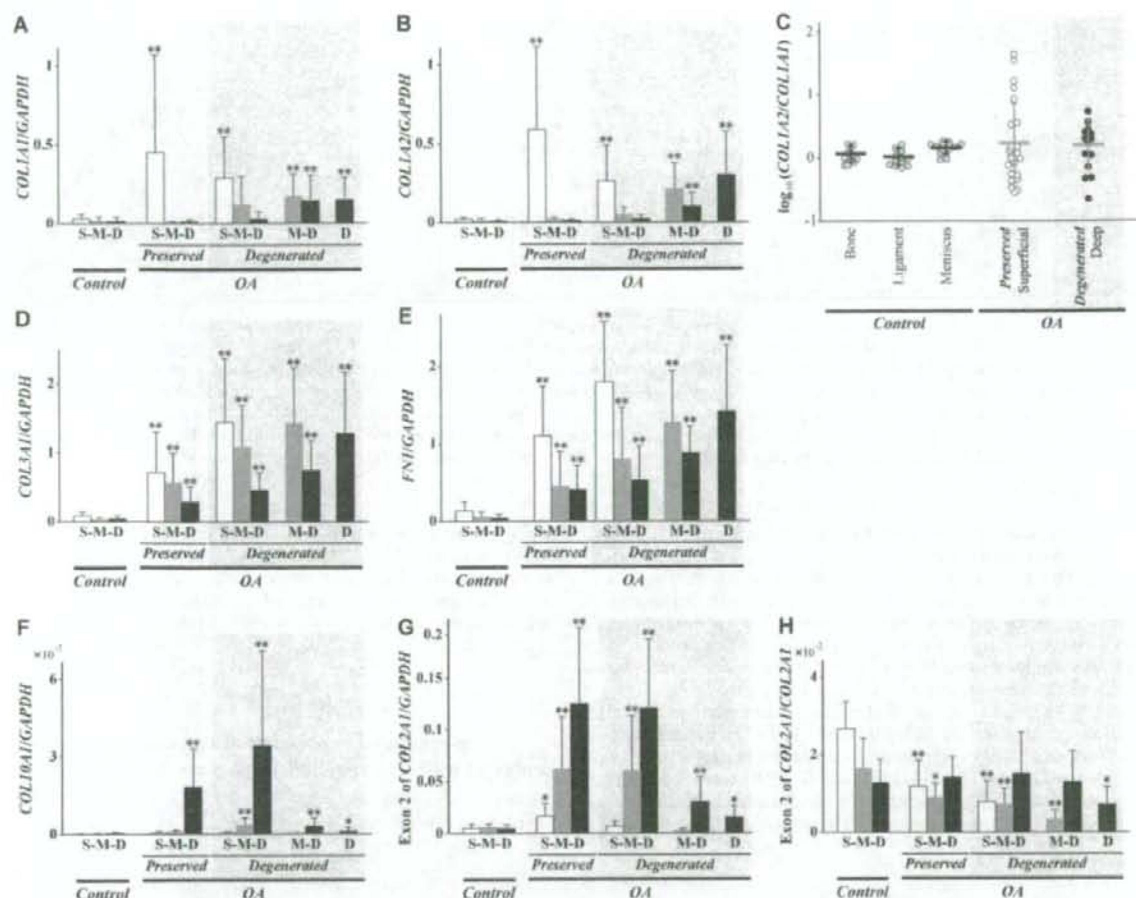

Figure 3. Expression of minor cartilaginous genes in OA and control cartilage. **A** and **B**, Expression of *COL1A1* (**A**) and *COL1A2* (**B**) in control and OA cartilage is shown as ratios of the expression of *GAPDH*, as described in Figure 2. **C**, The expression ratios of *COL1A2* to *COL1A1* were obtained in the superficial zone in preserved areas and in the deep zone in degenerated areas where the zone was directly exposed to the joint cavity, and were compared with those obtained in bone, ligaments, and menisci harvested from control joints. Ratios are shown in logarithmic values. **D–F**, Expression of genes coding type III collagen (*COL3A1*) (**D**), fibronectin (*FN1*) (**E**), and type X collagen (*COL10A1*) (**F**) is shown as ratios of the expression of *GAPDH*. **G** and **H**, Expression of exon 2 of *COL2A1* gene is shown as ratios of the expression of *GAPDH* (**G**) and by ratio to the total expression of *COL2A1* (**H**). S-M-D, M-D, and D under the respective groups of bars indicate the zone(s) retained in the samples. Each bar represents the results from at least 11 samples. Values are the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$, versus the corresponding zone in control cartilage. See Figure 2 for definitions.

from areas showing macroscopic signs of degeneration. In this study, such areas were designated "preserved" and "degenerated" areas, respectively. OA and control cartilage samples were separated into 3 cartilage zones by LCM, and gene expression was evaluated in the respective cartilage zones by real-time PCR, considering the zonal difference and the severity of cartilage degeneration.

Compared with that in the control cartilage, the expression of type II collagen was strongly up-regulated in all areas in OA cartilage (Figure 2A). The up-regulation was most apparent in the deep zone, where the expression was ~20-fold that in the corresponding zone of the control cartilage. In contrast, the level of up-regulation was considerably reduced in the upper part of the degenerated cartilage. Where the zones were

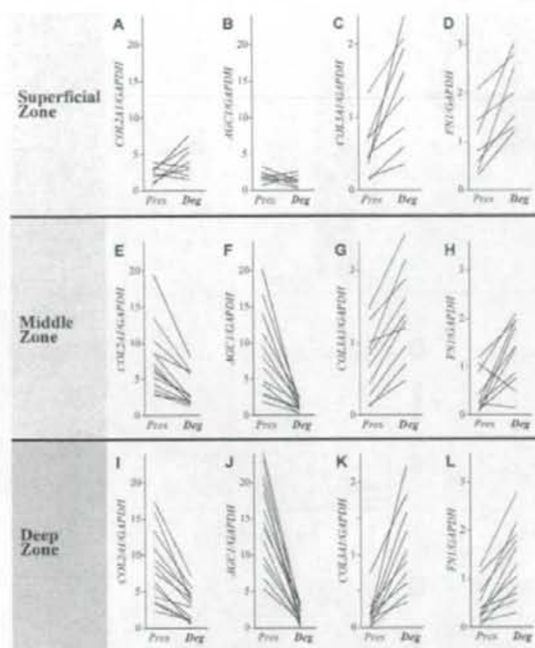

Figure 4. Comparison of gene expression between preserved (Pres) and degenerated (Deg) areas. In each osteoarthritic joint, the expression of 4 genes was compared in the respective cartilage zones between the preserved and degenerated areas. For the middle and deep zones, expression in the degenerated area was determined where the zones were directly exposed to the joint cavity due to the loss of the upper zone(s) to the disease. Expression of *COL2A1* (**A**, **E**, and **I**), *AGC1* (**B**, **F**, and **J**), *COL3A1* (**C**, **G**, and **K**), and *FN1* (**D**, **H**, and **L**) in the superficial, middle, and deep zones is shown. In these graphs, each line represents the expression in a single joint. Results from 7–13 joints are shown as the ratio of gene expression to *GAPDH* expression.

directly exposed to the joint cavity due to the loss of the upper zone(s) to the disease, the expression levels in the middle and deep zones were almost half of those in the preserved areas.

The expression of aggrecan was also enhanced in OA cartilage (Figure 2B). Similar to type II collagen, the increase was most obvious in the deep zone of the preserved area but was less intense in the degenerated area. In this gene, the regional change of expression was more obvious than that in type II collagen. Thus, in the middle and deep zones exposed to the joint cavity in degenerated areas, the expression was virtually unenhanced, and the expression levels were similar to those in the control cartilage. The expression of link protein presented a regional change similar to that of aggrecan, although the decline in the degenerated area was less apparent (Figure 2C).

Spatially distinctive patterns in OA cartilage shown by expression of minor cartilaginous genes induced by OA. In OA, there is enhanced expression of several genes that are not expressed at substantial levels in normal cartilage. Types I, III, and X collagen and fibronectin are among those genes (5,9,13,14,20–22), which are termed minor cartilaginous genes in this report. A change in alternative splicing also occurs in OA, and there is induced expression of exon 2 of type II collagen gene, which is not expressed in healthy adult cartilage (11,12). Therefore, we evaluated the expression of these genes and the exon in OA and control cartilage, paying special attention to regional differences.

