YKL-40 Promotes Proliferation of Cutaneous T-Cell Lymphoma Tumor Cells through Extracellular Signal–Regulated Kinase Pathways

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YKL-40, one of the chitinase-like proteins, is associated with the pathogenesis of a wide variety of human diseases through modulation of inflammation and tissue remodeling by its diverse roles in cell proliferation, differentiation, and survival. Emerging evidence shows that aberrantly expressed YKL-40 promotes the development of malignancies by inducing proliferation of tumor cells, cytokine production, and angiogenesis by acting on various stromal cells, immune cells, and tumor cells. In this study, we investigated the expression and function of YKL-40 in cutaneous T-cell lymphoma (CTCL). We first revealed that serum YKL-40 levels were increased in patients with CTCL and correlated with disease severity markers. We also found that YKL-40 was expressed by epidermal keratinocytes and tumor cells in lesional skin of CTCL by immunohistochemistry. Although YKL-40 did not affect cytokine production from CTCL cell lines, YKL-40 promoted the proliferation of Hut78 cells and HH cells in vitro, which was dependent on extracellular signal–regulated kinase 1/2 pathways. Moreover, exogenous YKL-40 administration enhanced tumor growth of HH cells in vivo. Our study has suggested that YKL-40 produced from epidermal keratinocytes and CTCL cells promoted the proliferation of CTCL cells through extracellular signal–regulated kinase 1/2 pathways in autocrine and paracrine manners, leading to development of CTCL.


INTRODUCTION
Nowadays, it becomes evident that tumor cells are surrounded by a heterogeneous cellular and molecular microenvironment that influences tumor cell properties such as proliferation, survival, invasion, and escape from immune surveillance (Balkwill et al., 2012; Whiteside, 2008). The dynamic tumor cell–microenvironment crosstalk is mediated by tumor cell– and stroma cell–derived soluble factors in an autocrine or paracrine manner. Accumulating evidence indicates that tumor microenvironment plays an essential role in tumor development not only in solid tumors but also in hematopoietic malignancies (Herreros et al., 2008). Cutaneous T-cell lymphoma (CTCL) is a malignancy of skin-trafficking T cells, and mycosis fungoides (MF) and Sézary syndrome (SS) are the most common types of CTCL (Hwang et al., 2008). MF is characterized by a proliferation of neoplastic CD4-positive T cells with epidermotropism and usually has a prolonged clinical course. SS is characterized by a triad of erythroderma, lymphadenopathy, and leukemic involvement and generally shows rapid disease progression. Similar to other malignancies, tumor microenvironment contributes to the development of CTCL through several mechanisms including promotion of cell proliferation (Miyagaki and Sugaya, 2014).

YKL-40, one of the chitinase-like proteins, is produced by multiple cell types including stromal cells, immune cells, and tumor cells and combines properties of both cytokines and growth factors (Kzhyshkowska et al., 2016; Lee et al., 2011). YKL-40 has diverse roles in proliferation, differentiation, and survival of various cells, resulting in modulation of inflammation and tissue remodeling, and its aberrant expression is associated with the pathogenesis of a wide variety of human diseases (Prakash et al., 2013). Concerning malignancies, YKL-40 expression and function have been investigated mostly in solid malignancies. Elevated YKL-40 levels in the circulation were found in a variety of solid tumors, which frequently correlates with disease severity or predicts poor outcome (Krogh et al., 2016; Kzhyshkowska et al., 2016; Schmidt et al., 2006). YKL-40 has been shown to activate cancer signaling pathways and promote proliferation, angiogenesis, or metastasis of glioblastoma and colon cancer (Francescone et al., 2011; Kawada et al., 2012; Ku et al., 2011; Shao et al., 2009). Serum YKL-40 levels were increased in patients with untreated Hodgkin lymphoma and correlated with more advanced stages, which decreased after
YKL-40 can be a potential biomarker for disease control following standard salvage treatment for relapsed non-Hodgkin lymphoma (El-Galaly et al., 2015). These reports suggest that YKL-40 can also be involved in development of hematopoietic malignancies, although there have been few reports on the function of YKL-40 in such malignancies. On the basis of these findings, we investigated YKL-40 expression in lesional skin and sera of MF/SS and possible roles of YKL-40 in the progression of MF/SS. Here we show that YKL-40 expressed by tumor cells and epidermal keratinocytes augments proliferation of CTCL cells through extracellular signal–regulated kinase (ERK) 1/2 pathways, suggesting that YKL-40 can be a possible target for treatment of MF/SS.

