in all tissues and in RA tissue alone (Figure 5D). These results suggested that the participation of TP in the induction of CXCL10 expression is clinically relevant.

DISCUSSION

In the present study, we demonstrated that TP is involved in the expression of CXCL10 in FLS. More-

over, the expression of TP in FLS was markedly up-regulated by the combination of TNFα and IFNγ. Because the induction of CXCL10 activated by TNFα and IFNγ in FLS was markedly suppressed by the knockdown of TP, TP may play a critical role in the induction of CXCL10 by these cytokines. To the best of our knowledge, this study is the first to demonstrate the augmentation of CXCL10 expression by TP in RA FLS.

CXCL10 levels in the synovial fluid and synovial tissue of patients with RA are increased, and serum levels of CXCL10 are correlated with RA disease activity (25,26). A phase II clinical trial using an anti-CXCL10 monoclonal antibody (MDX-1100) in patients with RA has been previously described (27). MDX-1100 administered every 2 weeks for 12 weeks in combination with weekly methotrexate led to a statistically significant improvement in the American College of Rheumatology 20% response (28) compared with placebo (27). These studies showed that CXCL10 contributed to the pathogenesis of RA and is a promising molecular target for anti-RA therapy.

CXCR3, a CXCL10 receptor, is expressed on several immune cell types, such as natural killer cells, plasmacytoid and myeloid dendritic cells, B cells, and, especially, Th1 cells. Accumulating evidence has suggested that CXCR3 ligands are involved in the selective recruitment of Th1 cells into the site of tissue inflammation (29,30). CXCL10 expression was increased in a mouse model of collagen-induced arthritis and played a critical role in the infiltration of Th1 cells (20). We observed that the induction of TP by TNFα and IFNγ increased the expression of CXCL10 in FLS, indicating that TP induced by these cytokines may contribute to the Th1 phenotype in RA. Meanwhile, Laragione et al reported that CXCL10 and its receptor CXCR3 enhanced the invasion of synovial fibroblasts in RA (14).

We previously demonstrated that TP is involved in the invasion and metastasis of some solid tumors (3). We also showed that TP increased the expression of MMP-1, MMP-9, IL-8, and VEGF in cancer cells (6). Leek et al suggested that TNFα produced by neighboring tumor-associated macrophages may play a role in the regulation of TP expression in tumor cells, as well as their metastatic behavior (31). TP may promote the invasive activity of FLS as well as carcinoma cells by enhancing the expression of CXCL10. Kwak et al demonstrated that CXCL10 played a critical role in the infiltration of Th1 cells and macrophages into inflamed joints (20). CXCL10 promoted the expression of RANKL in Th1 cells, which caused bone destruction by enhancing osteoclast differentiation (20). We demonstrated that TP induced the expression of CXCL10 in RA FLS. These findings suggested that TP is involved in the Th1 phenotype and bone destruction observed in the pathogenesis of RA.

Besides *CXCL10* and *CXCL11*, many genes, including *CCL3, IL32, CD40, ANGPTL4, CCL7*, and *ADAMTS4*, were also induced by TP. CCL3 as well as CXCL10 recruit immune cells, including Th1 cells. Genome-wide haplotype association and gene prioritization identified *CCL3* as a risk locus for RA (32). IL-32 was a potent inducer of prostaglandin E_2 release in mouse macrophages and human monocytes (33). The level of IL-32 in synovial tissue from patients with RA was correlated with the erythrocyte sedimentation rate, which is a marker of systemic inflammation (33). The interaction of CD40 on the surface of FLS with CD40L expressed on activated T lymphocytes enhanced the production of VEGF and, consequently, neovascularization in RA (34). ANGPTL4 also had angiogenic activity in a mouse model of CIA (35) and enhanced the expression of MMP-1 and MMP-3 in chondrocytes (36). TP may cause pleiotropic effects on RA pathogenesis by enhancing the expression of these genes.

Kusabe et al showed that antirheumatic drugs such as aurothioglucose and dexamethasone suppressed IL-1β–induced TP expression in FLS (37). These investigators also demonstrated that the immunosuppressant FK-506 (tacrolimus), which has been approved as an antirheumatic drug in Japan, inhibited the level of TP that was increased by TNFα (38). We confirmed that aurothioglucose and FK-506 suppressed the expression of *TYMP* and *CXCL10* mRNA in RA FLS (data not shown). These results suggested that the antirheumatic effect of these drugs may be attributable, at least in part, to antiangiogenic and antiarthriogenic activities following the down-regulation of TP.

In the present study, both *TYMP* siRNA and TPI inhibited the expression of TP as well as CXCL10 induced by TNFα, with or without IFNγ, in FLS. It is still unclear how the enzymatic activity of TP is involved in the expression of TP and CXCL10. Further study is required to clarify this mechanism.

TP generates ROS and increases proinflammatory cytokines in cancer cells (39). We previously reported that TP augments ROS generation in cancer cells, and that the enzymatic activity of TP was required for the generation of ROS (24). In the current study, we examined whether TP induces CXCL10 expression through ROS generation in RA FLS. The TP-induced CXCL10 expression was suppressed by the antioxidant EUK-8. Furthermore, the induction of TP expression by

TNFα in FLS was also suppressed with EUK-8 treatment. These findings suggested that TNFα augmented TP expression through ROS production, and TP also enhanced the ROS level and consequently increased CXCL10 expression.

