Human Cartilage Chitinase 3-like Protein 2: Cloning, Expression, and Production of Polyclonal and Monoclonal Antibodies for Osteoarthritis Detection and Identification of Potential Binding Partners

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Human cartilage chitinase 3-like protein 2 (CHI3L2 or YKL-39) is a member of family-18 glycosyl hydrolases that lacks chitinase activity. YKL-39 is known as a potential marker for the activation of chondrocytes and the progression of osteoarthritis. In this study, we cloned and expressed a functional form of human YKL-39 in the bacterial system. The *Escherichia coli* expressed YKL-30 was used as immugen for production of anti YKL-39 polyclonal and monoclonal antibodies. Both antibody types were highly selective, reacting only with YKL-39. Isotype mapping identified two hybridoma clones (so called clones 6H11 and 8H3) to be IgM isotype. Dot blot assay showed that the monoclonal antibody was strongly active with the synovial fluid of an osteoarthritis patient, human monocyte, and T lymphocyte cell lines. Database search for protein binding partners gave high hits with several glycoproteins that play particular roles in cartilage tissue scaffolding, connective tissue formation, and cell-cell interactions. In conclusion, anti YKL-39 polyclonal and monoclonal antibodies were raised and tested to be suitable for immunological applications, such as the investigation of the YKL-39 regulating pathway and the development of an immunosensing tool for sensitive detection of cartilage tissue destruction.

Introduction

HUMAN CHITINASES AND CHITINASE-LIKE PROTEINS (CLPs) are exclusive members of family-18 glycosyl hydrolases (GH-18) that play important roles in tissue remodeling, injury, or inflammation.^(1,2,3) These proteins include chitinase 3-like 1 protein (CHI3L1 or YKL-40 or HC gp-39),⁽⁴⁾ chitinase 3-like 2 protein (CHI3L2 or YKL-39),^(4,5) oviduct-specific glycoprotein (oviductin or Mucin 9),⁽⁶⁾ and stabilin-1 interacting chitinase-like protein (SI-CLP).⁽⁷⁾ All CLPs possess the (α/β)₈ TIM barrel domain that contains the GlcNAc binding groove, which permits such proteins to retain the ability to bind chitooligosaccharides with high affinity.^(8,9) Substitution of the essential residue (glutamic acid) at the end of the DxxDxDxE conserve motif with leu, ile, or trp results in a lack of chitinase activity in CLPs.⁽²⁾

Human YKL-40 and YKL-39 are homologous with 51% sequence identity. YKL-40 was initially isolated from the conditioned medium of human synoviocytes as a heparin-binding glycoprotein.^(1,10) The name YKL-40 was given based on its three N-terminal amino acids: tyrosine (Y), lysine (K), and leucine (L) and its apparent molecular weight of 40 kDa. High expression level of YKL-40 has been implicated in a

number of inflammatory diseases and various types of solid tumors.⁽¹¹⁾ Recently, YKL-40 has been suggested as a prognostic marker in cancer diseases.^(11,12) On the other hand, its closely related homolog (CHI3L2 or YKL-39) was identified as a non-glycosylated, secretory protein in the primary culture of human articular chondrocytes.⁽⁴⁾ Expression of YKL-39 mRNA was detected in various tissues, including heart, lung, brain, spleen, pancreas, and liver.⁽¹³⁾ YKL-39 mRNA was detected in macrophages under a strong stimulation by a combination of Th2 cytokine IL-4 and TGF- β growth factor.⁽¹⁴⁾ YKL-39 is now recognized as a specific biomarker for the activation of chondrocytes and the progress of osteoarthritis (OA),^(15,16) a degenerative joint disease involving degradation of articular cartilage and subchondral bone that affects 25% of adults over 65 years of age globally. Real-time PCR and DNA microarray analysis showed that YKL-39 mRNA was significantly upregulated in cartilage of patients with OA.⁽¹³⁾ Moreover, YKL-39 mRNA expression was correlated with collagen type 2 upregulation in both early and late stages of the disease.⁽¹⁵⁾ In addition, YKL-39 was found to induce an autoimmune response in patients with rheumatoid arthritis (RA),^(17,18) as well as in mouse models.⁽¹⁹⁾ The elevated mRNA level of YKL-39 was also detected in human

embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells through a signal transduction regulated by ERK1/ERK2 kinases.⁽²⁰⁾ Nevertheless, the detailed regulated pathway controlled by YKL-39 remains poorly understood.