In accordance with previous reports (6–8,23), the expression of type I collagen genes, *COL1A1* and *COL1A2*, was induced in OA cartilage (Figures 3A and

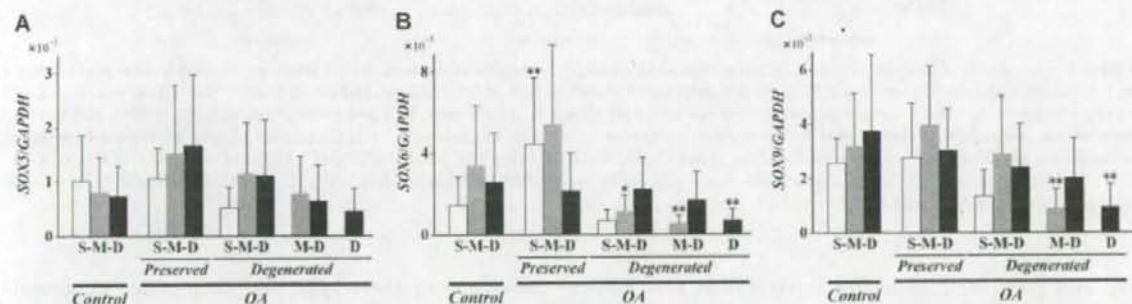

Figure 5. Expression of *SOX* genes in OA and control cartilage. Expression of *SOX5* (**A**), *SOX6* (**B**), and *SOX9* (**C**) in control and OA cartilage is shown as ratios of the expression of *GAPDH*, as described in Figure 2. S-M-D, M-D, and D under the respective groups of bars indicate the zone(s) retained in the samples. Each bar represents the results from at least 13 samples. Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, versus the corresponding zone in control cartilage. See Figure 2 for definitions.

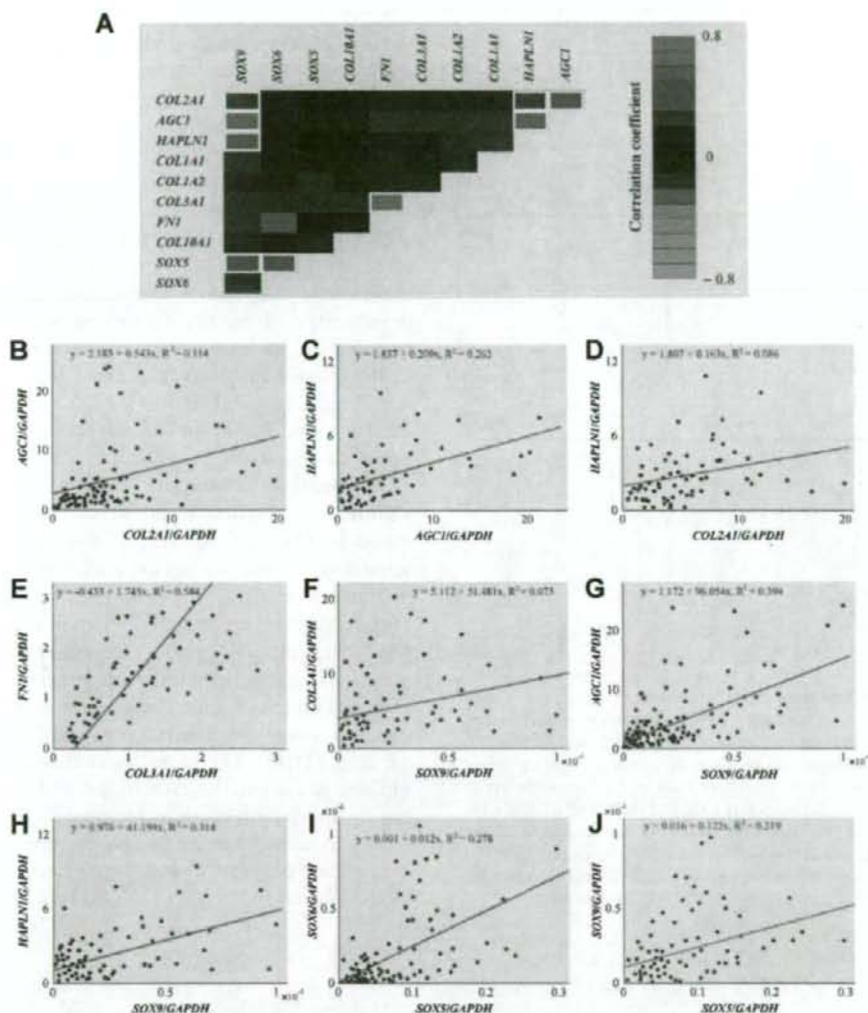

Figure 6. Correlation of gene expression in osteoarthritic (OA) cartilage. Expression of cartilage matrix genes, minor cartilaginous genes induced by the disease, and 3 cartilage-related *SOX* genes was determined at various sites of OA cartilage, and a correlation of expression was investigated among the genes. **A,** Correlation coefficients among the genes are shown by a heat map. Red and green colors indicate positive and negative correlations, respectively. Yellow square frames indicate significant correlations of expression. **B–J,** Correlation of gene expression is shown by scattergrams. Significant correlations were found between *COL2A1* and *AGC1* (**B**), *AGC1* and *HAPLN1* (**C**), *COL2A1* and *HAPLN1* (**D**), *COL3A1* and *FN1* (**E**), *SOX9* and *COL2A1* (**F**), *SOX9* and *AGC1* (**G**), *SOX9* and *HAPLN1* (**H**), *SOX5* and *SOX6* (**I**), and *SOX5* and *SOX9* (**J**), with the strongest correlation between *COL3A1* and *FN1*.

B). However, their induction levels varied markedly among samples, and practically no induction was observed in approximately half of the samples. Within the samples with detectable expression, these genes were expressed in the superficial zones in less degenerated areas and in the middle and deep zones in severely degenerated areas. Interestingly, although these genes showed similar patterns of expression within OA cartilage, their expression levels often differed considerably. The loss of coordinated expression was apparent when

the expression ratio of *COL1A2* to *COL1A1* was compared between OA cartilage and other normal tissues containing type I collagen as a major component (Figure 3C). While the expression ratio of *COL1A2* to *COL1A1* was between 0.7 and 1.7 in the bone, ligament, or meniscus tissues obtained from nonarthritic joints, the ratio in OA cartilage ranged widely from 0.2 to 44. The poor coordination in expression suggests that the expression of type I collagen genes could be induced by an aberrant mechanism(s) in OA cartilage.

In contrast to type I collagen, the induction of type III collagen messenger RNA (mRNA) was consistently observed in OA samples. Within OA cartilage, the expression of type III collagen was most intense in the upper region of degenerated cartilage (Figure 3D). The expression of another gene, fibronectin, was consistently induced in OA cartilage. The regional change of fibronectin expression was very similar to that of type III collagen expression (Figure 3E).

Unlike type I or type III collagen, the induction of type X collagen was observed primarily in the deep zone (Figure 3F). The induction was weaker than that of type I or type III collagen as judged by the ratios of expression to that of *GAPDH*, and the level of induction was considerably different among OA samples; the expression was virtually absent in approximately half of the samples. Interestingly, the expression of type X collagen was more obvious in the less degenerated areas than in the more degenerated areas where the superficial zone was lost to the disease.

Consistent with previous reports, the expression of exon 2 of the *COL2A1* gene was obviously increased in OA cartilage when evaluated by the ratio of its expression to that of *GAPDH* (Figure 3G). However, the expression of exon 2 relative to total *COL2A1* expression was rather reduced in OA cartilage (Figure 3H). Thus, it was assumed that the appearance of type IIA procollagen might not be the result of a phenotypic change in the chondrocytes as previously speculated (11,12), but is more likely to be associated with the up-regulation of type II collagen expression.

Chondrocytes at the upper part of degenerated cartilage undergo a phenotypic change. Next, we compared gene expression between preserved areas and degenerated areas in the respective cartilage zones of the respective OA joints. In the superficial zone, the expression was compared in each sample between the preserved and degenerated areas (i.e., between the 2 regions in the superficial zone without and with macroscopic degeneration). In the middle and deep zones, the comparison was performed in each sample between the preserved areas and the degenerated areas where the zones were directly exposed to the joint cavity.

The result clearly indicated that a shift occurred in the pattern of gene expression at the upper region of degenerated cartilage (Figure 4). In the degenerated areas in the middle and deep zones, the expression of cartilage matrix genes (type II collagen and aggrecan) was suppressed, while the expression of minor cartilaginous genes (type III collagen and fibronectin) was enhanced. In the superficial zone, the expression of minor cartilaginous genes was induced similarly in the degenerated areas, although the suppression of cartilage matrix gene expression was not apparent. In spite of considerable differences in expression levels among the samples, the shift of gene expression was consistently observed in almost all OA samples. Thus, the chondrocytes are considered to undergo a phenotypic change at the upper region of degenerated cartilage, no matter in which cartilage zone the cells reside.