In conclusion, we demonstrated that TP regulated the expression of CXCL10 in RA FLS via enzymatic activity. Therefore, compounds that inhibit TP activity, such as TPI, may be good candidates for new anti-RA agents.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nishioka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Toyoda, Tabata, Akiyama, Nishioka.

Acquisition of data. Toyoda, Tabata, Kuramoto, Mitsuhashi, Saijo, Horikawa.

Analysis and interpretation of data. Toyoda, Tabata, Kishi, Kawano, Goto, Aono, Hanibuchi, Horikawa, Nakajima, Furukawa, Sone, Akiyama, Nishioka.

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REVIEW

Role of surfactant protein A in non-infectious lung diseases

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Abstract : Surfactant protein A (SP-A) is a large multimeric protein found in the airways and alveoli of the lungs. SP-A is a member of the collectin family of proteins, characterized by NH_2-terminal collagen-like regions and COOH-terminal lectin domains. Although other surfactant proteins such as SP-B function to reduce surface tension in the lungs, SP-A as well as SP-D regulates the pulmonary immune response. To date, a number of studies have shown the immunoregulatory function of SP-A, mainly in the field of infectious diseases. By binding to a wide variety of pathogens, SP-A opsonizes and enhances pathogen uptake by phagocytes. In addition to the effect on pathogens, recent studies have shown that SP-A also modulates lung immune system in the area of non-infectious lung diseases. In this review, the potential role of SP-A in the multiple aspects of pulmonary host defense will be discussed, focusing mainly on non-infectious lung diseases such as acute and chronic pulmonary fibrosis and lung cancer. J. Med. Invest. 61 : 1-6, February, 2014

Keywords **:** *surfactant protein A, non-infectious lung injury, lung cancer*

INTRODUCTION

Pulmonary surfactant was initially identified as a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung (1, 2). This definition has been reassessed with recent studies showing that surfactant also functions in pulmonary host defense. Surfactant is mostly composed of phospholipids that are essential for reducing surface tension at the air-liquid interface of the lung. About 10% of surfactant consists of protein ; four surfactant proteins (SPs) have been defined : SP-A, SP-B, SP-C and SP-D. Among these proteins, SP-B and SP-C are small and hydrophobic, and SP-B is essential for the ability of surfactant to reduce surface tension (3). On the other hand, the host-defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of proteins.

Collectins are distinguished by their amino (N)-terminal collagen-like regions that have a repeating triple helix of Gly-X-Y triplets, where X denotes any amino acid and Y is often a hydroxyproline residue. The carboxy-terminal domains of the collectins all have C-type (calcium-dependent) lectin activity. The lectin domains mediate the interaction of collectins with a wide variety of pathogens. The most well-understood consequence of this interaction is pathogen opsonization and enhanced uptake by phagocytes. SP-A and SP-D are synthesized as primary translation products of approximately 26-36 kDa and 43 kDa, respectively. The collagen-like domain is N-terminal to a coiled-coil structure that precedes the lectin domain. The collectins are assembled as trimeric subunits, which multimerize to varying degrees. SP-A is mainly an octadecamer and forms a bouquet-like structure, whereas SP-D forms a dodecamer (Figure 1). SP-A structurally

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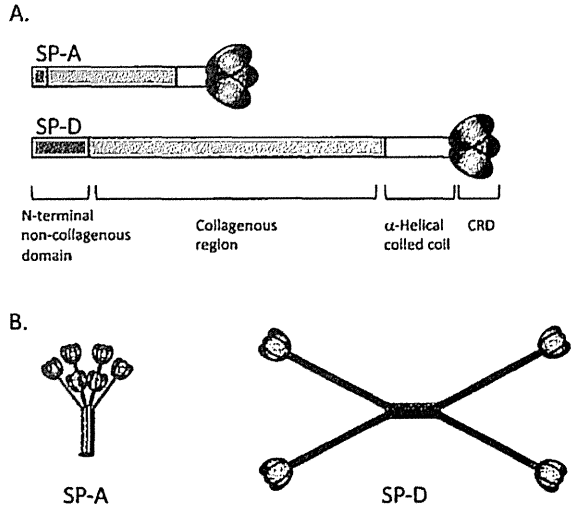

Figure 1. SP-A and SP-D are members of a family of proteins known as collectins. (A) Collectins have collagen-like amino (N)-terminal regions and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). Collectins consist of structural subunits that are composed of trimeric polypeptide chains. The trimers are assembled into oligomers. SP-A is octadecamer (18-mer), consisting of six trimeric subunits. SP-D is a dodecamer (12-mer), consisting of four trimeric subunits. The models are not drawn to scale.

resemble the first component of complement, C1q, however, C1q does not contain a lectin domain, although it does have a collagen-like N-terminal triple helix. In humans, the genes that encode SP-A and SP-D have been mapped to a cluster on the long arm of chromosome 10 (4-7).