In this study, we describe gene isolation and cloning of YKL-39 from human cDNA. The recombinant protein was expressed in large quantity in the *Escherichia coli* system as a fully functional form, hence allowing anti YKL-39 polyclonal and monoclonal antibodies to be generated. Both polyclonal and monoclonal antibodies proved to be highly specific for human YKL-39 and may be suitable for use in the investigation of protein-protein or protein-ligand interactions.

Materials and Methods

This research was conducted according to the internationally recognized guidelines for animal handling. The document requested for animal studies was viewed and approved from the Ethics Committee for Researchers Involving Animal Subjects, Suranaree University of Technology (Thailand). This research was conducted according to the guidelines for human handling based on the declaration of Helsinki. The document requested for human studies was viewed and approved from the Ethics Committee for Researchers involving Human Subjects, Suranaree University of Technology.

Gene isolation and cloning of human YKL-39

A set of specific oligonucleotides was designed to amplify the full-length *CHI3L2* gene encoding YKL-39 (GenBank accession no. NM_004000) by PCR technique using human cDNA (GenScript, Piscataway, NJ) as DNA template. The forward oligonucleotide, including a *Bam*HI cloning site and the reverse oligonucleotide, including an *Xho*I cloning site are:

*Bam*HI

YKL-39 (forward): 5'-GCGGATCCTACAACTGGTTTGCTAC-3'

YKL-39 (reverse): 5'-ACTCGAGTTACAGGGAGCCAAGGCTTCT-3'

*Xho*I

The PCR product of expected size (1.1 kb) was purified using the QIAquick Gel Extraction Kit (Qiagen, Singapore). The purified fragment was cloned into pET32a(+) vector, generating the recombinant plasmid, so-called pET32a(+)/*CHI3L2*. Employing this vector system, the recombinant YKL-39 was expressed as a fusion protein that contains thioredoxin (Trx) fragment, following the hexahistidine (His₆) residues attached at the N-terminus of the YKL-39 polypeptide. The Trx fragment was fused to the protein to enhance protein solubility, while the His₆ tag was included to aid protein purification. Both sense and anti-sense strands of the *CHI3L2* gene were confirmed by automated DNA sequencing (First Base Laboratories, Malaysia).

Recombinant expression and purification

Single colonies of the *E. coli* BL21 (DE3) cells harboring the recombinant plasmid pET32a(+)/YKL-39 were grown overnight in Luria Bertani (LB) medium containing 100 mg/mL ampicillin (LB/Amp) at 37°C with gentle agitation. The next day the starting *E. coli* culture was transferred to a larger volume (2–4 L) of the same medium, and grown until OD₆₀₀ reached 0.6. Expression of the recombinant YKL-39 was in-

duced by adding 0.5 mM IPTG into the culture medium, and the cells were grown at 25°C for an additional 18 h. Cell pellet, harvested by centrifugation, was resuspended in 30 mL of lysis buffer (50 mM Tris-HCl [pH 8.0] containing 50 mM NaCl, 1 mM PMSF, 1 mg/mL lysozyme, and 1% (v/v) triton X-100), and lysed on ice using a Sonopuls Ultrasonic homogenizer with a 6-mm-diameter probe. Crude supernatant containing soluble fusion protein obtained after centrifugation at 12,000 rpm for 45 min was applied on a Ni-NTA agarose column (10 cm × 1 cm; Qiagen, Hilden, Germany) by gravity flow. YKL-39 containing fractions eluted by 250 mM imidazole were pooled and then thoroughly dialyzed against 20 mM Tris-HCl buffer [pH 8.0] for a complete removal of imidazole. The protein solution was concentrated using Vivaspinn-20 membrane concentrator (*M_r* cut-off 10,000; Vivascience, Hannover, Germany). Further purification was performed using anion exchange chromatography on a 5 cm × 1 cm Hi-Trap Q FF pre-packed column (GE Healthcare, Bangkok, Thailand) connected to an ÄKTApriTM plus system (GE Healthcare). The purified fusion protein was cleaved by enterokinase for removal of thioredoxin. Afterwards, the Trx/His₆ fragment was removed from the YKL-39 protein by passing the protein mixture through a second Ni-NTA agarose column. Unbound fractions containing the purified YKL-39 were pooled and buffer was exchanged to 20 mM Tris-HCl (pH 8.0) and concentrated again using Vivaspinn-20 membrane concentrator (*M_r* cut-off 10,000). The purity of YKL-39 was verified by SDS-PAGE. Protein concentration was determined by Pierce BCA assay. Aliquots of the purified YKL-39 were stored at –30°C until use.