Expression of *SOX* genes in OA and control cartilage. During chondrogenic differentiation, the expression of cartilage matrix genes is regulated by the transcriptional factors *SOX5*, *SOX6*, and *SOX9* (24). In order to estimate the involvement of these molecules in the change of chondrocyte metabolism in OA, their expression was investigated (Figure 5). In OA cartilage, the expression of *SOX* genes tended to be reduced in the degenerated areas, particularly in the upper region of the degenerated cartilage. The reduction was most obvious with *SOX6*, followed by *SOX9*, and was least apparent with *SOX5*. In the preserved areas, the expression of *SOX5* and *SOX6* tended to be increased above control levels, although this trend was not observed with *SOX9*. These regional changes of *SOX* expression within OA cartilage suggested that the altered *SOX* gene expression might be related to the change in matrix gene expression in OA.

Correlation of gene expression in OA cartilage. In an attempt to understand the mechanism(s) underlying the altered gene expression in OA cartilage, a possible correlation of gene expression was investigated (Figure 6A). The expression of 3 cartilage matrix genes correlated significantly. The expression of type II collagen was significantly correlated with that of aggrecan ($r = 0.110$, $P = 0.0081$) (Figure 6B), and a stronger correlation was observed between aggrecan and link protein ($r = 0.512$, $P < 0.0001$) (Figure 6C). A significant correlation was also observed between type II collagen and link protein ($r = 0.294$, $P < 0.0001$) (Figure 6D), implying that the expression of these genes might be modulated by a common factor(s) in OA cartilage.

In contrast, no significant correlation was found between the expression of cartilage matrix genes and minor cartilaginous genes induced by the disease in any combination (from $P = 0.102$ to $P = 0.991$) (Figure 6A).

Among the 5 minor cartilaginous genes evaluated, a significant correlation was observed only between type III collagen and fibronectin ($r = 0.764$, $P < 0.0001$) (Figure 6E). Therefore, the expression of minor cartilaginous genes was assumed to occur without any association in OA cartilage, except for that of type III collagen and fibronectin. Interestingly, the correlation between type III collagen and fibronectin was stronger than any other relationship observed in this study, suggesting the presence of certain link(s) in their expression. In fact, we have obtained data indicating that the expression of type III collagen in human OA cartilage could be induced, at least partly, through the activation of $\alpha 5\beta 1$ integrin by fibronectin (Fukui N: unpublished observation).

Next, a possible correlation of expression was investigated between the *SOX* genes and the 3 cartilage matrix genes. Although no significant correlation was found between *SOX5* or *SOX6* and the matrix genes (from $P = 0.072$ to $P = 0.857$) (Figure 6A), the expression of all 3 matrix genes was significantly correlated with that of *SOX9* (Figures 6F–H). The correlation was strongest with aggrecan ($r = 0.627$, $P < 0.0001$), followed by link protein ($r = 0.560$, $P < 0.0001$), and was weakest with type II collagen ($r = 0.270$, $P = 0.013$). The expression of *SOX* genes was not correlated with that of the minor cartilaginous genes in any combination (from $P = 0.436$ to $P = 0.959$) (Figure 6A). Meanwhile, the expression of *SOX* genes was mutually correlated. Significant correlations were observed between *SOX5* and *SOX6* ($r = 0.527$, $P < 0.0001$) (Figure 6I) and between *SOX5* and *SOX9* ($r = 0.468$, $P = 0.001$) (Figure 6J), although the correlation between *SOX6* and *SOX9* was not significant ($P = 0.728$).

DISCUSSION

The result of this study has provided a comprehensive view of the change in metabolic activity of the chondrocytes in OA. The profile of gene expression differed considerably with the site, depending on the cartilage zone and the extent of cartilage degeneration. In the macroscopically intact areas of OA cartilage, the expression of cartilage matrix genes was markedly enhanced, particularly in the middle and deep zones. This observation was consistent with the results of previous studies using in situ hybridization (3,4,6,9), in which the enhanced matrix synthesis was considered to be a reparative response that attempts to reconstitute the impaired cartilage matrix (2–4). Meanwhile, the up-regulation of cartilage matrix genes was less obvious in the degenerated areas, particularly in the upper regions. Instead, at those regions, the expression of type III collagen and fibronectin was most enhanced. The shift in gene expression was apparent when the profile of gene expression was compared between preserved and degenerated areas in each OA joint (Figure 4).

This shift in gene expression could be significantly involved in the progression of the disease. First, in OA, cartilage matrix is lost primarily from the surface of degenerated cartilage (25), and that loss of matrix could be accelerated by the reduced cartilage matrix synthesis in the surface region (4,9). Second, matrix loss may be facilitated by the induction of type III collagen synthesis. Although this collagen could be a minor component of normal articular cartilage (26–28), it may diminish the quality of cartilage matrix when expressed in excess through the inhibition of proper matrix organization (28,29). Third, fibronectin is known to cause an intense catabolic response in chondrocytes and synoviocytes when cleaved into fragments (30). Therefore, the induction of this protein at the site of enhanced catabolism may be even more significant in the progression of the disease. Taking these findings together, the shift in matrix gene expression at the upper region of degenerated cartilage could be a critical event in OA pathology. Since the shift of gene expression was observed in virtually all OA samples, the regulation of cellular metabolism at that site may be an effective strategy in the future to delay or inhibit disease progression.

Compared with type III collagen and fibronectin, the expression of the other minor cartilaginous genes was less pronounced in OA cartilage in terms of areas, intensities, and frequencies. The induction of type I collagen mRNA was highly variable among OA samples, and, even when expressed, *COL1A1* and *COL1A2* mRNA were often induced at different intensities. The expression of type I collagen in human OA cartilage has remained controversial in previous studies. Although our result of *COL1A1* expression was consistent with several reports (4,6,9), it was discordant with another report regarding the area of expression (31). Further, while we observed the expression of *COL1A2* in human OA cartilage, it was not detected in an earlier study (9). The revealed discrepancy between *COL1A1* and *COL1A2* expression may account for these contradictions in the literature. Likewise, there has been a controversy regarding the induction of *COL10A1* ex-

pression in human OA cartilage: some investigators observed the expression in the upper part of OA cartilage (12,32), whereas others reported it in the deep zone (13,14,33–35). Our result is consistent with the latter finding, in that we identified its expression primarily in the deep zone. However, because the expression of *COL10A1* was relatively weak and fairly inconsistent among OA cartilage samples, we assume that the expression of type X collagen in OA cartilage might be of limited significance in the pathology of OA.

Previously, the appearance of type IIA procollagen mRNA or exon 2 of *COL2A1* in OA cartilage was considered to be the result of a phenotypic reversal of chondrocytes (11,12). However, this speculation is not supported by the present result. Since a result consistent with our own was reported in another recent study (23), a phenotypic reversal of chondrocytes may not be a dominant event in OA cartilage.

In light of these findings, the metabolic change of the chondrocytes in OA may be understood as follows. In the degenerated areas, a major change in the metabolism occurs in the upper region of degenerated cartilage. Such a change resembles that of the dedifferentiation process in the decline of type II collagen and aggrecan expression and the induction of type III collagen expression (Figures 2 and 3) (an illustration of the sequential changes of gene expression in articular chondrocytes during dedifferentiation is available at http://www.hosp.go.jp/~sagami/rinken/crc/index.html). However, the change is different from that process in the expression of link protein, fibronectin, and type I collagen genes. Thus, the metabolic change in the degenerated areas of OA cartilage was considered to be unique and not closely related to the one during the dedifferentiation process. Meanwhile, in the preserved areas, the expression of cartilage matrix genes is highly up-regulated. Although the phenotypic deviation is less obvious in those areas, the expression of type I collagen and type X collagen genes may be induced there in the superficial and deep zones, respectively.

Although the mechanism(s) for these metabolic changes remains entirely unknown, the change in *SOX9* expression may be related to the altered chondrocyte metabolism in OA. As shown in the correlation study, the regional difference in matrix gene expression within OA cartilage could be ascribed, at least partly, to the change in SOX9 expression. However, the present result also indicates that the general up-regulation of matrix gene expression in OA chondrocytes was not associated with the increase in *SOX9* expression. In this study, the amounts of SOX proteins were not assessed. Furthermore, the transcriptional activity of SOX9 is known to be modulated by the level of phosphorylation (36) and by the presence of coregulators (37,38). Thus, taking these factors into account may provide a better explanation of the significance of SOX proteins in the altered chondrocyte metabolism in OA.