Since SP-A and SP-D are the members of the collectin family, the immunoregulatory functions of SP-A and SP-D have been studied mainly in the field of infectious diseases. They are able to bind to a variety of bacteria, viruses, allergens and apoptotic cells and thereby function as opsonins to enhance the uptake of these cells and particles. Some pathogens are aggregated by SP-A and/or SP-D. They are also reported to have direct effects on immune cells and modulate the production of cytokines and inflammatory mediators (8, 9). On the other hand, recent clinical and experimental studies suggest that they are also involved in non-infectious lung diseases such as pulmonary fibrosis and lung cancer. In this review, we focus and discuss on diverse functions of SP-A in various lung diseases. An emphasis is placed on recent studies showing that SP-A has novel functions in regulating epithelial cell apoptosis in mouse bleomycin-induced acute lung injury model, and controlling the polarization of macrophages in lung cancer model.

SP-A AND ACUTE NON-INFECTIOUS LUNG INJURY

Acute respiratory distress syndrome (ARDS) is a clinical syndrome with acute lung injury characterized by significant hypoxemia with bilateral pulmonary infiltrates consistent with edema (10, 11). ARDS is caused by multiple etiologies including non-infectious lung diseases such as acute interstitial pneumonia and chemical pneumonia, as well as infectious diseases such as microbial pneumonia and sepsis. Despite decades of intense investigation, the fundamental mechanisms that initiate and control ARDS have not been elucidated, and conventional treatment plans such as high-dose glucocorticoid therapy is not effective in many cases and overall 28-day mortality of ARDS is fairly high (25-40%).

Several clinical studies reported the changes of SP-A expression in ARDS patients. They have demonstrated that SP-A concentration in the bronchoalveolar lavage (BAL) fluid was significantly lower in both established ARDS patients and patients at-risk for ARDS, compared to healthy volunteers (12, 13). On the other hand, SP-A levels were increased in the serum of patients with ARDS (12). These findings suggested that SP-A might play an important role in the pathogenesis of non-infectious lung injury. We therefore evaluated the contribution of SP-A in the pathogenesis of non-infectious lung injury using mouse bleomycin-induced lung injury model. We compared the development of bleomycin-induced acute lung injury in wild-type (WT) and SP-A -/- mice, and found that: (a) SP-A -/- mice were more susceptible to bleomycin induced death; (b) SP-A -/- mice exhibited higher level of inflammatory cytokine and high mobility group box (HMGB) 1 expression as well as increased vascular permeability compared to WT mice; and (c) exogenous SP-A administration rescued the phenotype of SP-A -/- mice. In combination with *in vitro* experiments, we have also shown that SP-A reduces apoptosis induced by bleomycin (14). These results suggested the importance of SP-A in the pathogenesis of non-infectious acute lung injury. Although the precise mechanism by which SP-A regulates epithelial cell apoptosis (especially the early stage of apoptosis; see ref. 14) needs to be further determined, this observation might also explain the increased vascular permeability and HMGB1 levels in SP-A -/- mice compared to WT mice subject to bleomycin treatment. As maintaining the integrity of the airway epithelium is a crucial step in the regulation of acute

lung injury, SP-A might have the defensive role in the pathogenesis of ARDS by regulating the epithelial cell apoptosis to maintain the integrity of the alveolar epithelium.

SP-A AND LUNG CANCER

Lung cancer is the major cause of malignancy-related death worldwide. The mortality rate is 80-90%, which makes this disease the leading cause of cancer-related death (15). The high mortality of this disease is primarily due to the difficulty of early diagnosis, the high metastatic potential, and poor responses to chemical or radiation therapy. Since there is no established curative therapy for advanced lung cancer to date, clinical management is palliative in many cases. Therefore, it is crucial to investigate and understand the underlying biological and molecular mechanisms of lung cancer progression.

In clinical studies, SP-A was expressed in approximately 49% of primary non-small cell lung carcinoma (16) and is used as specific marker of carcinoma that originates in type II pneumocytes. Additionally, a previous study demonstrated that deletion of the *SP-A* gene in non-small lung cancer cells was associated with tumor progression (17). Tsutsumida *et al.* found that lung adenocarcinoma patients with relatively high MUC1 mucin expression and low SP-A expression in cancer cells had poor outcome (18). These studies demonstrate that, in addition to use as a diagnostic marker, SP-A expression in lung cancer cells could be the useful biomarker of good prognosis. However, the role of SP-A in lung cancer has not been extensively studied and the mechanisms by which SP-A controls lung cancer progression is still unknown.

To determine the role of SP-A in lung cancer pathogenesis, we have generated SP-A over-expressing human lung adenocarcinoma cells, and showed that: (a) SP-A expression in cancer cells suppressed progression of lung adenocarcinoma in both xenograft and lung metastasis models; (b) SP-A inhibited lung cancer progression not by its direct effect on tumor cells but by regulating host microenvironment, including macrophages and natural killer (NK) cells; (c) SP-A increased the number of M1 tumor-associated macrophages (TAMs) in the tumor microenvironment, resulting in NK cell recruitment and activation within tumor tissue (Figure 2). These results suggested new immunoregulatory functions of SP-A, which is frequently expressed in

