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Original

Isolation of a mRNA Preferentially Expressed in Synoviocytes from Rheumatoid Arthritis That is Identical with Lumican, which Encodes a Collagen Binding, Extracellular Matrix Protein

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Abstract: Rheumatoid arthritis (RA) is a complex disease including autoimmune disorder and resulting in inflammation. It also shows progressive proliferation of synoviocytes, and these synoviocytes destroys articular structure. In order to understand the mechanism of this abnormal proliferation of RA-originated synoviocytes in molecular level, we analyzed the gene expression profiles by using DNA chips that contain more than 10,000 genes. Comparing the expression profiles of normal and RA-originated synoviocytes, we found several genes that are highly expressed in RA-originated synoviocytes than normal synoviocytes. Among these genes, we focused on one hypothetical protein, cDNA of which contains one reading frame in its DNA fragment, indicating that this fragment is a part of large mRNA structure. The expression of this gene in RA-originated synoviocytes is about three times higher than that in normal synoviocytes by DNA chip analysis. After cDNA cloning of this mRNA, we found that the gene is identical with lumican, which encodes a collagen binding, extracellular matrix protein. This mRNA was widely distributed in many tissues but its alternatively spliced forms are differently expressed in various tissues.

Key words: Rheumatoid arthritis, Synovial cells, DNA chip, Lumican

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that causes the immune system to attack the joints, arising inflammation and destruction of the joints. These joints are characterized by inflamed synovial hyperplasia with excessive inflammatory cell infiltration, leading to erosion of articular cartilage and marginal bone, with subsequent joint destruction. Much information over the past two decades contributed to the better understanding of the mechanism of inflammation. Nevertheless, the pathogenesis of synovitis has not been fully elucidated[1)]. In RA joints, synovial cells show abnormal growth, and these synoviocytes cover the articular structure destroying articular cartilage. Also the mechanism of these phenomena is not fully understood in molecular level. Recently, some cytokines, receptors and transcription factors were shown to contribute to development of this disease[2-6)]. It was also shown that some genetical background e.g. SNPs associate to the generation of this disease[7)]. We have also shown that increased expression of WISP genes might promote the abnormal proliferation of synoviocytes in RA[8)].

DNA chip technology[9)] made analysis of the expression of thousands genes possible at one time. This technology reveals the difference of gene expression between different types of cells or cells in different conditions. In addition, this technology contributes to the isolation of tumor-specific mutation in chromosome (CGH) or detection of genetic polymorphisms (SNPs)[10)]. As a result, this technology has provided novel diagnostic methods and therapeutic target for developing medicine.

Lumican is a small leucine-rich proteoglycan which belongs to the family including decorin, biglycan, fibromodulin, keratocan, epiphycan and osteoglycin. Lumican is highly expressed in corneal stroma as the major keratan sulfate proteoglycan. It is also expressed in other extracellular matrices (ECM), such as skin, muscle, and cartilage[11-16)]. The results of gene knockout studies showed that lumican plays an important role in the formation of structural phenotype of the mature collagen in various tissues. Another member of the small leucine-rich proteoglycan family, decorin showed the same function as lumican, because decorin-deficient mice were very similar to those of lumican-deficient

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mice[17, 18]. Lumican also showed suppressive effect on colony formation in soft agar or tumor growth of rat fibroblast F204 cells transformed by K-ras or v-src oncogene[19].

In this study, we compared the expression levels of about ten thousand of genes between synovial cells from RA and normal control by using DNA chip, and identified the three novel genes or uncharacterized genes which were up-regulated in RA-originated synoviocytes. Furthermore, we analyzed the expression and structure of lumican, one of these three genes, and also investigated the relationship of this protein with other proteins.