Although the present study has clarified the metabolic change of chondrocytes in OA cartilage, it also has several limitations. First, the metabolic change was evaluated primarily by mRNA expression, and protein synthesis was not determined. The major difference in mRNA expression levels among the samples posed another problem. A large variation among human cartilage samples has been reported repeatedly in previous studies (7,8,23). For OA samples, this might reflect the diversity of the pathology, while the variation among the controls might have stemmed from differences in joint physiology that could be related to the donor's condition before death. These points should be clarified by future studies. Despite these limitations, we believe that our study has revealed several novel aspects of OA pathology. We hope that the current results may offer another clue to eventually establishing a novel strategy to treat this tenacious disease.

AUTHOR CONTRIBUTIONS

Dr. Fukui 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 design. Fukui.

Acquisition of data. Ikeda, Ohnuki, Tanaka, Hikita, Mitomi, Juji, Katsuragawa, Yamamoto, Sawabe, Yamane, Suzuki.

Analysis and interpretation of data. Fukui, Mori, Sandell, Ochi.

Manuscript preparation. Fukui.

Statistical analysis. Fukui.

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SHORT COMMUNICATION

Replication of the association between a chromosome 9p21 polymorphism and coronary artery disease in Japanese and Korean populations

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Abstract Coronary artery disease (CAD) has become a major health problem in many countries. Recent genome-wide association studies have identified the association between rs1333049 on chromosome 9p21 and susceptibility to CAD in Caucasoid populations. In this study, we evaluated the associations of rs1333049 with CAD in Japanese (604 patients and 1,151 controls) and Koreans (679 patients and 706 controls). We found a significant association in both Japanese [odds ratio (OR) = 1.30, 95% confidence interval (CI); 1.13–1.49, $p = 0.00027$, allele count model] and Koreans (OR = 1.19, 95% CI; 1.02–1.38, $p = 0.025$, allele count model). These observations demonstrated that chromosome 9p21 was the susceptibility locus for CAD also in East Asians.

Keywords Coronary artery disease · 9p21 · Single nucleotide polymorphism · Case-control study · Japanese · Korean

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Introduction

Coronary artery disease (CAD), clinically manifested with angina pectoris (AP) or myocardial infarction (MI), has become a major health problem in many countries because of its increasing prevalence and high mortality. Although non-genetic factors such as smoking, hypertension, hypercholesterolemia and diabetes mellitus significantly contribute to development of CAD, considerable evidence indicates the involvement of genetic factors in the pathogenesis of CAD (Wang 2005). Recent large scale association studies have accumulated information on the susceptibility genes linked to CAD. Notably, genome-wide association studies repeatedly identified the association of the chromosome 9p21 locus with the susceptibility to CAD in Caucasoid populations (Wellcome Trust Case Control Consortium 2007; McPherson et al. 2007; Helgadottir et al. 2007; Samani et al. 2007). In general, one of the main problems in association studies is the lack of reproducibility, which indicates that contribution of the reported factor is not common or not large enough to be replicated in other studies (Morgan et al. 2007). Therefore, validation studies are indispensable to clarify the genes involved in the pathogenesis, though there might be ethnic differences

in the genetic backgrounds or regional differences in the environmental or lifestyle factors even in the same ethnic groups. In this study, we evaluated the association of rs1333049with CAD in Japanese and Korean populations.

Materials and methods

Subjects

The study protocol was approved by the Ethics Review Boards of the Medical Research Institute of Tokyo Medical and Dental University, Kitasato University School of Medicine, Tokyo Metropolitan Geriatric Medical Center, and Samsung Medical Center. Japanese subjects consisted of 604 patients and 1,151 controls, while Korean subjects included 679 patients and 706 controls. The Japanese control group was comprised of healthy volunteers without a history of CAD ($n = 633$) and consecutive autopsied persons without pathological findings of acute or old MI ($n = 518$). Korean control subjects were randomly selected from healthy individuals ($n = 182$) and cancer patients ($n = 524$) without a history of CAD. The diagnosis of CAD and classical risk factors was based on the standard criteria as described previously (Hohda et al. 2003). Severity of coronary atherosclerosis was classified according to the number of coronary vessels with significant stenosis (angiographic luminal stenosis >50%) as one-, two-, or three-vessel disease (VD).

Genotyping

TaqMan SNP genotyping assay (Applied Biosystems) was used to determine the genotype of rs1333049 in PCR products generated with primer pair rs1333049-F (5′-CCTTCATGCTATTTTGAGGAG) and rs1333049-R (5′-GGAAGATAAGTTGAGAATGTCA).

Statistical analysis

Genotype distributions and allele frequencies were compared between the cases and controls using a chi-square test. When the p value was less than 0.05, the association was considered to be significant. Strength of the association was expressed by odds ratio (OR). Significance of the association with coronary disease severity was examined by Mann–Whitney U-test.

Results and discussion

Five SNPs on the chromosome 9p21 locus, rs1333049, rs10757274, rs2383206, rs2383207, and rs10757278, were associated with CAD, and they were in tight LD in Caucasians. Because the structure of LD based on HapMap database information suggested that they were also in tight LD in Asians, we used rs1333049 in this study. As shown in Table 1, the genotype distribution was in Hardy–Weinberg equilibrium in all tested populations. There was significant association at the allele count model in both Japanese (OR = 1.30, 95% CI; 1.13–1.49, $p = 0.00027$) and Korean (OR = 1.19, 95% CI; 1.02–1.38, $p = 0.025$), confirming the association in East Asian populations. A replication study in Korean by using different SNPs was recently reported (Shen et al. 2008). These data strongly suggested that the chromosome 9p21 locus conferred susceptibility to CAD across racial lines.

Because previous studies did not address the correlation between the risk allele and phonotypic background of CAD, we investigated the association between rs1333049 and severity of atherosclerosis. According to the number of significantly affected vessels, CAD patients were classified into three groups, 1VD, 2VD and 3VD. As shown in Table 2, there was no trend of association between the rs1333049 genotype and severity of CAD in both Japanese and Koreans (Table 2). Stratified analyses of rs1333049 with risk factors of CAD such as diabetes mellitus, hyperlipidemia, or hypertension showed again no trend of association between rs1333049 and risk factors (data not

Table 1 Association of rs1333049 on chromosome 9p21 with CAD in Japanese and Koreans

Genotype	CAD ($n = 604$) n (%)	Control ($n = 1,151$) n (%)	OR (95%CI)	p value
(a) Japanese				
GG	114 (18.9)	286 (24.9)	0.70 (0.55–0.90)	0.0046
GC	312 (51.7)	606 (52.7)	0.96 (0.79–1.17)	ns
CC	178 (29.5)	259 (22.5)	1.44 (1.15–1.80)	0.0013
C allele frequency	0.55	0.49	1.30 (1.13–1.49)	0.00027
HWE (p)	0.54	0.19		
Genotype	CAD ($n = 679$) n (%)	Control ($n = 706$) n (%)	OR (95%CI)	p value
(b) Korean				
GG	158 (23.3)	192 (27.2)	0.81 (0.64–1.04)	ns
GC	335 (49.3)	353 (50.0)	0.97 (0.79–1.20)	ns
CC	186 (27.4)	161 (22.8)	1.28 (1.00–1.63)	0.049
C allele frequency	0.52	0.48	1.19 (1.02–1.38)	0.025
HWE (p)	0.96	1.00		

CAD coronary artery disease, *OR* odds ratio, *CI* confidence interval, *HWE* Hardy–Weinberg equilibrium, *ns* not significant, ($p > 0.05$)

Table 2 Association of rs1333049 on chromosome 9p21 with the severity of coronary atherosclerosis

	Japanese CAD		Korean CAD	
	CC *n* (%)	Non-CC *n* (%)	CC *n* (%)	Non-CC *n* (%)
1VD	77(31.4)	168(68.6)	64(27.5)	169(72.5)
2VD	54(31.0)	120(69.0)	41(25.0)	123(75.0)
3VD	46(25.6)	134(74.4)	31(27.4)	82(72.6)
Mann–Whitney *U*	$p = 0.22$		$p = 0.86$	

CAD coronary artery disease, *VD* vessel disease means significantly affected vessel (angiographic luminal stenosis >50%). Angiographic data were available for 599 Japanese patients and 510 Korean patients. Significance of the association with coronary disease severity was examined by Mann–Whitney *U*-test

shown). Though further studies are required to decipher the mechanism of involvement of this locus to the pathogenesis of CAD, the observations in this study strongly suggested that the chromosome 9p21 locus was a genetic risk factor independent of classical risk factors.

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Lymphotoxin-alpha polymorphisms and presence of cancer in 1,536 consecutive autopsy cases

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Abstract

Background: Lymphotoxin-alpha (LTA) is a pro-inflammatory cytokine with anti-tumor activity. The objective of this study was to determine whether *LTA* polymorphisms influence the presence of cancer.