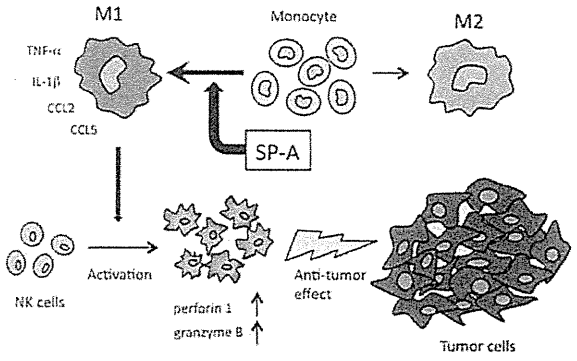

Figure 2. Possible role of SP-A on tumor progression. Although the precise mechanism by which SP-A regulates the polarization of TAMs is still unknown, SP-A increases the number of M1 TAMs in the tumor microenvironment, resulting in NK cell recruitment and activation within tumor tissue. Activated NK cells produce perforin 1 and granzyme B, which in turn can inhibit tumor growth. SP-A was confirmed not to have direct effect on NK cells (ref. 19).

pulmonary adenocarcinoma (19).

Considering the character of TAMs, it is now generally accepted that TAMs usually polarize to M2 and represent pro-tumoral functions (20). Indeed, we have seen in our study that approximately 60% of TAMs had M2 phenotype in vector-transduced tumors. However, when tumor cells expressed SP-A, this M1/M2 balance was reversed, and M1 anti-tumor (pro-inflammatory) macrophages became dominant. Subsequent analyses indicated that SP-A aided in making the TAMs M1-dominant by increasing the number of recruited M1 macrophages rather than shifting the M2 TAMs into M1 phenotype in the tumor microenvironment. In the field of infection, it is well known that the macrophages are one of the target cells that SP-A interacts with to regulate infectious inflammation. From this point of view, there might be a common pathways for SP-A to regulate macrophage-related inflammation between infectious lung diseases and lung cancer. For instance, SP-A may enhance the binding of cytokines to their respective receptors. SP-A is reported to bind to several receptors including Toll-like receptor (TLR) 2 and 4, and regulate inflammatory responses induced by pathogen-derived products such as peptidoglycan and LPS via TLRs (21-23). In addition to its role in TLR-mediated cellular responses induced by infectious challenges, it is very possible that SP-A regulates the function of TAMs in the tumor microenvironment through the interaction with TLRs. However, it is also considerable that the story is not so simple. We also showed in this study that SP-A activated only circulating monocytes/macrophages

while it showed no effect on resident alveolar macrophages (AMs) in cytokine expression. In the lung, the resident AMs are thought to acquire the tolerance against SP-A as they are continuously contacted by SP-A, which could be a plausible explanation, as the host needs to be protected from the overzealous inflammation in the lungs. Further studies are needed to understand the precise molecular mechanisms of the diverse and cell-specific function of SP-A against macrophages in the context of SP-A and lung cancer.

GENETIC VARIATIONS IN SP-A

Two functional genes of SP-A were detected in previous report (24) ; *SFTPA1* and *SFTPA2* that encode SP-A1 and SP-A2 protein, respectively. Although human SP-A1 and SP-A2 have a 96% degree of similarity at the protein level, these genes were differentially regulated by development (25) and have a minor difference in carbohydrate-binding activity (26), resulting in the functional differences. For example, SP-A2 exhibits a higher level of activity than SP-A1 in its ability to enhance inflammatory gene induction and pathogen clearance, potentially due to the increased stability of the protein (27, 28). Although a comprehensive documentation of the allelic variations of all SPs is beyond the scope of this review, we will introduce several common polymorphisms that occur in SP-A, as we are in the period to begin to understand the molecular mechanisms by which these polymorphisms affect SP function.

Selman *et al.* has reported that one SP-A1 ($6A^4$) allele and the single-nucleotide polymorphisms (SNPs) that characterize the $6A^4$ allele were found with higher frequency in idiopathic pulmonary fibrosis (29). More recently, Maitra *et al.* reported that BAL fluid from humans heterozygous for a missense mutation in *SFTPA2* which changes glycine at position 231 to valine (G231V) contained more transforming growth factor (TGF)-β1 than control samples, and expression of mutant SP-A2 in lung epithelial cells led to secretion of latent TGF-β1, which was capable of autocrine and paracrine signaling (30). These data suggest that therapeutic targeted to block the pathway induced by mutant SP-A2 might be especially beneficial for the molecularly defined subgroup of patients with pulmonary fibrosis.