Materials and Methods

Isolation and culture of synovial cells

Fresh synovial tissues were minced and digested with collagenase and DNase I. Tissue debris was removed through a cell strainer, and cells were washed twice with medium. The resultant single cells were dispended into the wells of a 24-well microtiter plate (Costar, Cambridge, MA) at a density of 2 x 10^6 cells /ml in 2 ml of Dubecco's modified Eagle's medium (DMEM; Life Technologies, Gaitherburg, MD) supplemented with 10% HEPES (Life Technologies), 100 IU/ml penicillin and 100 mg/ml streptomycin. The plates were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. Synovial tissue cells were split weekly, once primary cultures had reached confluence, and used after the third passage. These cells morphologically appeared to be homogeneous to fibroblast-like cells. They were cultured at a density of 1 x 10^5 cells /ml in 2 ml DMEM with 10% FCS in a 24-well microtiter plate and allowed to adhere overnight. After the third passage of cell culture, cells are harvested and RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations.

DNA chip analysis

High-density DNA chip (Toyobo Co. Ltd., Tokyo, Japan) containing over 10,000 known and unknown human genes were used for hybridization studies. Hybridization was performed following the instructions of manufacturer's protocol. The chips were aligned on an Imaging scanner (Applied Biosystems Inc, CA) and saved as digital images.

Reverse transcriptase-PCR

Total RNA from normal or RA-originated synoviocytes was reverse transcribed to cDNA using oligo dT_{16} primers with AMV-derived reverse transcriptase, and the resulted first-strand cDNA was amplified with each RASP (RA specific cDNA) specific primer set. The amplification condition was as follows: 95°C (1 min) - 57°C (1 min) - 72°C (2 min) for 35 cycles. The PCR products were applied on agarose gels (2 %) and electrophoresed.

Northernblot analysis

Human MTN blots I and III (Clontech, Labo. Inc., Mountain View, CA) were used for Northern blot analysis. A 240 bp fragment of lumican cDNA was amplified by RT-PCR and this fragment was radiolabeled by using Random primer DNA labeling kit (TAKARA Bio Inc., Shiga, Japan) and [α-^{32}P]dCTP (BD Bioscience, Franklin Lakes, NJ). Hybridization was performed following the instructions of manufacturer's protocol.

Structural analysis

Physico-chemical profiles of RASP were analyzed by using web platforms. For example, AAs sequence of RASP10 (1040 bp, 131AAs) was aligned in some square of analysis page of web analysis platform. Then the sequence was transmitted to the host computer site, and the results were soon retrieved from this host computer. The same operation was performed by using different programs on web platforms.

Results

DNA chip analysis

We utilized DNA chip technology to compare multiple gene expression profiles, representative of human adult normal (leg sarcoma) synoviocytes (n=5 individuals) and RA-originated synoviocytes (n=5 individuals). Total RNA was isolated from primary cultures of OA or RA synoviocytes and then hybridized to high density DNA chip including over 10,000 human genes. Fig. 1 showed typical hybridization patterns of the filters. Comparing the density of each spot between two different fluorescent color (Cy3 as normal and Cy5 as RA), genes differentially expressed between normal and RA-originated cells were isolated. Table 1 showed the ratio, signal intensity and the category of these isolated genes. The intensity values between normal and RA-originated cells were normalized by using the values of control (house-keeping) genes. Competitive comparisons between all normal and RA samples demonstrated a similar expression profile for almost all genes. Eighty-three genes for RA-originated and 131 genes for normal synoviocytes were highly expressed (Table 1). Among these genes, three unknown genes (EST clones) were highly expressed in RA-originated synoviocytes over two-folds. We focused on these cDNAs (RASP2, RASP3 and RASP10).