Polyclonal antibody production

Two male New Zealand rabbits (8 weeks old) were immunized subcutaneously with purified YKL-39 emulsified in PBS with 50% Freund's complete adjuvant (Sigma, St. Louis, MO). Immunizations were repeated twice every second week by the same antigen. Five days after the final boost, blood was collected from the central ear artery. The immunized serum was allowed to clot and retract at 4°C overnight, and the serum was retrieved by centrifugation at 4500 rpm for 15 min. The immunized serum was further purified by affinity chromatography using protein A agarose column (GenScript). After the purified antiserum was eluted with 0.1 M glycine (pH 2.5), the pH of the antibody solution was immediately raised to 7.4 with 1.5 M Tris base. The eluted fraction was then applied onto a HiPrep 26/10 desalting column (GE Healthcare, Uppsala, Sweden) for buffer exchanging to PBS. Aliquots of the purified antibody were stored at –30°C until use.

Antibody titers of the anti-YKL-39 pAb were determined by Western blot analysis. Briefly, the purified YKL-39 (5 µg) was resolved on 12% polyacrylamide gel, and then electrophoretically transferred onto a nitrocellulose membrane by the semi-dry transferring technique. After blotting, non-specific binding was blocked by incubation with 5% (w/v) skimmed milk in 1x PBS for 1 h at 28°C. The blocked membrane was rinsed once with 1x PBS, then incubated for an additional 1 h with anti-YKL-39 antiserum at a dilution of 1:20,000. Cross-reactivity of the purified pAb (10 ng) was tested by indirect ELISA against three different YKL-39 homologs. These proteins included YKL-40, acidic mammalian chitinase (AMCase), and bacterial chitinase. Signals

representing antibody-protein interactions were detected with the HRP-conjugated IgG using the enhanced chemiluminescence method.

Monoclonal antibody production

Two BALB/c mice were immunized intraperitoneally with 70 µg of YKL-39 protein at 2-week intervals. The mice were bled after the third week of immunization and checked for antibody production by indirect ELISA, as described above. Splenocytes of the immunized mice were mixed with P3X63Ag8.653 mouse myeloma cells at a ratio of 2:1 and then centrifuged at 4500 rpm for 10 min. Cell pellets were incubated at 37°C for 5 min before being fused together using 50% (v/v) polyethylene glycol (PEG), following standard hybridoma technique. After the fusion reaction was completed, the fused cells were collected by centrifugation, resuspended in hypoxanthine-aminopterin-thymidine (HAT) medium, and distributed into the 96-well plates. Positive hybridoma cells were screened from about 200 hybridoma clones by indirect ELISA. After limiting dilutions were carried out, five positive clones were further screened, and finally two single monoclones, namely 6H11 and 8H3, were obtained. The hybridoma cells obtained from both monoclones were counted, and cell concentrations were adjusted to 4, 2, and 1 cells per 150 µL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) BM condensed H1 and 10% (v/v) FCS (Gibco, Grand Island, NY). After 7–10 days of cultivation, sensitivity and specificity of the monoclonal antibody (MAb) were determined by indirect ELISA and Western blot analysis, as described above.