RESULTS

YKL-40 expression is upregulated in sera of CTCL

To investigate YKL-40 involvement in CTCL, we first measured serum YKL-40 levels in patients with CTCL. Serum YKL-40 levels in patients with CTCL (34.45 ± 7.29 ng/ml) were significantly higher than those of healthy controls (30.26 ± 6.33 ng/ml; Figure 1a). We also measured YKL-40 levels in the sera of patients with atopic dermatitis (AD), which is a representative T helper type 2 (Th2)-mediated inflammatory skin disease. Serum YKL-40 levels in patients with AD (29.71 ± 19.75 ng/ml) were comparable with those of healthy controls and significantly lower than those of patients with CTCL (Figure 1a). Serum YKL-40 levels in patients with early MF (stage IA–IIA, n = 21), advanced MF (stage IIb–IVb, n = 30), and SS (n = 7) were 29.73 ± 7.71 ng/ml, 36.69 ± 5.04 ng/ml, and 38.98 ± 6.82 ng/ml, respectively. YKL-40 levels in sera of patients with advanced MF and SS were significantly higher than those of healthy controls or patients with early MF (Figure 1b). We also compared serum YKL-40 levels with other clinical laboratory data, such as age; sex; and serum levels of lactate dehydrogenase, soluble IL-2 receptor, and thymus and activation-regulated chemokine. Serum YKL-40 levels significantly correlated with serum lactate dehydrogenase and soluble IL-2 receptor levels (r = 0.39, P < 0.01 and r = 0.41, P < 0.01, respectively; Figure 1c and d), which are regarded as disease severity markers of CTCL, but not with serum thymus and activation-regulated chemokine levels (data not shown). Thus, YKL-40 expression in sera of patients with advanced CTCL was upregulated and correlated with disease severity.

Tumor cells and epidermal keratinocytes expressed YKL-40 in lesional skin of CTCL

To investigate the source of YKL-40, we next stained the lesional skin of CTCL and AD and normal skin from healthy controls with antibody to YKL-40. In normal skin, YKL-40 was expressed on epidermal keratinocytes weakly (Figure 1e). Relatively strong YKL-40 expression in epidermal keratinocytes was seen in lesional skin of CTCL (Figure 1f and g). The expression became stronger as the disease progressed (Table 1). In addition, a large portion of dermal-infiltrating cells were also positive for YKL-40, and the number of YKL-40–positive cells and YKL-40 expression levels increased as the disease progressed (Figure 1f and g, Table 1). On the other hand, in AD skin, YKL-40 expression levels in epidermal keratinocytes were similar to those in normal skin, and there were a small number of YKL-40–expressing cells in the dermis (Figure 1h). These results suggested that CTCL cells expressed YKL-40. Consistently, YKL-40 mRNA expression levels in peripheral blood mononuclear cells were higher in patients with SS than those in healthy controls (Supplementary Figure S1). To clearly elucidate that, we performed hematoyxin-eosin, CD3, CD4, and CD30 staining with serial sections in CTCL lesional skin. We found that epidermotropic CD3+ CD4+ cells and dermal-infiltrating CD3+ CD4+ cells with atypical nuclei were positive for YKL-40 both in early MF and advanced CTCL (Figure 1i–k and data not shown). In addition, not only CD30+ cells but also CD30+ cells expressed YKL-40. We finally investigated whether YKL-40 expression was associated with large cell transformation. We found that YKL-40 expression levels were weak to moderate in some cases with large cell transformation, suggesting that YKL-40 expression was not associated with large cell transformation (Figure 1l, Table 1). The main sources of YKL-40 in lesional skin were epidermal keratinocytes and CTCL cells, and YKL-40 expression levels increased as the disease progressed.

YKL-40 induces cell proliferation but does not increase cytokine production from CTCL cells

YKL-40 has the capacity to induce proliferation and cytokine production from tumor cells, resulting in the development of a tumor, including colon cancer and glioblastoma (Chen et al., 2011; Francescone et al., 2011; Ku et al., 2011). We first focused on whether YKL-40 affects tumor cell production of Th2 cytokines and vascular endothelial growth factor (VEGF)-A, all of which are important factors in CTCL development and expressed from CTCL cells (Krejsgaard et al., 2006; Pileri et al., 2015; Takahashi et al., 2016). YKL-40 did not augment IL-4, IL-13, and VEGF expression from CTCL cell lines, Hut78 cells, and HH cells (Supplementary Figure S2a–c). We next assessed the effect of YKL-40 on proliferation and migration of CTCL cell lines. YKL-40 significantly increased cell numbers of Hut78 and HH cells in a dose-dependent manner (Figure 2a). In addition, YKL-40 increased proliferation of Hut78 and HH cells as shown by BrdU uptake (Figure 2b). Moreover, YKL-40 induced migration of Hut78 and HH cells (Figure 2c). Thus, YKL-40 did not affect Th2 and angiogenic cytokine production from CTCL cells but induced proliferation and migration of CTCL cells.