RT-PCR analysis of novel or unknown genes

Among the several genes that were differentially expressed between normal and RA-originated synoviocytes on DNA chips, we selected three RASP genes. They belonged to the EST clones and were characterized as unknown genes (RASP3) or unknown proteins (RASP2 and RASP10) that encode their proteins (Fig. 2). We also performed RT-PCR analysis of these cDNA clones to confirm their differential expression patterns between normal and RA-originated synoviocytes. When comparison of the expression

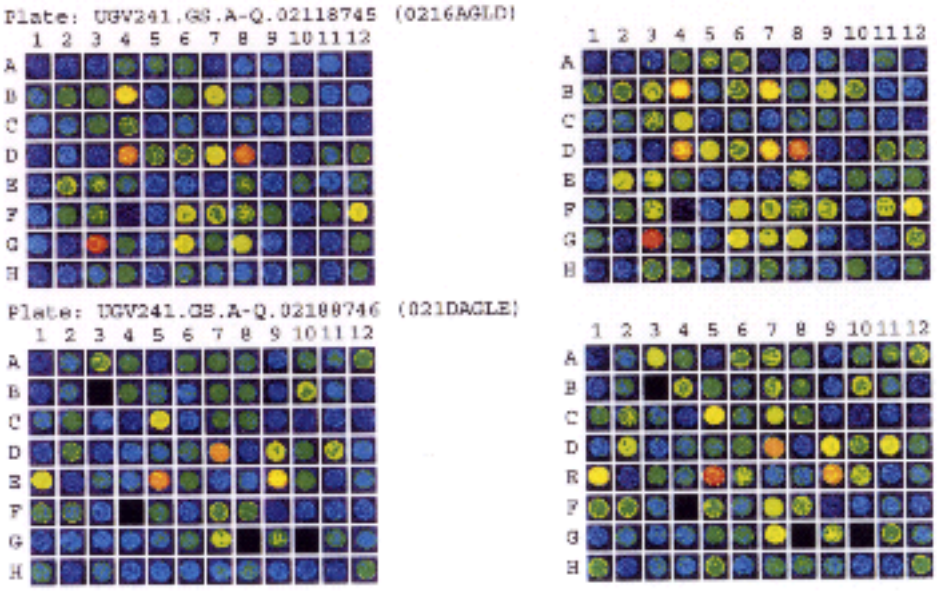

Figure 1. High density DNA chip analysis of mRNA expression in RA-originated and normal synoviocytes. The expression pattern was analyzed by using chip analyzer with two different wavelengths. Left panel shows the results of Cy3 (normal) and the right panel shows the results of Cy5 (RA). Red color shows high expression and yellow color shows medium expression.

of three RASP genes between normal and RA-originated synoviocytes was made, all three genes were highly expressed in RA-originated synoviocytes. (Fig. 2) as shown in the results of DAN chips. Among them, RASP2 and RASP10 showed high expression in RA-originated synoviocytes.

Structure analysis of RASP10

RASP10 cDNA (1040 bp) is included in human ovary tumor cDNA and encodes unknown protein with 131 AAs. We analyzed physico-chemical profiles of this hypothetical protein including secondary structure and hydrophobicity pattern by using some web-based platform for analysis of secondary structure of proteins. The prediction results showed that this protein has highly characteristic structure (Fig. 3). Based on predicted secondary structure and hydrophobicity, the N-terminal half of RASP10 was hydrophilic and C-terminal half of RASP10 was hydrophobic. Furthermore, there were typical helix structures and several phosphorylation sites by PKC and TK, indicating that RASP10 is a kind of membrane protein which recognizes some unknown ligands.

Northern blot analysis of RASP2

Next, we analyzed the expression pattern of RASP2 mRNA. RASP2 cDNA (1717 bp) is included in human ovary tumor cDNA and encodes a 338 AAs protein. This protein was found to be a keratin sulfate proteoglycan, named lumican. Lumican is a small

Table 1. Isolated clones by high density DNA chips. K means known genes and U means unknown genes. The ratio of RA/N or N/RA is indicated after the name of each gene.