Methods: *LTA* polymorphisms C804A (rs1041981, T60N) and T495C (rs2229094, C13R) were determined in 1,536 consecutive autopsy cases and were registered in the Japanese single-nucleotide polymorphisms (SNPs) for geriatric research (JG-SNP) Internet database. Tumors were systematically reviewed, pathologically confirmed, and assessed in relation to *LTA* genotype.

Results: The study population consisted of 827 males and 709 females, with a mean age of 80 years. Altogether, we studied 606 subjects without cancer and 930 subjects with cancer of the stomach (n = 183), lung (n = 164), colon or rectum (n = 143), or other sites. The presence of cancer was higher in males than in females. The C804A and T495C polymorphisms were associated with cancer in males (CA + AA: CC, adjusted OR = 0.72, 95% CI = 0.53 – 0.99; TC + CC: TT, adjusted OR = 1.45, 95% CI = 1.04 – 2.02; respectively) but not in females. In males, the C804A polymorphism was associated with lung cancer (CA + AA: CC, adjusted OR = 0.60, 95% CI = 0.37 – 0.97), whereas the T495C polymorphism was associated with gastric cancer (TC + CC: TT, adjusted OR = 1.68, 95% CI = 1.06 – 2.65).

Conclusion: We found some evidence of an association between *LTA* polymorphisms and cancer risk in elderly Japanese men. Further studies in larger populations should examine this hypothesis.

Background

Elderly people often experience chronic and low-grade inflammation, whereupon the levels of certain inflammatory serum markers (e.g., C-reactive protein) and pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha and interleukin-6) are elevated. However, increases in circulating inflammatory markers are often not noticeable in healthy elderly persons and are much below the levels experienced during acute infections [1,2]. Moreover, it is well known that the prevalence of cancer increases sharply

with age, and that the majority of cancer cases occur in patients over the age of 65 [3]. Persistent inflammation is thought to trigger certain cancers, particularly those of the stomach, colon, and lung [4]. Although it has been established that the risks of cancer and inflammation increase with age [2], the influence of inflammatory cytokine polymorphisms on the genetic predisposition to cancer remains unclear.

Lymphotoxin-alpha (LTA), a member of the tumor necrosis factor (TNF) family of cytokines, was initially isolated on the basis of an anti-tumor activity. Later, this cytokine was shown to have inflammatory and immunologic activities [5]. The *LTA* gene is located within the class III region of the major histocompatibility complex (MHC) in chromosome 6p21.3 [6]. LTA plays a key role in communication between lymphocytes and stromal cells, thereby eliciting cytotoxic effects on cancer cells [7,8]. LTA induces the expression of vascular cell-adhesion molecule 1 (VCAM1) on vascular endothelial cells and recruits natural killer (NK) cells to parenchymal organs and tumor lesions [9]. NK cells have nonspecific host-defense mechanisms that aid in tumor rejection and protection from metastases. Previous studies have shown that tumor growth and metastasis are enhanced in *LTA*-deficient mice, which produce NK cells that have reduced anti-tumor potential [10,11]. Thus, LTA signaling plays an important role in anti-tumor surveillance via the maturation and recruitment of NK cells.

Previous studies have examined the relationship between *LTA* polymorphisms and various cancers, and found that the *NcoI* restriction fragment length polymorphism (A252G) in the first intron is in tight linkage disequilibrium with C804A, resulting in the substitution of threonine with asparagine at codon 60 in exon 3 [12,13]. The *LTA* 252G allele increases LTA at the levels of mRNA and protein [12]. Previous studies have found that the relationship between the risk of cancer and the rate of survival among those with the *LTA* polymorphism varies according to the cancer type [14-17]. However, the effect of *LTA* polymorphisms on the presence of cancer in general is not known.

Toward this end, we searched for two non-synonymous polymorphisms, C804A (rs1041981, T60N) and T495C (rs2229094, C13R), in consecutive autopsy cases and determined whether they influenced cancer presence.

Methods

Subjects

The study population consisted of 1,536 consecutive autopsy cases performed at the Tokyo Metropolitan Geriatric Hospital between 1995 and 2004. *LTA* polymorphisms were registered in the Internet database of Japanese single-nucleotide polymorphisms (SNPs) for geriatric research (JG-SNP) [18]. Subjects were enrolled regardless of the cause of death and autopsies were performed on 40% of patients who died at the hospital. The study population included subjects who died from malignant disease (33%), coronary heart disease (20%), and pneumonia (13%). These proportions were similar to the causes of death reported in a survey conducted by the Ministry of Health, Labor and Welfare of Japan http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/tokusyu/gaikoku07/09d.html which found that 30% of the Japanese population died from malignant disease, 16% from coronary heart disease, and 10% from pneumonia. The presence, absence, and histopathology of cancers were determined from autopsy findings. Histories of smoking and alcohol drinking status were retrospectively determined from medical records, and subjects were classified as smokers (including ex-smokers) versus non-smokers, and alcohol drinkers versus non-drinkers. Information on smoking and alcohol drinking status was missing from 109 and 118 patient histories, respectively. Written informed consent was obtained from the family of each patient at the time of autopsy. The study protocol was approved by the ethical Committees of the Tokyo Metropolitan Geriatric Hospital and the Tokyo Medical and Dental University.

Genotyping

Genomic DNA was extracted from the renal cortex using a standard procedure. *LTA* polymorphisms were genotyped via melting curve analysis [19]. PCR primers and probes were as follows. For *LTA* C804A (rs1041981, T60N): 5'-GTT GGC CTC ACA CCT TCA-3' (forward primer), 5'-TGG ATG CTT GGG TTC CTG-3' (reverse primer), CAG CAC CCT CAA ACC TGC-Fluorescein (anchor probe), and LC Red 640-GCT CAC CTC ATT GGT AAA CAT CCA CCT GAC CTC C-Phosphate (detection probe). For *LTA* T495C (rs2229094, C13R): 5'-CTC TTT CTC TGC AGG TTC TC-3' (forward primer), 5'-GCT CTA GGG CTC AAG GTT T-3' (reverse primer), CTC CCA AGG GTG GGT G-Fluorescein (anchor probe), and LCRed640-CAC CAC CCT ACA CCT CCT CCT TCT GG-Phosphate (detection probe). The PCR amplification reaction was performed in 5 μl volume using the LightCycler PCR kit (Roche Diagnostics, Penzberg, Germany). Each well contained 1 × PCR buffer, 4 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.05 μM of forward primer, 0.5 μM of reverse primer, 0.2 μM of each anchor and detection probe, 0.1 U of Faststart Taq polymerase, and 10 ng of genomic DNA. The cycling protocol was performed as follows: initial denaturation at 94°C for 10 min; followed by 40 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 15 sec; and a final extension step at 72°C for 10 min. After completion of PCR, the plates were heated from 40°C to 90°C with a gradient of 0.1°C per second immediately before melting temperature analysis

on the LightCycler 480 Instrument (Roche Diagnostics). The genotype was determined based on melting profiles automatically classified by LightCycler Genotyping software (Roche Diagnostics). C804A and T495C polymorphisms were genotyped with success rates of 96% and 95%, respectively. Genotyping accuracy was confirmed by sequencing randomly selected samples. The melting temperature analysis correlated entirely with the direct sequencing results.

Statistical analysis

Statistical analysis was performed using SAS software, version 9.1.3 (SAS Institute Inc., Cary, NC). The distribution of subjects with and without cancer, and associations with other variables, were compared using Fisher's exact test. Hardy-Weinberg equilibrium (HWE) was assessed using a permutation test. Odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model that compared genotype frequencies in subjects with and without cancer, with respect to *LTA* polymorphisms. The OR was adjusted by age, sex, smoking, and alcohol drinking status. This study had a statistical power of 0.8 to detect an OR of 1.5 in carriers of at least one polymorphic allele, compared with carriers of homozygous wild-type alleles. *LTA* haplotype frequencies were estimated using Haploview software http://www.broad.mit.edu/mpg/haploview/. Chi-square values were calculated to determine the distribution of *LTA* haplotypes among subjects with and without cancer. A probability level of 5% was considered statistically significant for all analyses.

Results

Characteristics of the study subjects

Selected demographic variables, including the sites and histological types of cancers, and risk factors are shown in Table 1 (Additional file 1). The mean age of subjects was 80.2 ± 8.9 years. The study population consisted of 827 (54%) males and 709 (46%) females. Altogether, we studied 606 subjects without cancer and 930 subjects with cancer. When the total number of subjects was divided by the number of subjects over or under the median age of 80 years, the distributions of subjects with or without cancer did not significantly differ. The frequency of cancer was significantly higher in males than in females. No differences in smoking and alcohol drinking status between cancer-bearing and cancer-free subjects were observed. The most frequent sites of cancer were the stomach (n = 183), lung (n = 164), and colon or rectum (n = 143).