Proliferative effect of YKL-40 in CTCL cells is dependent on ERK1/2 pathways

To understand the precise mechanism of the proliferative effect of YKL-40, we further investigated the downstream of YKL-40. YKL-40 induced proliferation and survival of various cell types through ERK1/2 and/or protein kinase B (Akt) pathways (Areshkov and Kavsan, 2010; Francescone et al., 2011; He et al., 2013). In our experiments, YKL-40 significantly increased phosphorylation of ERK1/2 at 30 minutes but not Akt in HH cells (Figure 3a and b). We also examined whether YKL-40 activated p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase, and NF-kB pathways, only to find that activation of those pathways was not induced (Supplementary Figure S3). To determine whether ERK1/2 phosphorylation is involved in the proliferative effect of YKL-40, HH cells were pretreated with U0126 (MAPK/ERK
Figure 1. YKL-40 expression is increased in sera and lesional skin of CTCL. (a) Serum YKL-40 levels in patients with CTCL and AD and in healthy controls. The measured values from individual patients are plotted by dots. Means are presented as bars. **P < 0.01. (b) Serum YKL-40 levels in patients with early MF, advanced MF, and SS and in healthy controls. The measured values from individual patients are plotted by dots. Means are presented as bars. **P < 0.01. (c) Correlation between serum YKL-40 levels and serum LDH levels. (d) Correlation between serum YKL-40 levels and serum sIL-2R levels. (e–h) Immunohistochemistry of (e) normal skin and lesional skin of (f) early MF, (g) advanced MF, and (h) AD for YKL-40. Representative results are shown. Bar = 25 μm. (i–l) Representative HE, CD4, CD30, and YKL-40 stainings with serial sections in patients with (i) early MF with epidermotropism, (j) SS with epidermotropism, (k) SS with large cell transformation, and (l) early MF with large cell transformation with weak YKL-40 positivity. Bar = 12.5 μm. AD, atopic dermatitis; CTCL, cutaneous T-cell lymphoma; HE, hematoxylin and eosin; LDH, lactate dehydrogenase; MF, mycosis fungoides; sIL-2R, soluble IL-2 receptor; SS, Sézary syndrome.
kinase 1/2 inhibitor) before YKL-40 stimulation. U0126 suppressed phosphorylated ERK1/2 expression in HH cells after YKL-40 administration without interfering with the expression of phosphorylated Akt (Figure 3c). In addition, the effect of YKL-40 to enhance proliferation of HH cells was completely abrogated by U0126 (Figure 3d), suggesting that ERK1/2 phosphorylation is essential for the proliferative effect of YKL-40 in CTCL cells. Thus, YKL-40 induced proliferation through activation of ERK1/2 pathways in CTCL cells.

YKL-40 enhances tumor growth of HH cells in vivo

On the basis of the data described previously, we assessed the effects of YKL-40 on HH cells in vivo. HH cells were injected subcutaneously into the back of SCID-beige mice, followed by repeated treatment with YKL-40. Significantly larger tumors were formed in mice treated with YKL-40, compared with those treated with phosphate buffered saline (PBS) (Figure 4a). As YKL-40 has the capacity to promote vascular endothelial cell proliferation (Shao et al., 2009), we decided to investigate angiogenesis in the tumors using CD34 staining. The number of CD34-positive vessels was higher in the tumors treated with YKL-40 than in those treated with PBS (Figure 4b and c). Anti–YKL-40 blocking antibody suppressed YKL-40-induced promotion of tumor formation and angiogenesis in the tumor in vivo (Figure 4d–f). Thus, YKL-40 promoted HH cell tumor growth in vivo by not only directly promoting proliferation of HH cells but also inducing angiogenesis.

**DISCUSSION**

In this study, we have first found that serum YKL-40 levels were increased in patients with advanced MF and SS and correlated with disease severity markers, suggesting that YKL-40 can be involved in the development of MF/SS. These results are similar to the previous reports describing that circulating YKL-40 expression levels are associated with disease severity or outcome in hematopoietic malignancies, such as acute myeloid leukemia, Hodgkin lymphoma, and non-Hodgkin lymphoma (Bergmann et al., 2005; Biggar et al., 1998). The results described in this article support the hypothesis that YKL-40 can be used as a biomarker for MF and SS.

**Table 1. YKL-40 Expression in Epidermal Keratinocytes and Tumor Cells in Normal Skin and Lesional Skin of AD, Early MF, and Advanced MF/SS**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Epidermal Keratinocytes</th>
<th>Tumor Cells</th>
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<tr>
<td></td>
<td>Negative</td>
<td>Weak</td>
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<tr>
<td>Normal</td>
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<td>3</td>
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<tr>
<td>AD</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Early MF</td>
<td>0</td>
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<tr>
<td>Advanced MF/SS 2</td>
<td>0</td>
<td>6</td>
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Abbreviations: AD, atopic dermatitis; MF, mycosis fungoides; SS, Sézary syndrome.

1The number in parentheses is the number of cases with large cell transformation in each group.