Ratio		cDNA clones	
RA > N	1.6 – 2.0	K: 41	α2-actin (1.9), keratin 7 (1.6), FGF9 (1.6)
Control 3		U_1: 18	ESTs
2.1, 2.2, 8,3		U_2: 14	ESTs
(mean 2.5)	>2.0	K: 7	Igκ (5.0), $Igγ_3$ (2.8), Igl (2.0)
		U_1: 1	EST
		U_2: 2	ESTs
N > RA	1.6 – 2.0	K: 65	BMP4 (1.8), IL-13 (1.7), MMP-9 (1.7)
Control 10		U_1: 15	ESTs
11.4, 7.6, 6.4, 6.0		U_2: 13	ESTs
(mean 7.9)	>2.0	K: 31	IL-13R (2.8), IGFBP6 (2.5), PLA_2 (2.2)
		U_1: 2	ESTs
		U_2: 5	ESTs

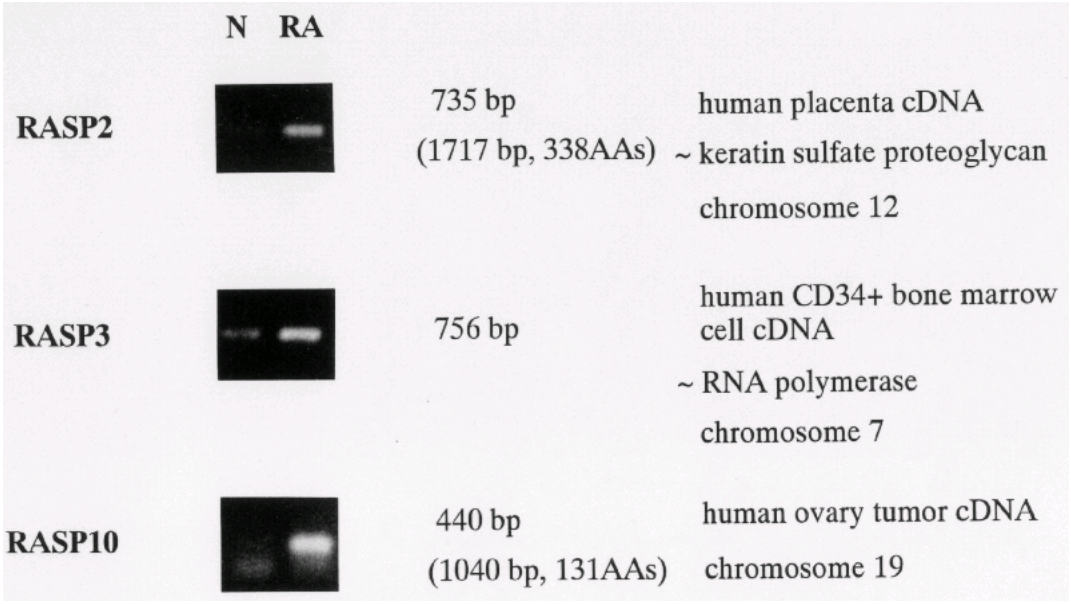

Figure 2. The expression of three novel genes (RASP2, 3 and 10) isolated from the results of high density DNA chips of normal and RA-originated synovial cells analyzed by RT-PCR, their sequence analysis and functional information. The results of RT-PCR reflected the results of expression levels in DNA chips. N means normal.