Isotype mapping

The specific isotype of clones 6H11 and 8H3 was determined by captured ELISA technique using IsoQuick™ Kits for Mouse Monoclonal Isotyping (Sigma). Each well of ELISA plate was coated with 50 µL of different goat anti-mouse Ig isotypes prepared in carbonate/bicarbonate buffer (pH 9.6) at 4°C for 18 h. The coated plate was blocked with 2% (w/v) skim milk in PBS (pH 7.2) for 1 h at 28°C. Then, the plate was washed four times with 0.05% (v/v) Tween-20 in PBS (washing buffer). Fifty µL of 10 µg anti-YKL-39 MAb were added into the well and further incubated for 1 h at 28°C. After the washing step, a rabbit anti-mouse Ig conjugated with HRP (Dako, Glostrup, Denmark) at 1:2000 dilution was added and incubated for an additional 1 h. To develop color of the peroxidase reaction, 100 µL TMB substrate (Invitrogen, Carlsbad, CA) was added into each well after washing four times. The enzyme reaction was then terminated by adding 100 µL of 1 N HCl. The intensity of the developed color was determined spectrophotometrically using Anthos MultiRead 400 Microplate Reader (Biochrome, Cambridge, United Kingdom) at a wavelength of 450 nm (A_{450}).

Cell culture

All human cell lines were from American Cell Culture Collection (Manassas, VA). Human T cell lines (Jurkat) and myeloid cell lines (U937 and THP-1) were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated FCS (Gibco), 40 µg/mL gentamycin, and 2.5 µg/mL amphotericin B (Biolab, Smutprakarn, Thailand) in a humidified atmo-

sphere of 5% CO₂ at 37°C. Hela and COS-7 (Monkey, kidney cell line) cells were cultured in DMEM (Gibco) with the condition as mentioned above. After cell passages three times a week, the cells were collected, and washed three times with PBS by centrifugation at 1500 rpm for 5 min. Then 1×10^7 cells were lysed on ice for 30 min in 1 mL of lysis buffer (50 mM Tris-HCl [pH 8.2], 100 mM NaCl, 2 mM EDTA, 0.02% (w/v) NaN₃) containing 1% (v/v) TritonX-100, 1 mM phenylmethylsulphonylfluoride (PMSF), 5 mM iodoacetamide, and 10 µg/mL aprotinin at 4°C for 30 min. Clear lysate was collected by centrifugation at 13,000 rpm, 4°C for 30 min, and used for functional assays.

Dot blot analysis

Purified YKL-39 or whole cell lysate prepared from various cell lines was spotted onto a nitrocellulose (NC) membrane and then allowed to dry. Non-specific membrane binding was blocked by 5% (w/v) skim milk in 1% (v/v) Tween-20 in PBS-T for 1 h at 28°C. The membrane was incubated for an additional 1 h with the MAb from clone 6H11. The membrane was washed five times in PBS-T, 5 min each, and incubated with HRP-conjugated IgG antibody (Promega, Madison, WI) at a dilution of 1:15,000 for 1 h. After thoroughly washing, the signals were detected by enhanced chemiluminescence method.

Results and Discussion

A database search showed that the *CHI3L2* gene encoding YKL-39 was located in human chromosome 1 and locus 1p13.3. We employed the nucleotide sequence of *CHI3L2* cDNA in the GenBank database (www.ncbi.nlm.nih.gov/genbank) to design a set of oligonucleotides and then amplify the full-length *CHI3L2* gene. The PCR fragment of an expected size (1.1 kbp) was directly cloned into pET32a(+) expression vector so that the recombinant YKL-39 polypeptide lacking a 26-aa signal sequence could be expressed in *E. coli* BL21(DE3) host strain. The deduced amino acid sequence of YKL-39 was found to be identical to the 390-aa sequence of *CHI3L2* isoform 1 (identifier: Q15782-4) reported in the UniProtKB/Swiss-Prot database. The exception is that a single amino acid residue in our sequence at position 318 had Trp instead of Arg. The discrepancy of this amino acid has been suggested previously to be a natural variation that occurs due to a single nucleotide polymorphism in relation to tissue specificity.⁽²¹⁾