2Skin biopsy of one case with advanced MF from ulcer lesion did not contain epidermal keratinocytes.

| Figure 2. YKL-40 induces cell proliferation and migration of CTCL cell lines. (a) Hut78 and HH cells were cultured with medium only or with YKL-40 (30 or 100 ng/ml) for 12 or 24 hours. Viable cells were counted by trypan blue exclusion. The cell numbers of each cell line cultured with medium were set at 1, and the proliferation levels were shown as a fold difference. (b) BrdU cell proliferation assay of Hut78 and HH cells cultured with medium only or YKL-40 (100 ng/ml) for 24 hours. The rate of BrdU uptake was determined based on the absorbance at 450 nm. One representative result from three independent experiments (n = 4) with similar findings. (c) Hut78 and HH cells were assessed in a migration assay for 24 hours at 37 °C with medium only or with YKL-40 (30 or 100 ng/ml). The percentage of migrating cells relative to input was determined (n = 10). Data are presented as mean ± SD. *P < 0.05, **P < 0.01. CTCL, cutaneous T-cell lymphoma; SD, standard deviation.
et al., 2008; El-Galaly et al., 2015). By immunohistochemistry, increased YKL-40 expression was found in epidermal keratinocytes and CTCL cells. Similar to our results, YKL-40 is expressed in various epithelial cells, such as bronchial epithelial cells, colonic epithelial cells, and nasal epithelial cells (Chen et al., 2011; Park et al., 2013, 2010). In addition, considering that YKL-40 is expressed on tumor cells of many types of cancers including adenocarcinoma, squamous carcinoma, melanoma, glioblastoma, and multiple myeloma (Johansen et al., 2007; Ku et al., 2011; Mylin et al., 2006), it is no wonder that tumor cells of MF/SS expressed YKL-40. Consequently, upregulated YKL-40 expression from epidermal keratinocytes and tumor cells in lesional skin can cause increased YKL-40 levels in sera in patients with MF/SS. YKL-40 has the capacity to induce tumor cell proliferation, tissue remodeling, cytokine production, and angiogenesis by acting on various stromal cells, immune cells, and tumor cells, resulting in development of tumor. We first focused on Th2 polarization, which is an important factor in the development of CTCL (Miyagaki and Sugaya, 2014), as YKL-40 is involved in the initiation of antigen-induced Th2 inflammation in mice (Lee et al., 2009). However, YKL-40 did not augment Th2 cytokine production from CTCL cell lines. In the previous report by Lee et al. (2009) YKL-40 induced Th2 response through augmenting IgE production, activating dendritic cells and alternative macrophages, and inhibiting apoptosis in inflammatory cells but not enhancing Th2 cytokine production from T cells. Thus, YKL-40 might not have the capacity to induce Th2 cytokine production from T cells. We also focused on angiogenesis, because angiogenesis, assessed either by microvessel density or by microvessel number in lesional skin, is increased in MF/SS (Mazur et al., 2004; Vacca et al., 1997). In addition, expression levels of several angiogenic factors, such as VEGF, angiogenin, angiopoietin-2, and placental growth factor, are increased in patients with MF/SS and correlated with disease severity (Kawaguchi et al., 2014; Krejsgaard et al., 2006; Miyagaki et al., 2017, 2012; Pileri et al., 2015), suggesting that angiogenesis is important for progression of MF/SS. As YKL-40 is known to induce VEGF in glioblastoma cell lines (Francescone et al., 2011), we measured VEGF expression in CTCL cell lines after YKL-40 stimulation. However, we could...
YKL-40 Involvement in CTCL Progression

not find any effect of YKL-40 on VEGF expression from CTCL cell lines. Other than VEGF induction from tumor cells, YKL-40 has the capacity to enhance migration and tube formation of human microvascular endothelial cells, resulting in increased vasculature in many malignancies (Kawada et al., 2012; Shao et al., 2009) and YKL-40-induced angiogenesis is supposed to be caused by this mechanism, because VEGF induction by YKL-40 is only reported in glioblastoma (Francescone et al., 2011). Consistently, YKL-40 administration increased tumor vasculature in an in vivo tumor inoculation model using HH cells. In addition, we found that YKL-40 induced proliferation of CTCL cell lines in vitro and HH cells in vivo. These results suggest that YKL-40 can be involved in the development of MF/SS through promoting tumor cell proliferation and angiogenesis but not inducing cytokine production from tumor cells. Moreover, we found that YKL-40 induced migration of CTCL cell lines similar to glioma cells (Ku et al., 2011). Considering that epidermal keratinocytes expressed YKL-40, YKL-40 can also be involved in epidermotropism in CTCL.