CONCLUDING REMARKS

From decades ago, surfactant was recognized as a soap-like substance that reduced surface tension in the lung and made breathing easier. With the development of molecular and biological techniques, it was discovered that SP-A was structurally homologous to an immune protein of the complement cascade, C1q. Since then, an entire family of proteins has been identified, and the role of the innate immune system has garnered increasing attention. After the generation of knock-out mice, *in vivo* and *in vitro* data accumulation was accelerated, and now it is well known that SP-A (and SP-D) has the defensive role in infectious lung diseases by mediating various immune-cell functions. More recently, studies have shown novel roles for these proteins in non-infectious lung diseases. As presented in this review, SP-A plays a role in regulating apoptosis in the model of acute lung injury and controlling the polarization of macrophages in lung cancer model. Modern high molecular technologies would make us possible to explore more deeply in the genetic variances in SPs in various diseases, which also has a potential to accelerate the understanding of SP function. Although there are still many obstacles, the studies described in this review support the intriguing possibility that therapeutic strategy targeting SP-A might be efficacious for the treatment of non-infectious lung diseases as well as infectious diseases.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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Abstract. *Aim: The aim of the present study was to investigate the prognostic impact of central nervous system metastases (CNS) after acquired resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) in EGFR-mutant non-small cell lung cancer (NSCLC). Patients and Methods: We defined CNS-collapse as death due to uncontrolled and progressive CNS metastases. Post-progression survival (PPS) after initial TKI failure and T790M status were retrospectively compared in 92 patients with or without CNS collapse. Results: The median PPS in 32 patients with CNS-collapse (16.7 months) was significantly shorter than that of 60 without (26.8 months) (p=0.0002). T790M was detected in four (12%) out of the 32 CNS-collapse patients and in 26 (43%) out of 60 without (p=0.0026). Median PPS in 39 patients with leptomeningeal metastases (LM) (11.4 months) was significantly shorter versus 53 without (26.8 months) (p=0.0006). The median PPS was 25.1 months in 40 patients with brain metastases and 11.2 months in 52 without (p=0.0387). T790M was detected in 4/5 resected brain tumors (80%) and in 1/26 cerebrospinal fluid (CSF) samples (4%) (p=0.0008). Conclusion: CNS-collapse represented poorer prognosis, which was associated with T790M-negative status and LM. Controlling CNS metastases, especially LM, is important to achieve longer survival.*

Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancers and the majority are already unresectable and metastatic upon their initial diagnosis. Cytotoxic chemotherapies, such as platinum-based regimens, were once the primary therapeutic option for metastatic NSCLC but their advancement has reached a plateau. Molecular-targeted therapies have been developed recently and they have provided a remarkable benefit to patients harboring specific genetic alterations. Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene have been identified in patients with radiographic responses to EGFR-tyrosine kinase inhibitors (TKIs) (1, 2). Currently, the efficacy of up-front EGFR-TKIs has been established for patients harboring *EGFR*-sensitive mutations in prospective randomized phase III trials and the median progression-free survivals (PFSs) are approximately 12 months (3-7).

Despite an initial dramatic response, most patients harboring *EGFR* mutations acquire resistance to EGFR-TKIs. Approximately one-third of the patients appear to develop central nervous system (CNS) metastases, such as brain metastases (BM) and leptomeningeal metastases (LM) after the initial response to an EGFR-TKI (8-10). CNS metastases are generally associated with poor prognosis in NSCLC (11-13) but little is known regarding the prognostic impact of CNS metastases after acquired resistance to EGFR-TKI.

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Key Words: Central nervous system, epidermal growth factor receptor-tyrosine kinase inhibitor, acquired resistance, T790M, leptomeningeal metastases, brain metastases.

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Several acquired resistance mechanisms to EGFR-TKI have been identified (14-19) and the secondary *EGFR* mutation, a point-mutation in exon 20 (T790M), accounts for approximately one-half of the cases of acquired resistance to EGFR-TKI. Recent reports have demonstrated that the presence of T790M predicts a favorable prognosis and indolent progression compared to the absence of T790M after EGFR-TKI failure (20, 21). Notably, T790M is rarely detected in CNS lesions (21). T790M-negative rapid growth cancer cells invading CNS lesions may induce a poorer prognosis (22). We, therefore, consider the low incidence of T790M in CNS lesions to be associated with poorer prognosis after acquired resistance to EGFR-TKI.

The aim of the present study was to investigate the prognostic impact of CNS metastases in *EGFR*-mutant NSCLC patients after acquired resistance to EGFR-TKI. We also examined the association between T790M prevalence and prognosis in patients with CNS metastases, such as BM and LM.

Patients and Methods

Patients. We retrospectively reviewed the cases of 92 *EGFR*-mutant NSCLC patients whose T790M status had been confirmed by re-biopsy after acquired resistance to an EGFR-TKI (gefitinib, erlotinib or afatinib) between May 2008 and October 2013 at our Institutes. Acquired resistance was defined as Jackman *et al.* proposed (23). In their criteria, response or durable stable disease (≥6 months) was confirmed on EGFR-TKI followed by progression while receiving EGFR-TKI. The interval between the initial EGFR-TKI failure and rebiopsy varied among the patients. BM diagnoses were confirmed by magnetic resonance imaging (MRI). LM diagnoses were judged by MRI findings and/or cytology of cerebrospinal fluid (CSF). Informed consent regarding the *EGFR* mutational analysis was obtained from all patients.

EGFR mutational analysis. Re-biopsy was performed for the 92 patients at various sites using a variety of procedures at our institutes. We isolated tumor DNA from these 92 specimens, and we analyzed *EGFR* mutations using the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method, as described by Nagai *et al.* (24). Twenty patients received rebiopsies at multiple sites and five underwent plural rebiopsies; we adopted the first result of T790M status. Almost all mutation analyses were performed in malignant cell-confirmed specimens but three cytology-negative CSFs revealed *EGFR* mutations. No other acquired resistant molecular mechanisms (*e.g.*, MET) were examined.