Genotype and allele frequencies of LTA *polymorphisms*

C804A and T495C were genotyped in all subjects. The genotype frequencies of C804A were 37% for CC, 48% for CA, and 15% for AA. The minor allele frequency was 39%, and the allele distribution was consistent with HWE (p = 0.65). The genotype frequencies of T495C were 66% for TT, 31% for TC, and 3% for CC. The minor allele frequency was 19% and the allele distribution was consistent with HWE (p = 0.48).

Association of LTA *polymorphisms with cancer overall*

The associations between C804A and T495C polymorphisms and the presence of cancer are shown in Table 2 (Additional file 1). Among cancer-free subjects, the frequencies of the C804A genotypes CC, CA, and AA were 33%, 49%, and 18%, respectively, in males, and 33%, 54%, and 13%, respectively, in females (Table 3) (Additional file 1). In comparison with the CC genotype, the CA genotype was associated with a significantly lower presence of cancer in all subjects (adjusted OR = 0.78, 95% CI = 0.61 – 0.99). In males, the CA + AA genotype was associated with a significantly lower presence of cancer compared with the CC genotype (adjusted OR = 0.72, 95% CI = 0.53 – 0.99). The association between the C804A polymorphism and cancer in females was not significant (CA + AA: CC, adjusted OR = 0.92, 95% CI = 0.66 – 1.29). Additionally, cancer frequency was not associated with C804A (data not shown).

In cancer-free subjects, the frequency of the T495C genotypes TT, TC, and CC were 70%, 28%, and 2%, respectively, in males, and 66%, 31%, and 3%, respectively, in females (Table 3 in Additional file 1). Compared with the TT genotype, the CC genotype was associated with a significantly higher presence of cancer (adjusted OR = 2.24, 95% CI = 1.09 – 4.61). In males, the TC + CC genotype was associated with a significantly higher presence of cancer, compared with the TT genotype (adjusted OR = 1.45, 95% CI = 1.04 – 2.02). The association between the T495C polymorphism and cancer in females was not significant (TC + CC: TT, adjusted OR = 1.08, 95% CI = 0.77 – 1.50).

Additionally, we explored the relationship between the presence of C804A and T495C haplotypes and tumor formation. C804A and T495C appeared to be present in moderate linkage disequilibrium (r^2 = 0.15, D' = 1.0) and three major haplotypes were identified (495T-804C, 495T-804A, and 495C-804C). None of these haplotypes were associated with the presence of cancer (data not shown).

Association of LTA *polymorphisms with specific types of cancers*

Significant associations between *LTA* polymorphisms and the presence of cancer in males prompted us to explore the relationships between these polymorphisms and the sites of cancer. After analyzing subjects with stomach, lung, colorectal, prostate, breast, liver, biliary tract, kidney or urinary tract, and hematopoietic malignancies, we

observed a positive association between *LTA* polymorphisms and lung and stomach cancers (Table 4 in Additional file 1). Among male subjects with lung cancer, the C804A CA + AA genotype was associated with a significantly lower presence of cancer than was the CC genotype (adjusted OR = 0.60, 95% CI = 0.37–0.97). The association between C804A polymorphism and lung cancer in females was not significant (CA + AA: CC, adjusted OR = 0.65, 95% CI = 0.35 – 1.23).

The T495C polymorphism was not associated with the presence of lung cancer, but the TC + CC genotype was associated with a significantly higher presence of gastric cancer in males, compared with the TT genotype (adjusted OR = 1.68, 95% CI = 1.06–2.65). The association between T495C polymorphism and gastric cancer was not significant in females (TC + CC: TT, adjusted OR = 1.16, 95% CI = 0.61 – 2.21).

Discussion

Our findings demonstrate that the *LTA* polymorphism C804A is associated with a lower presence of cancer, particularly lung cancer, in elderly Japanese men, consistent with previous studies showing that LTA has anti-tumor activity.

Several laboratories have recently published a functional analysis of the *LTA* C804A (T60N). Compared with the C allele, the A allele is more bioactive with regards to the induction of VCAM1 in cultured human coronary-artery smooth muscle cells [13]. Furthermore, C804A is present in very high linkage disequilibrium with another polymorphism, *LTA* A252G. These two polymorphisms have been shown to be completely concordant in Japanese subjects [16,17]. Compared with the A allele, the G allele of the A252G genotype confers higher transcriptional activity in Jurkat cells [13] and phytohemagglutinin-stimulated peripheral blood mononuclear cells [12]. Thus, the C and A alleles are considered low and high bioactive alleles in *LTA*, respectively. Accordingly, the data suggest that the lower presence of cancer in our study population may reflect enhanced LTA activity because of the presence of high-bioactive alleles.

The C804A and A252G polymorphisms have been studied with regards to cancer survival rates and risks of developing various type of cancer, including lung [14], stomach [15,20,21], colorectal [22], breast [23], cervix [16], endometrium [17] and bladder [24] cancers, as well as leukemia [25], lymphoma [26,27], and myeloma [28]. The high-bioactive genotype was associated with the risk of developing cancers of the lung [14], colon or rectum [22], non-Hodgkin lymphoma [26,27], and myeloma [28]. Low-bioactive genotypes were associated with the risk of developing cervical [16] and endometrial cancers [17], but not gastric [15,20,21], breast [23], bladder cancers [24], or leukemia [25]. These differences may be partially explained by the multi-functionality of LTA. LTA can promote cell growth and adhesion, and can potentially favor the growth of certain tumors.

It remains unclear why C804A is associated with cancer in males but not in females. However, gender is known to play an important role in the development of various cancers. Recent studies also suggest that cytokine secretion and innate immunity differ between the genders [29,30].

The C13R (T495C) polymorphism has not been as thoroughly studied as the T60N (C804A) polymorphism. Previous study reported there was not an association between the T495C polymorphism and lung cancer [31]. The T495C polymorphism is a haplotype component associated with altered LTA expression and increased levels of vascular- and autoimmune-mediated inflammation [32]. These findings suggest that the T495C polymorphism is also associated with cancer. We observed an association between the C allele of the T495C polymorphism and the presence of cancer, particularly gastric cancer. However, as the minor allele frequency for this polymorphism was low, these results must be confirmed in a larger study population.

The *LTA* gene is located within the class III region of the MHC, in the 6p21.3 chromosome [6]. This region contains many other genes, including that encoding the proinflammatory cytokine, TNF. It is well-known that this region shows high degrees of linkage disequilibrium. Thus, associations identified in this study may reflect the effects of other gene variants in this region.

As this was a hospital-based autopsy study, we had limited access to information on lifestyle variables that could potentially influence the development of cancer. Furthermore, although autopsies were performed on many of the deaths (40%) that occurred in the hospital, and the causes of death among our autopsy cases were similar to those reported in a national survey, we cannot rule out the possibility of selection bias. Such bias can arise from chance of admission, consent to autopsy, cause of death, and autopsy practice. Survival bias is also a possibility, as particular genotypes may be associated with other diseases or influence the lifespan of certain subjects, thereby introducing bias into the study population. However, the C804A genotype frequency was similar to that reported in other Japanese population studies [16,17], and any effects of such possible bias did not cause our results to deviate from HWE.

Conclusion

We found some evidence of an association between *LTA* polymorphisms and cancer risk in elderly Japanese men. Further studies in larger populations should examine this hypothesis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT performed the statistical analysis and drafted the manuscript. SI participated in the design and performed the statistical analysis. TA performed the pathological analysis. NT assessed the data integrity. MM coordinated the study and helped draft the manuscript. MS performed the pathological analysis and helped coordinate the study. All authors read and approved the final manuscript.