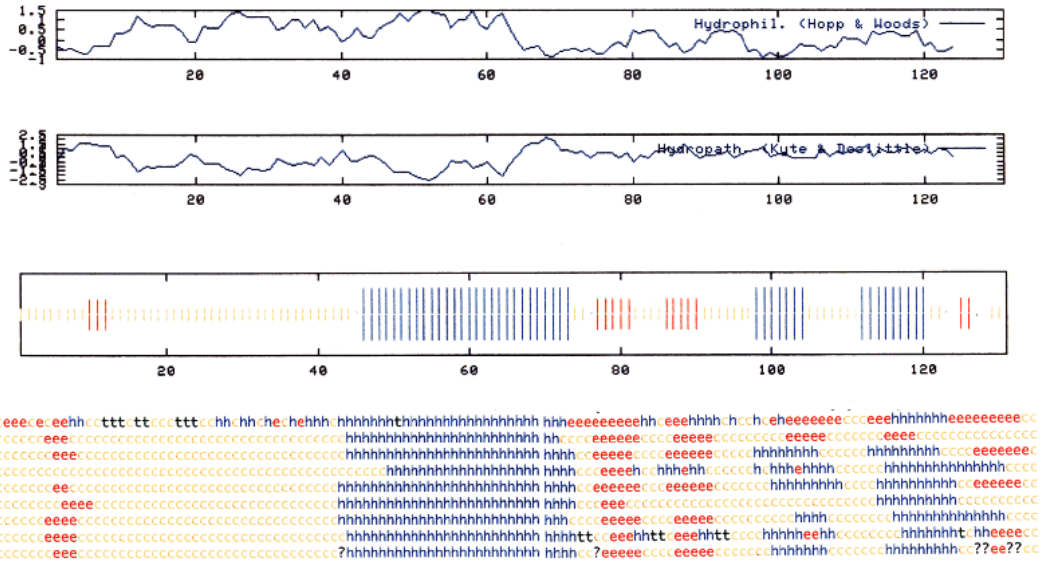

Figure 3. Prediction of secondary structure of the RASP10 protein. Of note, its N-half was hydrophilic and C-half was hydrophobic. In addition, there were several phosphorylation sites by PKC and TK. H indicates alpha helix and E indicates beta sheet.

leucine-rich proteoglycan and is highly expressed in corneal stroma as the major keratan sulfate proteoglycan. It plays an important role in the formation of structural phenotype of the mature collagen in various tissues. Northern blot analysis of RASP2 mRNA in various tissues showed that there were two kinds of mRNA with different sizes (1.7kb normal mRNA and small mRNA), and these two RNAs were differentially distributed in various tissues (Fig.4). The small mRNA (about 0.3kb) showed high expression in heart, skeletal muscle, adrenal gland, lymph node, stomach and thyroid. Of note, the small mRNA showed highest expression in heart and skeletal muscle. Contrary, the normal mRNA was highly expressed in heart and placenta.

Discussion

In the present study, we compared representative gene expressed profiles between normal and RA samples, using high density DNA chips. This chip technology offers the advantage of comparing multiple profiles generated from different subjects, although it