Post-progression survival and T790M analysis. To investigate the patients' prognoses after initial EGFR-TKI failure, we examined the periods of post-progression survival (PPS) after initial EGFR-TKI failure and the T790M prevalence in each clinical factor. CNS-collapse was defined as death due to uncontrolled and progressive CNS metastases, which caused performance status (PS) deterioration that prohibited further cytotoxic chemotherapies except for EGFR-TKIs. We compared the PPS and T790M status in the patients with and without CNS-collapse. We also compared the PPS and T790M status in the patients with BM or LM to analyze the prognostic and biological distinction between BM and LM. PPS was herein defined as the period from progressive disease (PD) on initial EGFR-TKI therapy to death.

Statistical analyses. The PD of initial EGFR-TKI therapy was judged by each physician in charge according to clinical progression or objective progression as described by the Response Evaluation Criteria in Solid Tumors, version 1.1. PFS was defined as the length of time from the initiation of the first EGFR-TKI therapy until PD or death. PPS was defined as the date of the PD on initial EGFR-TKI until death. Each patient's characteristics were compared between T790M-positive and -negative patients using the Fisher's exact test. PPS curves were estimated according to the Kaplan-Meier method. PPSs were compared using the log-rank test. A *p*-value less than 0.05 was considered significant. The statistical analyses were performed using JMP 7 (SAS Institute, Inc., Cary, NC, USA).

Results

Patients' characteristics and T790M prevalence. Between May 2008 and October 2013, we retrospectively investigated the prognostic impact of CNS metastases in 92 EGFR-mutant patients whose T790M status had been confirmed after acquired resistance to EGFR-TKI. The patients' characteristics and T790M prevalence are shown in Table I. At the initial mutational analyses, the types of *EGFR* mutation observed before the initial TKI included 45 (49%) deletional mutations in exon 19, 44 (48%) L858R point-mutations in exon 21 and three (3%) point mutations in exon 18 (G719X). Re-biopsy was performed in 31 (34%) CNS lesions (26 CSFs and five brain tumoral tissues), 58 (63%) thoracic lesions (30 lung tissues and 28 pleural effusions) and three (3%) lymph nodes. The median interval between initial TKI progression and re-biopsy was 4.7 months (range=0-60.1 months).

Only two clinical factors were significant for T790M prevalence; the presence of LM and the biopsy site. T790M was identified in five (16%) of 31 CNS specimens and in 25 (41%) of the other 61 lesions (p=0.0191). Six (16%) of the 39 patients with LM harbored T790M, as did 24 (45%) of the 58 patients without LM (p=0.0325). Other characteristics had no significant association with the detection of T790M.

Post-progression survivals and T790M prevalence in patients with and without CNS-collapse. The comparison of the PPS of the patients with and without CNS-collapse is shown in Figure 1. The median PPS with CNS-collapse (n=32) was 16.7 months (95% confidence interval (CI)=9.6–20.1 months) and that without CNS-collapse (n=60) was 26.8 mo (95% CI=14.5-37.3 months) (p=0.0002). Among the 32 patients with CNS-collapse, 31 (97%) out of the 32 patients developed CNS-collapse due to LM and only one (3%) of

Table I. *Patients' characteristics and T790M prevalence.*

Characteristics	Number	T790M (%)	*p*-Value
Age			
≥70	31	13 (42%)	0.2399
<70	61	17 (28%)	
Gender			
Male	31	11 (35%)	0.8144
Female	61	19 (31%)	
Smoking history			
Never	63	21 (33%)	0.8270
Former/Current	29	9 (31%)	
Histology			
Adenocarcinoma	85	30 (35%)	0.0913
Squamous/Large	7	0 (0%)	
Performance Status (ECOG)			
0-1	42	16 (35%)	0.3737
2-4	50	14 (28%)	
Types of *EGFR* mutation			
Exon 18 (G719X)	3	1 (33%)	
Exon 19 (deletion)	45	18 (40%)	0.3200
Exon 21 (L858R)	44	11 (25%)	
Initial TKI			
Gefitinib	73	27 (37%)	0.1021
Erlotinib/Afatinib	18/1	3 (16%)	
Response to Initial TKI			
CR/PR	67	24 (36%)	0.3269
SD	25	6 (24%)	
Line of initial TKI			
First	33	11 (33%)	0.9117
Second or later	59	19 (32%)	
PFS with initial TKI			
≥10 months	51	17 (33%)	0.8686
<10 months	41	13 (32%)	
Interval between TKI failure and rebiopsy			
≥4 months	49	17 (35%)	0.6637
<4 months	43	13 (30%)	
Leptomenigeal metastases			
+	39	6 (15%)	0.0325
–	53	24 (45%)	
Brain metastases			
+	40	12 (30%)	0.6614
–	52	18 (35%)	
Biopsy site			
CNS (Brain/CSF)	5/26	5 (16%)	0.0191
Thoracic/Other	58/3	25 (41%)	

ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CR, complete response; PR, partial response; SD, stable disease; PFS, progression-free survival; CNS, central nervous system; CSF, cerebrospinal fluid.

the 32 cases was due to BM ($p<0.0001$). T790M was detected in four (12%) out of the 32 patients with CNS-collapse and in 26 (43%) of the 60 patients without CNS-collapse ($p=0.0026$). In contrast, CNS-collapse was observed in 28 (45%) of the 62 T790M-negative and four (13%) out of the 30 T790M-positive patients ($p=0.0026$).