It is widely supposed that YKL-40 functions through binding to a cell surface receptor, based on many reports describing that YKL-40 induced cytokine production, migration, proliferation, and activation of several signaling pathways in many targeted cells (Kzhyshkowska et al., 2016; Lee and Elias, 2010). The ability of YKL-40 to induce migration and proliferation of CTCL cells also suggests the existence of the receptors for YKL-40 on the cell surface. However, receptors for YKL-40 are incompletely characterized and only little has been reported. YKL-40 was shown to bind the surface protein RAGE, resulting in proliferation and survival of intestinal epithelial cells (Low et al., 2015). Binding of YKL-40 and IL-13 receptor α2 was also demonstrated in a human macrophage cell line and airway epithelial cells (He et al., 2013). In addition, YKL-40 had the capacity to induce the coupling of CD138 and αvβ3 integrin on a human glioblastoma cell line, leading to VEGF production and tumor growth (Francescone et al., 2011), and the coupling of CD138 and αvβ3 integrin on human microvascular endothelial cells, resulting in tube formation (Shao et al., 2009). On the other hand, in most reports, the receptors for YKL-40 have been still unknown. In CTCL, absence of CD138 expression by immunohistochemistry was reported in 2004 (O’Connell et al., 2004). Consistently, we also found that CD138 was not expressed on Hut78 and HH cells by flow cytometry (data not shown). In addition, flow cytometric analysis also revealed that IL-13 receptor α2 and RAGE did not express on the surface of Hut78 and HH cells.

Figure 4. YKL-40 enhances tumor growth of HH cells in vivo. (a) HH cells were injected into shaved abdomen of SCID-Beige mice with PBS or recombinant YKL-40 (2.5 μg/ml). Each reagent was injected on days 0, 4, 7, and 11. Tumor size was calculated on days 4, 7, 11, and 14. Data are presented as means ± SEM (n = 7). **P < 0.01, ***P < 0.001. (b) Numbers of CD34-positive vessels in mouse tumors on day 14 per high power field. Values are means and SD (n = 5). *P < 0.05. (c) Immunohistochemistry of CD34 in mouse tumors on day 14. Arrows indicate CD34-positive vessels. Representative results are shown. Bar = 12.5 μm. (d) HH cells were injected into shaved abdomen of NOD/SCID interleukin-2 receptor α-chain-deficient mice with PBS, recombinant YKL-40 (2.5 μg/ml), or combination of recombinant YKL-40 and anti–YKL-40 blocking antibody (1.0 μg/ml). Each reagent was injected on days 0 and 4. Tumor size was calculated on days 4 and 7. Data are presented as means ± SEM (n = 6). *P < 0.05, ***P < 0.001. (e) Numbers of CD34-positive vessels in mouse tumors on day 7 per high power field. Values are means and SD (n = 6). *P < 0.05. (f) Immunohistochemistry of CD34 in mouse tumors on day 7. Arrows indicate CD34-positive vessels. Representative results are shown. Bar = 12.5 μm. Ab, antibody; PBS, phosphate buffered saline; SD, standard deviation; SEM, standard error of the mean.
(data not shown). Thus, we hypothesize that YKL-40 promotes proliferation and migration of CTCL cells through unknown receptors. Concerning angiogenesis, YKL-40 might affect dermal endothelial cells through the coupling of CD138 and αvβ3 integrin as described previously. Consistent with our hypothesis, we found that anti-YKL-40 blocking antibody suppressed YKL-40-induced promotion of tumor formation and angiogenesis in vivo.

Recently, YKL-40 has been shown to activate various pathways, such as NF-kB, ERK1/2, Akt, Wnt/β-catenin, and signal transducer and activator of transcription 3 pathways (Francescone et al., 2011; He et al., 2013; Low et al., 2015; Shao et al., 2009). Among them, ERK1/2 and Akt signaling pathways play an important role in YKL-40-mediated cell proliferation and survival. YKL-40 stimulates the proliferation of a human embryonic kidney cell line and a human glioblastoma cell line by activating ERK1/2 signaling pathway (Areshkov and Kavan, 2010). Moreover, in airway epithelial cells, YKL-40 inhibits apoptosis through activation of ERK1/2 and Akt signaling pathways (He et al., 2013). Similarly, in this study, we found that YKL-40 induced cell proliferation of HH cells in an ERK1/2 pathway—dependent manner. Although constitutive activation of ERK1/2 signaling pathways are reported in tumor cells of MF/SS (Levidou et al., 2013; Mao et al., 2008), mutations on MAPK/ERK pathways are rare in CTCL (Bastidas Torres et al., 2018; Kiesling et al., 2011). In addition, RAS mutation was not found in HH cells (Kiesling et al., 2011). Thus, activation of ERK1/2 in MF/SS could be mostly induced by signaling from molecules such as YKL-40 but not by constitutive activation because of mutations on the pathways. In any case, ERK1/2 signaling pathways can play an important role in proliferation of tumor cells in MF/SS.

In conclusion, YKL-40 expression was elevated in lesional skin and sera in patients with MF/SS. YKL-40 induced proliferation of MF/SS tumor cells both in vitro and in vivo through phosphorylation of ERK1/2. These results suggest that blocking of YKL-40 can potentially be a therapeutic tool for MF/SS.