The deduced amino acid sequence of human YKL-39 was compared with that of YKL-40 (*CHI3L1*, P36222), acidic mammalian chitinase (AMCase, Q9BZP6-1), human chitotriosidase (CHIT1, Q13231), human oviduction (oviduction, Q86YN0), and stabilin-1 isoform 1 (SI-CLP, Q9NY15). The YKL-39 sequence is closest to CHIT1 (55.2% identity), followed by YKL-40 (51.3%). On the other hand, YKL-39 showed lowest identity to SI-CLP (17.5%). Figure 1 is the structurally based alignment of YKL-39 generated by ESPript⁽²²⁾ with other GH-18 homologs with known 3D structures. The secondary structure elements of YKL-39 comprise eight helices and eight β -strands that form an $(\alpha/\beta)_8$ TIM barrel domain. This domain is responsible for binding to chitoooligosaccharides. The amino acid residues S143 and I145 located at the end of DxxDxxSxE motif (Fig. 1, black asterisks) are reported to suppress chitinase activity. In true chitinases like AMCase⁽²³⁾ and CHIT1,⁽²⁴⁾ these two residues are found as Asp and Glu

FIG. 1. Structure-based alignment of human YKL-39 and its homologs. The sequence alignment was generated by CLUSTAL W. The amino acid sequence of human YKL-39 obtained from this study has the sequence identical to the database sequence (SwissProt: Q15782) with a genetic variant of R → W at position 318. The YKL-39 sequence was aligned with the amino acid sequence of human acidic mammalian chitinase, AMCase (Swiss-Prot: Q9BZP6); human macrophage chitinase, CHIT1 (Swiss-Prot: Q13231); and human chitinase-3-like protein 1, CH13L1 or YKL-40 (SwissProt: P36222). The secondary structural components of AMCase (pdb 3FXV), CHIT 1 (pdb 1GUV), and YKL-40 (pdb 1HJW) were executed, edited, and displayed as a PostScript output using the program ESPript, v 2.2.⁽²⁴⁾ The amino acid residues that are important for binding to GlcNAc units are indicated by pink triangles. The last three acidic residues in the DxxDxDxE motif (yellow letters), which are important for chitinase activity of AMCase and CHIT1, are identified by black asterisks.

instead. Double mutations of S143 to D143 and I145 to E145 in YKL-39 created pseudo-chitinase activity for this protein.⁽⁹⁾

YKL-39 was successfully expressed in *E. coli* BL21 (DE3) cells using pET32a(+) expression vector as a fusion protein with thioredoxin (Trx), followed by hexahistidine (His₆) residues attached at the N-terminus. The total molecular weight of the fusion protein was estimated to be 58.6 kDa. Figure 2 is the chromatographic profile of the Trx/His₆/YKL-39 fusion protein after two purification steps using Ni-NTA agarose affinity chromatography followed by HiTrap Q FF pre-packed column (5 × 1 mL, GE Healthcare). Bound fractions (Fig. 2, inset) eluted with a linear gradient of 0–100% of 1 M NaCl yielded a single protein band that migrated near 55 kDa. After enterokinase cleavage to remove the Trx/His₆ fragment, the YKL-39 polypeptide with seven extra residues (A-M-A-D-I-G-S) preceding the YKL sequence was seen on SDS-PAGE gel at the molecular weight between 35–45 kDa, which corresponded to the predicted mass of 41.5 kDa. After a second Ni-NTA agarose column, the final yield of the purified YKL-39 obtained from 1 L of bacterial culture was ~3.4 mg (Table 1).

The purified YKL-39 protein was used as immunogen for production of anti-YKL-39 polyclonal antibody. After final immunization, the immunized serum was collected and purified using protein A column (Genscript). Titers of the purified antibody were subsequently determined by indirect

FIG. 2. Purification of *E. coli* expressed YKL-39. The *E. coli* expressed YKL-39 fusion protein was applied onto a Ni-NTA agarose column, followed by a HiTrap Q FF pre-packed column (5 cm × 1 cm, GE Healthcare). Inset shows SDS-PAGE of the eluted fractions containing YKL-39. After removal of Trx/His₆ fragment with enterokinase, highly purified YKL-39 was obtained from the second Ni-NTA agarose column.