Post-progression survival in patients with and without leptomeningeal metastases. The comparison of PPS in patients with and without LM is shown in Figure 2. The median PPS in patients with LM (n=39) was 11.4 months (95% CI, 10.1–23.4 months) and that in the patients without LM (n=53) was 26.8 months (95% CI=16.2-37.3 months) ($p=0.0006$). Six (16%) of the 39 patients with LM harbored T790M and 24 (45%) of the 58 patients without LM harbored T790M ($p=0.0325$). Thirty-one (79%) out of the 39 patients with LM developed CNS-collapse.

Post-progression survival in patients with and without brain metastases. The comparison of PPS in the patients with and without BM is shown in Figure 3. The median PPS in the patients with BM (n=40) was 25.1 months (95% CI=20.4-34.0 months) and that in the patients without BM (n=52) was 11.2 months (95% CI=10.1–23.4 months) ($p=0.0387$). Fifteen (38%) of the 40 patients with BM developed CNS-collapse and the cases of 14 of these 15 (93%) patients were complicated with LM.

T790M status in CSF and brain tumoral tissue. T790M status was examined in five (13%) brain tumoral tissues of the 40 patients with BM and in 26 (67%) CSF samples from 39 patients with LM. T790M was detected in four (80%) out of the five brain tumoral tissues and in one (4%) of the 26 CSF samples ($p=0.0008$).

Discussion

Our data demonstrated that NSCLC patients with CNS-collapse, defined as death due to uncontrolled and progressive CNS metastases after acquired resistance to EGFR-TKI, had poorer prognoses compared to the patients without CNS-collapse (median PPS: 16.7 *vs.* 26.8 months, $p=0.0002$). Approximately one-third of NSCLC patients after initial response to EGFR-TKI appear to develop CNS metastases, such as BM and LM (8-10). CNS metastases are a relatively late complication in the clinical course of patients with advanced NSCLC and its prevalence increases gradually. This increasing prevalence was observed in our cohort of *EGFR*-mutant NSCLC patients; the prevalence of BM and LM was 43% (40/92) and 42% (39/92), respectively. The longer the clinical course, the higher the prevalence of CNS metastases became. Therefore, the control of CNS metastases is extremely important to achieve longer survival after acquired resistance to EGFR-TKI.

The incidence of T790M in patients with CNS-collapse was lower than in those without, whereas T790M-negative patients frequently developed CNS-collapse. We previously demonstrated that the emergence of T790M in CNS is rare compared to other lesions (21). The low incidence of T790M implies the existence of other specific resistance mechanisms

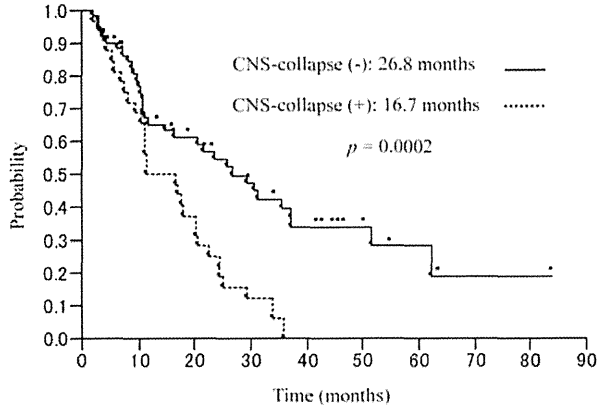

Figure 1. *Post-progression survival of patients with and without CNS-collapse.*

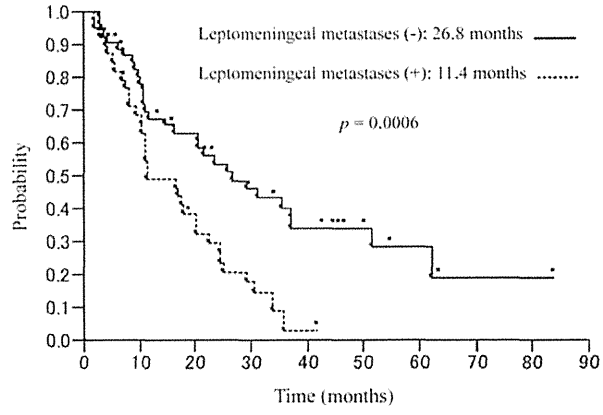

Figure 2. *Post-progression survival of patients with and without leptomeningeal metastases.*

in CNS. This is partially due to poor EGFR-TKI penetration into the CNS, which is called "pharmacokinetic failure." Pre-clinical data demonstrated that T790M-positive cancer cells are mediated by TKI exposure (22). T790M-negative cancer cells have rapid growth potential compared to T790M-positive cancer cells and they frequently metastasize to extrathoracic sites, including the CNS (20, 22). Poor TKI exposure in the CNS may induce a T790M-negative rapid growth cell invasion resulting in poor prognosis. Thus, sufficient drug exposure to the CNS may induce the indolent growth of T790M-positive cancer cells even in the CNS, which may contribute to a better prognosis. In fact, some recent reports demonstrated the efficacy of high-dose EGFR-TKIs in refractory CNS lesions after the failure of standard-dose EGFR-TKIs (25-31).