MATERIALS AND METHODS

Tissue and serum samples

Serum samples were obtained from 58 patients with CTCL (51 MF cases and 7 SS cases; mean ± standard deviation age, 59.19 ± 15.14 years, 42 males and 16 females). The distribution of patients by stage was as follows: 12 cases at stage IA, 10 cases at stage IB, 1 case at stage IIA, 22 cases at stage IIB, 3 cases at stage IIIA, 1 case at stage IIIB, 7 cases at stage IVA and 2 cases at stage IVB. Samples were also obtained from 22 patients with AD (36.41 ± 7.71 years, 19 males and 3 females) and 24 healthy controls (57.20 ± 12.17 years, 17 males and 7 females). The clinical features of 7 patients with SS are summarized in Supplementary Table S1. Skin samples for immunohistochemistry were collected from 20 patients with CTCL (14 MF cases and 6 SS cases). The distribution of patients by stage was as follows: 3 cases at stage IA, 3 cases at stage IB, 5 cases at stage IIB, 8 cases at stage IVA and 1 case at stage IVB. Samples were also collected from 10 patients with AD. We used normal skin adjacent to benign skin tumors from 15 patients as healthy controls. Most serum and skin samples were taken from different patients in this study. All patients with MF and SS were given diagnoses according to International Society for Cutaneous Lymphomas/European Organisation for Research and Treatment of Cancer criteria (Willemze et al., 2005). Patients with MF were subgrouped into early stage (IA–IIA) and advanced stage (IIB–IVB) according to disease staging with skin, lymph nodes, visceral involvement, and blood classification. All patients with AD were given diagnoses according to the criteria of Hanifin and Rajka (Hanifin and Rajka, 1980). The healthy controls had no history of allergy, CTCL, or any inflammatory skin disease. All samples were collected after informed consent during daily clinical practice. The medical ethics committee of the University of Tokyo approved all described studies, and the study was conducted according to the principles of the Declaration of Helsinki. All serum samples were stored at –80°C until use. Written informed consent was obtained to use blood and skin samples from patients and healthy controls.

Cell lines

Hut78 (SS cell line) and HH (aggressive CTCL cell line) cells were kind gifts from Dr Kazuyasu Fujii (Department of Dermatology, Kagoshima University, Kagoshima, Japan). The cells were cultured in RPMI (Millipore, Billerica, MA) containing 10% fetal bovine serum, penicillin G sodium, streptomycin sulfate, and amphotericin B.

Mice

SCID-Beige mice and NOD/SCID IL-2 receptor γ-chain-deficient mice were purchased from Charles River Laboratories (Wilmington, MA). They were free of pathogenic bacteria and viruses. All experiments were performed using male mice between 7 and 10 weeks of age. All animal experiments were approved by the Animal Experiment Committee of the Graduate School of Medicine of the University of Tokyo guided by the Bioscience Committee of the University of Tokyo.

ELISA

Immunoreactive YKL-40 in sera was quantified by Human Chitinase 3-like 1 DuoSet ELISA (R&D Systems, Minneapolis, MN). These assays employed the quantitative sandwich enzyme immunoassay technique. Optical densities were measured at 450 nm using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). The concentrations were calculated from the standard curve generated by a curve-fitting program.

Immunohistochemistry

Briefly, 5-µm-thick tissue sections from formaldehyde-fixed and paraffin-embedded samples were dewaxed and rehydrated. These sections were stained with mouse anti-human YKL-40 monoclonal IgG antibody (R&D Systems), mouse anti-human CD3 monoclonal IgG antibody (Leica Biosystems, Wetzlar, Germany), rabbit anti-human CD4 monoclonal IgG antibody (Roche Tissue Diagnostics, Tokyo, Japan), mouse anti-human CD30 monoclonal IgG antibody (Roche Tissue Diagnostics), or goat IgG (control) followed by ABC staining (Vector Lab, Burlingame, CA). Diaminobenzidine was used for visualizing the staining and counterstaining with Mayer hematoxylin was performed, according to the manufacturers’ instructions. The positivity of YKL-40 staining was scored as negative, weak, moderate, and strong.

Analysis of the effects of YKL-40 on CTCL cell lines

CTCL cell lines were stimulated with recombinant YKL-40 (ATGen, Seoul, South Korea). After indicated time points, proliferation assays, migration assays, real-time quantitative reverse transcriptase–PCR and western blotting were performed. Detailed methods are given in Supplementary Materials and Methods.
In vivo animal experiments

HH4 cells (1.0 x 10^7 cells) in 100 μl of PBS were injected subcutaneously into the shaved back of SCID-Beige mice or NOD/SCID IL-2 receptor γ-chain-deficient mice. On days 0, 4, 7, and 10, human YKL-40 (2.5 μg/ml; ATGen) in 100 μl of PBS was injected in the YKL-40 group, whereas 100 μl of PBS was injected in the control group. On days 4, 7, 10, and 14, the tumor volume was calculated using the equation:

\[ V = L_1 \times L_2 \times H_1, \]

where \( V \) = volume (mm^3), \( L_1 \) = longest diameter (mm), \( L_2 \) = shortest diameter (mm) and \( H_1 \) = height (mm).