TABLE 1. PURIFICATION OF HUMAN YKL-39

Purification step	Total volume (mL)	Total protein (mg)	Yield (%)	Protein form
Crude extract	30	105	100	Trx/His ₆ /YKL-39
First Ni-NTA affinity column	15	6.8	6.5	Trx/His ₆ /YKL-39
Anion exchange column	5	5.1	4.8	Trx/His ₆ /YKL-39
Second Ni-NTA affinity column	5	3.4	3.3	YKL-39

E. coli BL21 (DE3) host cells harboring recombinant plasmid pET32a(+)/YKL-39 were cultured in LB/Amp medium until OD₆₀₀ reached 0.6. Expression of YKL-39 fusion protein was induced by 0.5 mM IPTG under 25°C for 18 h. Crude extract was prepared after the cell pellet was collected after centrifugation. The yields were obtained from 1 L of bacterial culture.

ELISA. Our raised antibody was found to be highly sensitive, reacting strongly with the YKL-39 protein even when only 5 ng of the purified antibody was used. For further biochemical characterization, 10 ng of the YKL-39 antiserum was selected as the optimum concentration. Figure 3A is the Ag:Ab binding curves, showing concentration dependence of anti-YKL-39 antiserum towards the recombinant YKL-39 and other CLP homologs. The hyperbolic feature, mimicking the Michaelis-Menten plot, between the interaction signals versus YKL-39 concentrations indicated specific interactions between the YKL-39 antiserum and antigen (Fig. 3A, red curve). When YKL-40 (green), AMCase (purple), and bacterial chitinase A (blue) were used as immunogens, no significant increase in the signal was observed. In addition, the antibody did not react with the pre-immune serum even at the maximum amount of YKL-39 used (gray line). The results indicate that the raised antibody was highly specific to the YKL-39 antigen. The binding intensity of each antigen was further

tested at a fixed concentration of 500 ng/well and the YKL-39 antibody of 10 ng/well (Fig. 3B). The absorbance obtained for YKL-39 was 15–19 fold greater than that for human YKL-40, AMCase, and bacterial chitinase. This further confirmed a high specificity of the purified antibody towards YKL-39.

YKL-39 is recently known as an osteoarthritis marker, since expression of YKL-39 was highly upregulated at mRNA and protein levels in cartilage from patients with osteoarthritis.^(13,15) Therefore, the availability of in-house polyclonal and monoclonal antibodies will open up an opportunity for the development of immunological tools (i.e., specific YKL-39 ELISA kit and YKL-39 immunosensor) for a sensitive detection of the disease progression. We further employed the hybridoma technique^(27,28) to raise monoclonal antibody specific for the *E. coli*-expressed YKL-39. Two positive clones, namely 6H11 and 8H3, were obtained after a limiting dilution screen of about 200 hybridoma clones. Isotyping mapping clearly suggested that both monoclones belonged to IgM isotype (Fig. 4A). To date, there is only one report on the monoclonal antibody against YKL-39 but its isotype has not been identified, although the MAb was purified using protein G column.⁽²⁵⁾ Otherwise there is no availability of specific YKL-39 MAb on the market. Because the 6H11 clone exhibited greater immunoactivity, we then selected this clone for further characterization. Crude anti-YKL-39 MAb was purified using the HiTrap IgM purification HP column (GE Healthcare), according to the manufacturer's instruction, and the antibody titers were tested against recombinant YKL-39 by Western blot analysis. As seen in Figure 4B, the strongest titer was seen at a ratio of 10:5 (μg/μg) of MAb:Ag, while the slightest titer of the antibody that recognized the YKL-39 Ag was 2.5:5(μg/μg). We further determined specificity of the newly raised MAb against various GH-18 homologs using Western blot analysis. As shown in Figure 4C, the antibody recognized both fused (lanes 2 and 3) and bare (lane 4) forms of human YKL-39, but did not recognize YKL-40 (lane 5), AMCase (lane 6), and bacterial chitinase (lane 7). The