Additional material

Additional file 1

Tables 1–4. Table 1 – Distribution of selected demographic variables and risk factors. Table 2 – Associations between LTA polymorphisms and overall cancer. Table 3 – Distribution of LTA genotypes in cancer-free and cancer-bearing subjects, lung and gastric cancers. Table 4 – Associations of LTA polymorphisms with lung cancer and gastric cancer.
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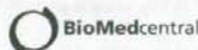

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ORIGINAL ARTICLE

Lewy Body Pathology Involves Cutaneous Nerves

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Abstract

Involvement of the peripheral autonomic nervous system is a core feature of Lewy body (LB) diseases, including Parkinson disease (PD), PD with dementia, and dementia with LBs. To investigate the potential use of skin biopsy for the diagnosis of LB diseases, we assessed anti-phosphorylated α-synuclein immunoreactivity in peripheral nerves in samples of skin from the abdominal wall and flexor surface of the upper arm in 279 prospectively studied consecutively autopsied patients whose data were registered at the Brain Bank for Aging Research between 2002 and 2005. Positive immunoreactivity was demonstrated in the unmyelinated fibers of the dermis in 20 of 85 patients with LB pathology in the CNS and the adrenal glands, the latter representing a substitute for peripheral autonomic nervous system sympathetic ganglia; no reactivity was seen in 194 patients without CNS LB pathology. In 142 retrospectively studied patients autopsied from 1995 onward who had subclinical or clinical LB disease, the sensitivity of the positive skin immunoreactivity was 70% in PD and PD with dementia and 40% in dementia with LBs. Skin immunoreactivity was absent in cases of multiple-system atrophy, progressive nuclear palsy, and corticobasal degeneration. We demonstrate for the first time that the skin is involved and may be a highly specific and useful biopsy site for the pathological diagnosis of LB diseases.

Key Words: α-Synuclein Adrenal gland, Dementia with Lewy bodies, Dermis, Immunohistochemistry, Lewy bodies, Parkinson disease.

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INTRODUCTION

Lewy body (LB) diseases (LBDs) (1) are defined by neuronal degeneration related to the presence of LBs; they include Parkinson disease, either with normal cognition (PD) or with dementia (PDD), dementia with LBs (DLB), and LB-related progressive autonomic failure (LBPAF) (2). Involvement of the peripheral autonomic nervous system (PANS) is a key feature of LBD and is a presenting clinical feature in some cases of PD, PDD, DLB, and LBPAF. Autonomic dysfunction can greatly influence the patient's prognosis and quality of life.

Pathological studies of the PANS demonstrate LB pathology involving the sympathetic and enteric nervous systems (3–8). Recently, ^{123}I-metaiodobenzylguanidine cardiac scintigraphy has become widely accepted in Japan as a tool for the diagnosis of LBD (9, 10); the pathological basis of this test is the presence of LB pathology of the sympathetic nerve fibers that supply epicardial fatty tissue (11).

There is a general consensus among neuropathologists that the standard organ for pathological evaluation of the PANS in LBD is the sympathetic ganglion (12), but autopsy sampling of the sympathetic ganglia can be difficult. We recently reported that the adrenal gland—one of the organs routinely examined at general autopsy—can be used as a substitute for the PANS sympathetic ganglia because it has similar pathological findings in LBDs (2).

The anatomical structures identified to date as having LB pathology are not appropriate biopsy sites for the premortem diagnosis of LBDs. In 2002, we examined skin excised near a decubitus ulcer at autopsy from a patient with DLB and found positive immunoreactivity with anti-phosphorylated α-synuclein antibodies in an unmyelinated fiber in the dermis. This observation prompted us to examine skin sampled from consecutively autopsied patients whose data were registered in the Brain Bank for Aging Research (BBAR).

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MATERIALS AND METHODS

Tissue Sources

The study consisted of 2 parts. The first part was a prospective study to determine the specificity of LBD in the skin in relation to LBD in the CNS and PANS. The second part was a retrospective study to determine the sensitivity of detection of LBD in the skin in relation to LBD in the CNS and PANS. The data registered in BBAR were from consecutively autopsied patients from a general geriatric hospital; informed consent was obtained from the relatives at autopsy (2, 13, 14).

In the prospective study, we used routinely sampled abdominal skin and prospectively sampled brachial skin from 279 consecutive autopsy patients whose data were registered in BBAR between 2002 and 2005. The patients' ages ranged from 52 to 104 years (mean, 80.8 ± 8.6 [SD] years); the male-to-female ratio was 167:112. The postmortem interval ranged from 52 minutes to 88 hours (mean, 13 hours). This series included 8 patients with progressive supranuclear palsy and 3 patients with corticobasal degeneration.

A wedge-shaped brachial skin sample, 1 cm × 0.5 cm in area and including the dermis and subcutaneous fatty tissue, was directly fixed in 4% paraformaldehyde for 48 hours and then embedded in paraffin. The abdominal skin had been routinely sampled since 1995, fixed in 10% buffered formalin for at least a week, and then embedded in paraffin. We used fixation in 4% paraformaldehyde for 48 hours because this fixation increased sensitivity for Lewy neurite detection in the CNS. We also continued to fix abdominal skin in 10% buffered formalin because the fixation is generally accepted and more applicable to potential biopsy in the clinic.

In the retrospective study, from among 1,594 patients whose data had been consecutively registered in BBAR between 1995 and 2005, we used 142 cases with the postmortem diagnosis of CNS LB stage II or greater (see later). The results of abdominal skin samples from 33 patients, who had been registered from 2002 onward and were also used in the prospective study, were included in the retrospective study to increase the case number for statistical analysis. Archival paraffin blocks of abdominal skin from the additional 109 patients from the period between 1995 and 2001 were stained as in the prospective study. The age range of the 142 patients was from 48 to 100 years (mean, 83.7 ± 7.6 years); the male-to-female ratio was 72:70. Abdominal skin samples from 3 cases with multiple-system atrophy (MSA; 1 case of MSA-P and 2 cases of MSA-C) from this period were also examined for comparison because no patient of MSA was included in the prospective studies. This study was approved by the institutional review boards of the Tokyo Metropolitan Institute of Gerontology and the Tokyo Metropolitan Geriatric Hospital.

Clinical Information

Clinical data, including information on the presence or absence of parkinsonism and cognitive state, were obtained from medical charts, as previously reported (13, 14). Final locomotive activity was classified into 4 levels: bedridden, wheelchair-bound, cane-assisted, and independent walking. In addition, the presence of decubitus ulcers was noted from the prosecutors' records at autopsy.

Pathological Examination of the Skin

Six-micrometer-thick serial paraffin sections of the skin were stained with hematoxylin and eosin and by immunohistochemistry with an autoimmunostainer (20NX; Ventana, Tucson, AZ) for single or double immunolabeling, as previously reported (15). All antibodies used are listed in Table 1.

For LB pathology, the antibodies used were anti-phosphorylated α-synuclein (psyn) (psyn no. 64 [16] monoclonal and PSer129 [17] polyclonal). One section each of 2 serial sections was used for the staining of monoclonal and polyclonal anti-psyn antibodies. In addition, selected sections were double-immunostained with psyn and anti-phosphorylated neurofilament monoclonal antibody (SMI31).

TABLE 1. Antibodies

Antibody	Clone	Epitope	Source	Animal	Dilution Ratio	Antigen Retrieval
psyn no. 64	Monoclonal	α-Synuclein phosphorylated ser129	T. Iwatsubo (available from Wako, Tokyo, Japan)	Mouse	1:20000 for CNS	Formic acid
					1:10000 for skin	Formic acid
PSer129	Polyclonal	α-Synuclein phosphorylated ser129	T. Iwatsubo	Rabbit	100	none
Ubiquitin	Polyclonal	Ubiquitin	DAKO, Glostrup, Denmark	Rabbit	1:1000	Microwave in citrate buffer
AT8	Monoclonal	τ-Phosphorylated Ser-202 and Thr-205	Innogenetics, Temse, Belgium	Mouse	1:1000	None
12B2	Monoclonal	Aβ11–28 a.a.	IBL, Maebashi, Japan	Mouse	1:50	Formic acid
GFAP	Polyclonal	Glial fibrillary acidic protein	DAKO	Rabbit	1:10	None
CD68	Monoclonal	CD68	DAKO	Mouse	1:100	Microwave in citrate buffer
TH	Monoclonal	Tyrosine hydroxylase	Calbiochem-Novabiochem, Darmstadt, Germany	Mouse	1:10000	Microwave in target retrieval solution (DAKO)
SMI31	Monoclonal	Phosphorylated neurofilament	Sternberger Immunochemicals, Bethesda, MD	Mouse	1:20000	None

GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase.

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Double labeling immunofluorescence studies were performed by incubating sections with anti-psyn antibody (PSer129, polyclonal) and anti-tyrosine hydroxylase (TH) antibody; for labeling, anti-rabbit Alexa 546 Fluor (Molecular Probes, Eugene, OR) (red) and immunoglobulin G Alexa 488 (green) were used. Sections were viewed under a Zeiss confocal laser scanning microscope (model LSM5 PASCAL; Jena, Germany).

For immunoelectron microscopy, we used selected paraffin sections stained with PSer129 and visualized using diaminobenzidine. The sections were evaluated by light microscopy and washed in a 0.1% (pH 7.4) phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Plastic capsules filled with fresh epoxy resin were placed on the appropriate area of the sections and after polymerization of the resin, the target tissues were stripped off from the glass slides to the top of the epoxy resin. Ultrathin sections were then obtained and were examined under an H-7500 electron microscope (Hitachi, Japan) without a counterstain.