On days 7 or 14, the tumor tissues were harvested and stained with anti-CD34 monoclonal antibody (Nichirei Biosciences, Tokyo, Japan) to identify vessels in and around the tumor. In some experiments, anti-human YKL-40 blocking antibody (1.0 μg/ml; HyAc Biotech, Wayne, PA) was injected in addition to human YKL-40 in the YKL-40 + anti-YKL-40 antibody group on days 0 and 4.

Statistical analysis

Statistical analysis between the two groups was performed using the Mann-Whitney U test. For testing equality of population means among three or more groups, the Kruskal-Wallis test was used. Correlation coefficients were determined by using the Spearman’s rank correlation test. \( P \)-values of <0.05 were considered statistically significant.

Data availability statement

This study does not include large data sets, such as gene expression arrays, single nucleotide polymorphism arrays, proteomic data sets, high throughput sequencing, or genome-wide association study data.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: TM; Data Curation: HS, HB; Formal Analysis: HS, HB, TM; Funding Acquisition: TM; Investigation: HS, HB; Methodology: HS, HB; Project Administration: TM; Resources: HB, TM, HK, RN, TO, NST, HS, MS; Supervision: TM, SS; Validation: HS, HB; Visualization: HS, HB, TM; Writing - Original Draft Preparation: HS, HB, TM; Writing - Review and Editing: TM, HK, RN, TO, NST, HS, MS, SS

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.09.007.

REFERENCES


www.jidonline.org


SUPPLEMENTARY MATERIALS AND METHODS

Real-time quantitative reverse transcriptase–PCR assay
HH or Hut78 cells were plated onto six-well plates at 1 × 10^6 cells per well, and recombinant YKL-40 (30 or 100 ng/ml) with or without phorbol 12-myristate 13-acetate (20 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (200 ng/ml; Sigma-Aldrich) was added. After 24 hours, total mRNA was extracted from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). In some experiments, cDNA of peripheral blood mononuclear cells obtained from three patients with Sézary syndrome, whose percentage of tumor cells in peripheral blood mononuclear cells was more than 50%, and three healthy controls by density centrifugation over Ficoll-Paque (GE Healthcare, Buckinghamshire, United Kingdom) was also synthesized. Quantitative reverse transcriptase–PCR was performed based on SYBR Green assay. The mRNA levels were normalized to those of the GAPDH gene. The relative change in the levels of genes of interest was determined by the 2^{-\Delta\Delta CT} method. Primers for human vascular endothelial growth factor-A, IL-4, IL-13, YKL-40, and GAPDH were as follows: vascular endothelial growth factor-A forward, 5′-AGCCTTGCTTGCTCTCTAC-3′ and reverse, 5′-TCCTCCTCTGCAATGCTG-3′; IL-4 forward, 5′-CACAGGCAAGCAGCTGAT-3′ and reverse, 5′-CTCTGGTGGCTCTGCCTACA-3′; IL-13 forward, 5′-CGAGAAGACCAGAGATTCCAC-3′ and reverse, 5′-GGGCCACCTGTTTTGG-3′; YKL-40 forward, 5′-AAGATAGCCTCAACACCCAG-3′ and reverse, 5′-ATGCTGTTTGTCCTCCGCACCACTCTGCAGATGCTG-3′ and reverse, 5′-ACCCACCTCCACCTTTGA-3′ and reverse, 5′-GCTTGACAAATGAC-3′.

Proliferation assays by cell count
HH or Hut78 cells were plated onto six-well plates at 1 × 10^6 cells per well and recombinant YKL-40 was added. Viable cells were counted by trypan blue exclusion.

BrdU cell proliferation assay
HH or Hut78 cells (7.5 × 10^4) were cultured for 24 hours with recombinant YKL-40 at 100 ng/ml. Cells were stained with BrdU for 2 hours and reacted with anti-BrdU antibody peroxidase conjugate followed by peroxidase substrate using Cell Proliferation ELISA, BrdU (Roche Applied Science, Penzberg, Germany). Sulfuric acid was added to the solution to terminate enzyme activity. Optical densities were measured at 450 nm using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories).

Migration assay
The migration assay was done by using a 48-well micro chemotaxis chamber and filters with 12-μm pores (Neuro Probe, Gaithersburg, MD). HH or Hut78 cells (2.5 × 10^4 cells per 48-well plate) were placed on top of the 12-μm pore size filters, whereas RPMI 1640 with or without recombinant human YKL-40 protein (30 or 100 ng/ml) was placed into the lower chamber. Following 24 hours at 37 °C, migrated cells that had fallen to the bottom of the plate were counted by a hemocytometer.