Our NSCLC patients with LM had a poorer prognosis than those without LM (median PPS: 11.4 *vs.* 26.8 months, p=0.0006). Notably, the PPS curves of the patients with and without LM are similar to the PPS curves of the patients with or without CNS-collapse. Out of the 32 patients with CNS-collapse, 31 (97%) developed CNS-collapse due to LM and only one (3%) developed CNS-collapse due to BM. In contrast, approximately 80% (31/39) of patients with LM developed CNS-collapse. Although the patients with BM had a better prognosis than those without, 15 (38%) of the 40 patients with BM developed CNS-collapse and the cases of 14 of these 15 (93%) patients were complicated with LM. These findings suggest that most patients with LM finally progress to CNS-collapse indicating a relative difficulty to achieve long survival. Even if the patients had only BM without LM in their early clinical courses, complication with LM induces a poor prognosis. We need to explore more effective therapeutic strategies for refractory LM, including high-dose EGFR-TKI, to obtain better prognoses of patients after acquired resistance to EGFR-TKI.

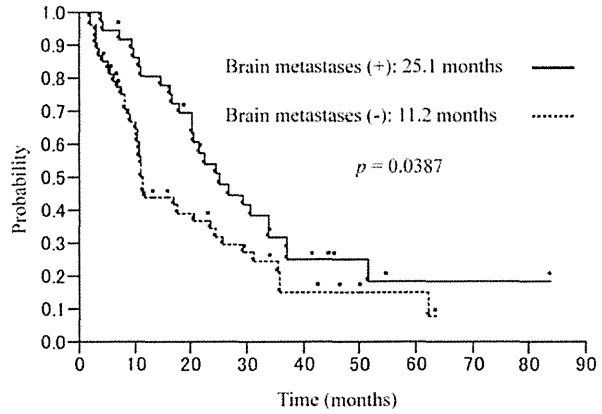

Figure 3. *Post-progression survival of patients with and without brain metastases.*

Interestingly, in our cohort, after acquired resistance to EGFR-TKI, the patients with BM had a better prognosis than those without BM, although BM is generally a poor prognostic factor in patients with NSCLC (11-13). We hypothesize two probable causes. First, BM is treatable in the majority of cases by frequent follow-up with MRI. In our Institutes, MRI is routinely performed every 3-4 months in patients with BM after acquired resistance to EGFR-TKI. Close follow-up using MRI enables the early detection of BM within the stereotactic radiation therapy (SRS) indication window. Early intervention with SRS may be useful to maintain the patient's neurological functions and EGFR-TKI

administration. In disseminated or multiple metastases without SRS indication, whole-brain radiation therapy (WBRT) can be applied. Moreover, some investigators recently reported the efficacy of local therapies with continued EGFR-TKI (32, 33). In patients with a symptomatic solitary metastasis, neurosurgery can be performed. BM in various situations is, thus, treatable in accordance with optimal procedures. Second, BM in patients with *EGFR*-mutant NSCLC may have an indolent nature after acquired resistance to EGFR-TKI. In our cohort, T790M status was examined in five (13%) brain tumoral tissues of 40 patients with BM and T790M was detected in four (80%) of these five tissues. This result suggests that EGFR-TKI exposure is sufficient in cerebral parenchyma, in contrast to CSF. Sufficient exposure of EGFR-TKI can mediate T790M-positive indolent-growing cancer cells in brain metastases. Conversely, T790M-negative rapid-growing cancer cells invade the medullary space due to the insufficient exposure to EGFR-TKI. Notably, we observed an early drop in the PPS curve of the group of patients without BM, which included many patients with LM. These patients with LM had extremely poor prognoses and rarely harbored T790M. We speculate that T790M-negative cancer cells tend to invade the medullary space and induce LM, is was related to poorer prognoses. T790M-positive cancer cells in BM may have a fundamentally indolent nature after acquired resistance to EGFR-TKI.

Our study includes several limitations. First, our cohort is relatively small in size and the data are retrospective. The intervals for the re-staging imaging were highly variable and this represents a bias for PFS assessment of initial TKI. Second, our cohort was limited to patients who had a targetable lesion to undergo rebiopsy. Cases without targetable lesions were not included, which would probably have a relatively small tumor burden and, thus, would have a better prognosis than those with targetable lesions. Third, the presence or absence of CNS-collapse in some patients was difficult to be distinguished if the patients simultaneously had uncontrolled and progressive CNS metastases and systemic disease deterioration. We, thus, had to judge which parameter was more influential in this respect, CNS metastases or systemic progression for PS deterioration.

In conclusion, CNS-collapse represented poorer prognosis, which was associated with T790M-negative status and LM. The patients with LM had a significantly poorer prognosis than those without LM. Conversely, the patients with BM had a better prognosis than those without. In available samples after acquired resistance to EGFR-TKI, T790M was frequently detected in brain tumoral tissue but rarely in CSF. BM and LM appear to have distinct clinical courses and tumor biologies. Since most of the patients with CNS-collapse were due to LM, more effective treatments for refractory LM are required. Future studies are warranted to develop better therapeutic strategies for CNS metastases after acquired resistance to EGFR-TKI.

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Conflicts of Interest

The Authors have declared no conflicts of interest.

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