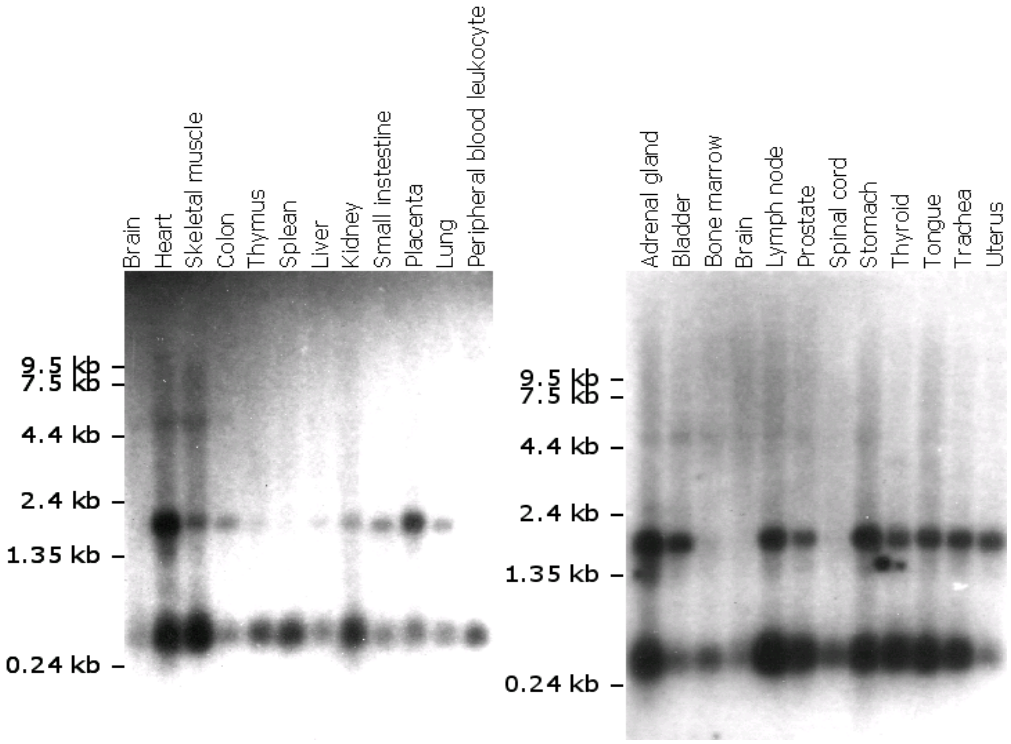

Figure 4. The expression of RASP2 in various tissues. Northern blot analysis was performed by using MTN blots I and III (Clontech). Of note, two kinds of RASP2 transcripts were observed.

still has some technical difficulties[9]. During the analysis, competitive comparisons were performed between normal and RA synovial samples from several different individuals to minimize the variation between each sample. Concerning its quantitative aspects, repeated experiments showed reproduced consistent data of chip analysis. The sensitivity was relatively low comparing with Northern blot, and it sometimes failed to detect the difference between two samples when Northern blot could detect such difference[20]. Nevertheless, data obtained with DNA chips show almost the same and consistent results comparing with RT-PCR in this case, and this method is thought to be one of the most powerful tools for integral analysis of gene expression.

Some groups have tried to analyze gene expression in RA using microarray technique[20]. Heller et al. randomly selected 1,000 human genes from a peripheral human blood cell library and also selected genes significant in inflammation, and designed microarrays with 96 elements. They compared gene expression of macrophages, chondrocytes and synoviocytes of human RA tissue, and showed known participants of TNF, IL-1, IL-6, IL-8, G-CSF and VCAM and novel participation of the IL-3, C-X-C chemokine Gro alpha and the matrix metallo-elastase.

In this study, we used high density DNA chips with over 10,000 human genes. We prepared fluorescent-labeled cDNA probes from total RNA isolated from synoviocytes of normal (Cy3)- or RA (Cy5)-derived articular tissues, and competitively hybridized these probes to one DNA chip. As a result, we succeeded to show known molecules, which were mainly related to inflammation and novel molecules for RA. Some of the known molecules were already shown in previous studies in relation to inflammation or abnormal growth of synoviocytes. (FGF, BMP, NMP, Ils and Igs) but we also found additional new molecules for RA (actin and keratin). FGF are known growth factors for fibroblasts. MMP is one of the matrix metalloproteases related to degradation of type I, II, III, VII, VIII and X collagens that promote cell growth and movement. Overexpression or up-regulation of all these factors is thought to be involved in the abnormal promotion of synovial cell tissue differentiation and the function of small RNA should be explained. These problems would also have some relation to the cause of some diseases including RA.

RASP10 (1040 bp, 131AAs) also has very special characters. Physico-chemical profiles of this protein (Fig.3) showed this protein has a character of membrane binding protein because hydrophobicity of N terminal half and C terminal half is quite different. N terminal half is hydrophilic and C terminal half is hydrophobic, indicating that this protein would bind to cell membrane with its C terminal half. It also has several phosphorylation sites by PKC and TK, showing that it might play some signal transducible function on cell membrane. Although the function of this protein is unknown, this protein will be quite important for cell proliferation, and the increase of this protein in RA synovial cells would cause its abnormal growth.

Further investigation for analysis of the function of these

proteins in the process of abnormal growth of RA synoviocytes is warranted.

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