FIG. 3. Production of anti-YKL-39 polyclonal antibody. (A) Anti-YKL-39 antiserum was raised against the purified YKL-39. Specificity of the purified anti-YKL-39 antibody (10 ng/well) towards different concentrations (0–500 ng/well) of the CLPs was tested using indirect ELISA assay. Red, YKL-39; green, YKL-40; purple, AMCase; blue, bacterial chitinase. Pre-immune serum tested with YKL-39 is presented (gray line). (B) Bar chart showing immunological interaction between a fixed concentration (500 ng/well) of YKL-39 homologs and purified YKL-39 antiserum (10 ng/well). Values are averaged from the data of triplicate trials.

FIG. 4. Monoclonal antibody production and characterization. Highly purified YKL-39 was used as immunogen to produce anti-YKL-39 MAb (see Materials and Methods section). (A) Isotype of the two hybridoma clones, 6H11 and 8H3, obtained after limiting dilutions was tested using the IsoQuick™ Kits for Mouse Monoclonal Isotyping (Sigma, St. Louis, MO). (B) Western blot analysis showing titration of purified YKL-39 MAb reactivity with YKL-39 at different concentrations: lane 1, 10 μg; lane 2, 5 μg; lane 3, 2.5 μg; lane 4, 1.25 μg; lane 5, 0.625 μg. (C) Cross-reactivity of anti-YKL-39 MAb with other human chitinases and chitinase-like proteins was analyzed by SDS-PAGE (left panel) in comparison with Western blot analysis (right panel). Lane M, protein molecular weight marker; lane 1, cell lysate of uninduced *E. coli* BL21 (DE3) host containing pET32a(+)/YKL-39; lane 2, cell lysate of the cells induced with IPTG; lane 3, elution of thioredoxin/YKL-39 from the first Ni-NTA affinity column; lane 4, purified YKL-39 after ion exchange chromatography, followed by thioredoxin removal, and further purified by the second Ni-NTA affinity column; lane 5, YKL-40; lane 6, AMCase; lane 7, bacterial chitinaseA; lane 8, human serum.

antibody also did not cross-react with proteins in human serum (lane 8). The results suggested that the MAb was highly specific for YKL-39.

We tested our MAb with the synovial fluid of a patient with osteoarthritis, and various human cell lines, including Jurkat, U937, Hela, COS-7, and THP-1. Figure 5A (SDS-PAGE) and 5B (immunoblot) show that anti-YKL-39 MAb reacted strongly with a protein band in the whole cell lysate of Jurkat cells (lane 2). This protein migrated to the same position of recombinant YKL-39 (lane 1) on SDS-PAGE. Jurkat cells have been reported previously to express SI-CLP, but not other CLPs.⁽⁸⁾ However, Santa Cruz Biotechnology (Santa Cruz, CA) used the whole cell lysate of Jurkat cells to determine the specificity of their commercially available anti-YKL-39 polyclonal antibody (H-80).⁽²⁶⁾ Figure 5C is dot blot assay showing strong cross-reactivity of anti-YKL-39 MAb with YKL-39, the

synovial fluid of a volunteer with osteoarthritis, and the whole cell lysate of U937 cells. However, the MAb did not recognize all proteins in the whole cell lysates of Hela cells, COS-7 cells, and THP-1 cells. Negative control for which IgG secondary antibody was added in the absence anti-YKL-39 MAb did not produce a positive signal. Such results suggested that our monoclonal antibody was functionally active and exhibited cell type specificity. Detection of YKL-39 in Jurkat cells (a model for differentiation of T lymphocytes) and U937 cells (a model for the differentiation of monocytes and macrophages) indicated that YKL-39, in some way, involves both innate and adaptive immune systems in response to the likelihood of infection or tissue inflammation.