Western blotting
HH cells (1 × 10^7 cells/ml) were cultured in six-well plates with recombinant YKL-40 (100 ng/ml) for 5, 15, or 30 minutes. After collecting proteins from HH cells, equal amounts of proteins were subjected to 4–12% NuPage Bis-Tris Gels (Invitrogen) at 120 V for 1 hour. The proteins were then transferred onto polyvinylidene fluoride membranes (Invitrogen) and blocked in 2% bovine serum albumin with 0.05% Tween-20 (Sigma-Aldrich) in Tris-buffered saline. The membranes were probed with p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal–regulated kinase [ERK]1/2), phosphorylated p44/42 MAPK (ERK1/2), protein kinase B, phosphorylated protein kinase B, p38 MAPK, phosphorylated p38 MAPK, c-Jun N-terminal kinase, phosphorylated c-Jun N-terminal kinase, NF-κB p65, or phosphorylated NF-κB p65 (Cell Signaling Technology) as primary antibody overnight at 4 °C, followed by incubation in secondary antibody for 45 minutes at room temperature. Visualization was performed by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA). In some experiments, HH cells were preincubated with 10 mmol/liter U0126 for 45 minutes. The density of some bands was quantified with Image J software (National Institutes of Health).
Supplementary Figure S1. YKL-40 mRNA expression levels were elevated in peripheral blood mononuclear cells from patients with SS compared with healthy controls. Peripheral blood mononuclear cells were isolated from peripheral blood of three patients with SS and three healthy controls. Quantitative RT-PCR was performed to measure YKL-40 expression relative to GAPDH \((n = 3-4)\). Data are presented as mean ± SD. RT-PCR, reverse transcriptase–PCR; SD, standard deviation; SS, Sézary syndrome.

Supplementary Figure S2. YKL-40 does not augment VEGF-A, IL-4, and IL-13 expression from CTCL cell lines. (a, b) Hut78 and HH cells were cultured with medium only with PMA (20 ng/ml) and ionomycin (200 ng/ml) or with PMA, ionomycin, and YKL-40 (100 ng/ml) for 24 hours. Quantitative RT-PCR was performed to measure (a) IL-4 and (b) IL-13 expression relative to GAPDH. (c) Hut78 and HH cells were cultured with medium only or with YKL-40 (100 ng/ml) for 24 hours. Quantitative RT-PCR was performed to measure VEGF-A expression relative to GAPDH. One representative result from three independent experiments \((n = 4)\) with similar findings. Data are presented as mean ± SD. * \(P < 0.05\). CTCL, cutaneous T-cell lymphoma; PI, phorbol 12-myristate 13-acetate + ionomycin; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcriptase–PCR; SD, standard deviation; VEGF, vascular endothelial growth factor.
Supplementary Figure S3. YKL-40 does not induce phosphorylation of p38 MAPK, JNK, or NF-κB p65 in HH cells. HH cells were cultured with YKL-40 (100 ng/ml) for 0, 5, 15, or 30 minutes. Phosphorylation of p38 MAPK, JNK, and NF-κB p65 was assessed by western blotting. A representative picture of three independent experiments is shown. JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; p-JNK, phosphorylated c-Jun N-terminal kinase; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase; p-NF-κB, phosphorylated NF-κB.

Supplementary Table S1. Clinical Features of Patients with Sézary Syndrome Whose Sera were Analyzed (n = 7)

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Stage</th>
<th>Total lymphocytes (/μl)</th>
<th>Sézary cells by peripheral blood smear (/μl)</th>
<th>Total CD4+ cells (/μl)</th>
<th>CD4/CD8 ratio</th>
<th>TCR clonality</th>
<th>Serum YKL-40 levels (ng/ml)</th>
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<tr>
<td>1</td>
<td>54</td>
<td>M</td>
<td>IVB</td>
<td>12,831</td>
<td>10,152</td>
<td>6,287</td>
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<tr>
<td>2</td>
<td>78</td>
<td>M</td>
<td>IVA</td>
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<td>4,880</td>
<td>6,823</td>
<td>22.6</td>
<td>Positive</td>
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<tr>
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<td>M</td>
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<td>1,769</td>
<td>1,571</td>
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<td>Positive</td>
<td>23.02</td>
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<td>47.49</td>
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Abbreviation: M, male; TCR, T-cell receptor.

*These cases had been treated with narrow band UVB therapy at the diagnosis of Sézary syndrome. Diagnosis was made based on the following findings: (1) they presented erythroderma; (2) clonally identical neoplastic T cells were detected both in skin and peripheral blood; and (3) Sézary cell counts by peripheral blood smear were >1,000/μl.