The BBAR Protocols for Evaluating Pathology of the CNS

The brains and spinal cords from all the patients of both the retrospective and the prospective studies were examined as previously reported (14). Briefly, 6-μm-thick sections were stained with hematoxylin and eosin and by the Klüver-Barrera method; selected sections were further examined with modified methenamine and Gallyas-Braak silver staining for senile changes, Congo red for amyloid deposition, and elastica Masson trichrome stain for vascular changes. In addition, selected sections were stained using the anti-α-synuclein and anti-ubiquitin antibodies (Table 1). To evaluate other senile changes, antibodies against phosphorylated tau, amyloid β (Aβ), glial fibrillary acidic protein, CD68, and phosphorylated neurofilament were also used.

Lewy body-related pathology in the CNS was examined at several levels of the thoracic spinal cord and in the medulla oblongata at the level of the dorsal motor nucleus of the vagus, the upper pons at the level of the locus ceruleus, the midbrain, the cerebellum (including the dentate nucleus), the basal ganglia (including the basal nucleus of Meynert), the amygdala, and the posterior hippocampus. In addition, areas were evaluated for LB scores according to the first and revised consensus guidelines for DLB (18, 19). These areas included the anterior cingulate gyrus, the entorhinal cortex, the second frontal and temporal gyri, and the supramarginal gyrus. Samples from these areas were stained by immunohistochemistry and classified into 7 CNS LB stages according to previously reported criteria (2, 14, 16) as follows: LB stage 0, no LBs; LB stage 0.5, Lewy neurites alone or diffuse or fine granular cytoplasmic staining lacking any focal aggregates in sections stained with anti-psyn antibodies; LB stage I, scattered LBs without cell loss (incidental LBD); LB stage II, abundant LBs with macroscopic loss of pigmentation in substantia nigra and locus ceruleus and/or gliosis demonstrated by glial fibrillary acidic protein immunohistochemistry in areas containing LBs but without attributable parkinsonism or dementia (subclinical LBD); LB stage III, PD without dementia; LB stage IV, DLB or PDD, transitional (limbic) form (DLBT or PDDT); and LB stage V, DLB or PDD, neocortical form (DLBN or PDDN). Parkinson disease with dementia was differentiated from DLB based on the definition in the consensus guidelines that "[PDD] dementia appears more than 12 months after the onset of parkinsonism." The CNS LB stages are shown in Table 2. We subcategorized CNS stages I and II into primary and secondary α-synucleinopathy on the basis of our previous work (14, 16). Primary α-synucleinopathy involved the intermediolateral column of the spinal cord or the preganglionic sympathetic neurons and was further subdivided into brainstem, transitional, and neocortical forms according to the LB score or distribution. Secondary α-synucleinopathy spared the

TABLE 2. Lewy Body Stages in the CNS

Stage	Substantia Nigra and Locus Ceruleus: Loss of Pigmentation	LB					Dementia	PA	Diagnosis
		Nigrostriatal	Limbic-neocortical	Spinal Cord	LB Score	Adrenal (2)			
0	−	−	−	−	0	+/−			
0.5	−	+/−	+/−	+/−	0	+/−			
I	−	+/−	+/−	+/−	0	+/−			Incidental LBD
II	+	+	+/−	+/−	0–10	+/−	−*	−*	Subclinical LBD
III	+	+	+	+	0–10	+	−	+	PD
IV P	+	+	+	+	3–6	+	+	+	PDDT
IV D	+	+	+	+	3–6	+/−	+	+/−	DLBT†
V P	+	+	+	+	7–10	+	+	+	PDDN
V D	+	+	+	+	7–10	+/−	+	+/−	DLBN†

*No dementia or parkinsonism associated with LB-related α-synucleinopathy.
†Differential diagnosis of PDD and DLB was based on the "1-year rule" in the consensus guidelines (19).
All patients with PD or PDD had adrenal LBD (2).
DLBN, dementia with LBs, with an LB score corresponding to the value for the neocortical form; DLBT, dementia with LBs, with an LB score corresponding to the value for the transitional form; LB, Lewy body; LBD, LB disease; PA, parkinsonism; PD, Parkinson disease; PDDN, PD with dementia, with an LB score corresponding to the value for the neocortical form; PDDT, PD with dementia, with an LB score corresponding to the value for the transitional form.

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preganglionic sympathetic neurons, preferentially involved the amygdala, and was termed *amygdala variant.*

Evaluation of Pathology Related to Other Senile Changes

Neurofibrillary tangles were classified into 7 stages and senile plaques into 4 stages according to Braak criteria (20). Argyrophilic grains were classified into the 4 stages that we previously reported (13). National Institute on Aging-Reagan criteria modified by us were adopted for the diagnosis of Alzheimer disease (AD) (21). Diagnoses of "dementia with grains" and "neurofibrillary-tangle-predominant form of dementia" were based on Jellinger's definitions (22, 23).

Pathological Study of the PANS

The adrenal glands were evaluated in the 279 patients in the prospective study, as previously reported (2). The adrenal glands from the additional 109 patients in the retrospective study were also examined for adrenal LB pathology. Data on 47 of these 109 patients were included in our previous report (2).

Statistical Analysis

Statistical analysis was performed by the χ^2 test for comparisons of categorical data. Statistical significance was established at $p < 0.05$.

RESULTS

Prospective Study

LB Pathology in the Skin

Immunohistochemical staining with anti-psyn antibodies demonstrated positive neurites and dots in nerve fascicles of the dermis and subcutaneous tissue (Figs. 1A, B). In some cases, psyn-positive nerve fibers showed swellings (Figs. 1C, D). Psyn-positive small dots or thin linear structures were also found around blood vessels (Figs. 1E, F). These psyn-positive structures seemed to colocalize with the

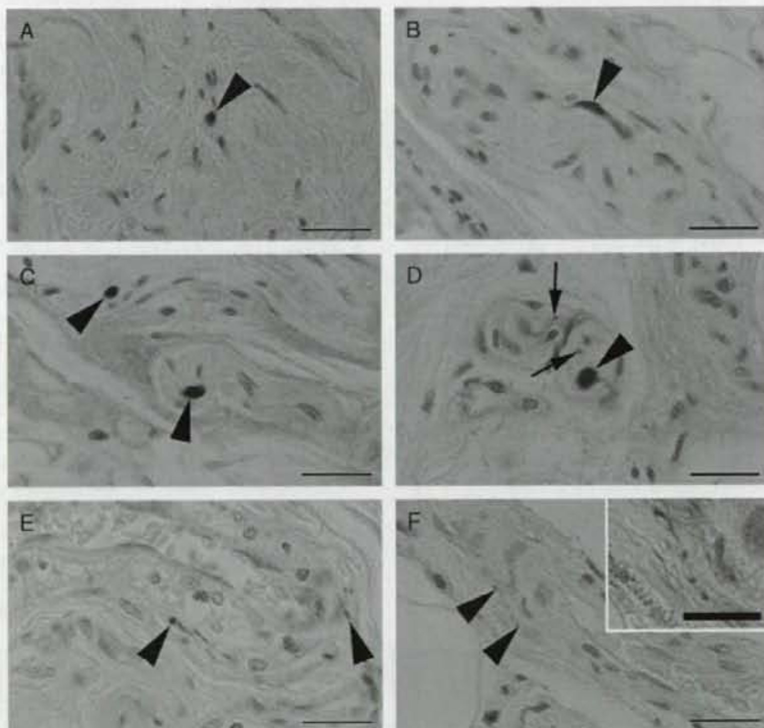

FIGURE 1. Immunohistochemical staining of nerve fascicles and vascular walls in the skin with anti-phosphorylated α-synuclein (psyn) antibodies. **(A)** Dotlike immunoreactivity (arrowhead) in a cross section of a nerve fascicle. **(B)** Longitudinal section of a cutaneous nerve with threadlike psyn immunoreactivity with a focal swelling (arrowhead). **(C)** Oval areas of immunoreactivity (arrowheads) scattered in nerve fascicles. **(D)** An oval structure connected to a linear structure (arrowhead) and small dotlike and linear staining (arrow). **(E)** Polyclonal anti-psyn antibody reveals a thin linear structure (arrowheads), extending from a nerve fascicle to the wall of blood vessel. **(F)** Monoclonal anti-phosphorylated α-synuclein antibody detects several positive thin linear structures in a vessel wall (arrowheads). Inset shows a higher power. **(A–E)**: PSer129, polyclonal; and **(F)**: psyn no. 64, monoclonal. Scale bars = **(A–F)** 25 μm; (inset in **F**) 10 μm.

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