We further analyzed which molecules in human cells possibly react specifically to human YKL-39. The deduced amino acid sequence of human YKL-39 was subjected to the STRING

FIG. 5. Cross-reactivity of anti-YKL-39 MAb. Detection of YKL-39 expression using anti-YKL39 MAb in whole cell lysate of Jurkat cells. (A) SDS-PAGE analysis. (B) Western blot analysis. Lane 1, YKL-39; lane 2, whole cell lysate of Jurkat cells. (C) Dot blot analysis showing antigen-antibody interactions with human sample or human cell lines. Purified YKL-39 or whole cell lysate was spotted onto nitrocellulose membrane, and then the membrane was allowed to dry. After blocking with 5% (w/v) skim milk, the membrane was incubated for 1 h with 6H11 MAb. The signals were detected by enhanced chemiluminescence using rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase at 1:5000 dilution.

search (http://expasy.org/proteomics/protein-protein_interaction). The top five hits identified the binding partners of YKL-39 to be augurin precursor (C2 or f40), followed by cartilage intermediate layer protein nucleotide pyrophosphohydrolase (CLIP), proline/arginine-rich end leucine-rich

repeat protein (PRELP), cartilage acidic protein 1 (CRCAT1), and stabilin 1 (STAB 1). The specific roles of these potential partners are listed in Table 2. Apart from C2, which is a small peptide with a potential function as a hormone stimulating tumor growth, other identified proteins are reported to be

TABLE 2. POTENTIAL BINDING PARTNERS OF HUMAN YKL-39

Abbreviation	Possible binding partner	GenBank code	Major location/function
C2 or f40	Augurin precursor (esophageal cancer-related gene 4 protein)	AAG42321	<ul style="list-style-type: none"> Found as cytoplasmic vesicles. Acts as a peptide hormone involved in physiological homeostasis.
CLIP	Cartilage intermediate layer protein nucleotide pyrophosphohydrolase	AB022430	<ul style="list-style-type: none"> Found in extracellular matrix. Plays a role in cartilage scaffolding. Suppresses IGF1-induced proliferation and sulfated proteoglycan synthesis. Inhibits TGFβ1-mediated induction of cartilage matrix genes. Impairs chondrocyte growth and matrix repair and indirectly promotes aging and osteoarthritis.
PRELP	Proline/arginine-rich end leucine-rich repeat protein	AAH32498	<ul style="list-style-type: none"> Found in connective tissue extracellular matrix. Anchors basement membranes to the underlying connective tissue.
CRTAC1	Cartilage acidic protein 1	CAI12583	<ul style="list-style-type: none"> Found in the interterritorial matrix of articular deep zone cartilage.
STAB1	Stabilin 1	AA150251	<ul style="list-style-type: none"> Involved in cell-cell or cell-matrix interactions. Found in cellular membrane. Functions in angiogenesis, lymphocyte homing, cell adhesion. Acts as a homeostatic receptor, which links signals from the extracellular environment to intracellular vesicular processes.

FIG. 6. Identification of potential binding partners of YKL-39. These functional partners of YKL-39 were identified using the program STRING freely available in the category Proteomics of the ExPASy Bioinformatics Resource Portal (<http://expasy.org/tools/>). Amino acid residues are numbered. The position of glycosylation sites is indicated with a vertical line with number.

highly glycosylated (Fig. 6). These proteins are particularly found as part of the extracellular matrix or involved in cartilage scaffolding or connective tissue remodeling (Table 2). Glycosylation of such proteins with GlcNAc units acting as the sugar core explains why these proteins could recruit YKL-39 with a defined binding groove for GlcNAc moieties at localized areas, such as connective or articular cartilage tissues.

Conclusions

In our study, we cloned and expressed human *CHI3L2* gene that encodes YKL-39, the recently determined osteoarthritis marker. The *E. coli*-expressed YKL-39 was used to produce anti-YKL-39 polyclonal and monoclonal antibodies highly specific for a functional form of human YKL-39. Two hybridoma clones were isolated and found to produce IgM monoclonal antibody. Both polyclonal and monoclonal antibodies were highly specific and active with various types of immunological assays, making them suitable for functional studies, including identification of the YKL-39 regulated pathways involving cartilage tissue degeneration and development of an immunosensing tool for monitoring the progress of degenerative joint disease.

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Author Disclosure Statement

The authors declare that they have no competing interests.

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