SPANDIDOS PUBLICATIONS

CD44 expression during tumor progression of follicular lymphoma

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Abstract. Follicular lymphoma often progresses to diffuse type lymphoma. To elucidate the mechanisms of the diffuse evolution of follicular lymphoma, we investigated the expression pattern of CD44 in 28 cases of follicular lymphomas (FLs) using an immunohistochemical method and semi-quantitative PCR-Southern blot analysis. The FLs were divided into four groups: i) intrafollicular (IF); ii) infiltrative (INF); iii) partially follicular (PF); and iv) minimally follicular (MF), according to the histological classification by Lukes and Collins. Immunohistochemical analysis of CD44 using antibodies against CD44 common (CD44C) epitopes showed that CD44 was expressed in the diffuse area in the INF (0/8 cases), PF (12/12 cases), and MF (2/2 cases) lymphomas, whereas CD44 was not expressed in the lymphoma cells within the area of follicular growth of IF (0/6 cases) and INF (0/8 cases). Semi-quantitative PCR-Southern blot analysis showed that CD19-selected B cells from the FLs were expressed as a product of 482 base pairs (bp) corresponding to a CD44 standard form (CD44s) (5/5 cases). Additionally, the lymphoma cells from the PF were expressed as products of 600 and 1100 bp and the cells from MF were expressed as products of 600, 900, and 1100 bp with the CD44 exon 10 or 11 probes. The results indicated that the expression of CD44s and CD44 variants containing exon 10 and 11 were up-regulated according to the diffuse evolution of the follicular lymphoma.

Introduction

Adhesive protein CD44 is a transmembrane glycoprotein with phosphorylation sites in its cytoplasmic domain (1,2). CD44 is expressed on many different cell types including hematopoietic cells, fibroblasts, some epithelial and endothelial cells, and cells in the central nervous system (3,4). It is encoded by a single gene, with alternative splicing providing the potential for multiple isoforms (5). The standard form of CD44 (CD44s) is ubiquitously expressed in epithelial and mesenchymal tissues, whereas the variant isoforms of CD44 (CD44v) generated by alternative RNA splicing are found in a restricted distribution (6-9). It has been shown that in a variety of human carcinomas (bladder, colon, breast, and stomach) there are certain overexpressed CD44v isoforms (10-14). Since differences in the expression of CD44v were detected between tumors with and without clinical evidence of metastasis formation, analysis of CD44v may serve as a prognostic and diagnostic marker (11).

Follicular lymphomas (FLs) constitute the majority of low-grade lymphomas, and often pursue an indolent course, but diffuse follicular center cell lymphomas display aggressive behavior and have a poor prognosis. Follicular lymphomas have either a follicular or diffuse growth pattern, and they frequently change from a follicular to a diffuse pattern over time (15).

Many studies of CD44 expression in non-Hodgkin's lymphoma (NHL) have been reported (16-21). Recently, upregulation of CD44v6 in NHLs was observed in highly aggressive lymphomas and CD44v6 expression could be shown to correlate with survival and the stage of the disease (22). In this study, we investigated the expression pattern of CD44 in 28 cases of follicular lymphomas (FLs) to elucidate the mechanisms of diffuse evolution of follicular lymphoma.

Materials and methods

Cases. Twenty-eight fresh lymph node specimens from 28 people with FLs were obtained from the Department of Pathology of Chiba University School of Medicine. Samples were snap-frozen in liquid nitrogen within 10 min of arrival at our pathology facilities, and kept frozen until use. Part of the samples was used for immunohistochemical staining. Non-neoplastic tissues were obtained from specimens surgically resected for the treatment of cancer or removed for non-neoplastic conditions (3 lymph nodes and 2 tonsils). The histological patterns of FLs were divided into four groups: i) intrafollicular (IF); ii) infiltrative (INF); iii) partially follicular (PF); and iv) minimally follicular (MF), according to the classification by Lukes and Collins (15). The study was

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conducted in accordance with the Helsinki Declaration. The University Ethics Committee approved this study and all patients provided written informed consent.

Immunohistochemistry. Tissue distribution of the different forms of CD44 was determined by streptavidin-biotin complex technique (Dakopatts Co., Inc., Denmark), in addition to identification of the immunophenotypes of the lymphomas. Cryostat sections were fixed in acetone for 10 min, washed in PBS, and pre-incubated with normal rabbit serum. After pre-incubation, the sections were incubated for 1 h at room temperature or overnight at 4˚C with the following primary antibodies (Abs): bcl-2 (Dakopatts Co., Inc.), murine monoclonal antibodies Bu52 (Binding Site Ltd., Birmingham, UK) and F10/44-2 (gift from Dr J. Farbre) against the common epitopes of CD44 molecules (CD44C), the Abs VFF-7 (Bender Med Systems, Vienna, Austria), 10 and 2C5 (R&D Systems Europe Ltd., Abingdon, UK) against the V6 variant, and affinity purified polyclonal rabbit serum against the 20 amino-terminal peptides of exon 11 (CD44EX11). Before incubating the secondary biotinated Ab for 30 min (antimouse F(ab)2; Dakopatts Co., Inc.), endogenous peroxidases were blocked with 0.3% H_2O_2 in methanol. All antibodies were titrated for optimal staining results. For detection, a streptavidin-biotin peroxidase complex was used, and then the samples were developed using diaminobentidine-HCl. The sections were counterstained with hematoxylin, dehydrated and cover-slipped. In negative control experiments, BALB/c mouse ascites were applied to the sections in all cases.

Polymerase chain reaction (PCR)-Southern blotting. Total RNA was extracted from CD19 (clone 4G7, Becton-Dickinson, CA) positive cells from two cases of infiltrative FL (one mixed cell and one large cell type), and a case of partially follicular FL (large cell type), and two cases of minimally FL (one mixed and one large cell type). Total RNA was also extracted from CD19 and CD3 (clone SK7, Becton-Dickinson) positive cells from 3 cases of reactive lymph node. Selection of CD19 and CD3 positive cells was done from dispersed neoplastic or non-neoplastic lymph nodes using immunomagnetic Dynabeads 450 SH/MS IgG (Dynal A.S., Oslo, Norway) according to the manufacturer's instructions. Then, cDNA was synthesized with reverse transcriptase followed by amplification using an RNA PCR Kit (Perkin-Elmer Cetus, Norwalk, CT).

To determine the expression levels of different CD44s, semiquantitative PCR was employed. The conditions were 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 2 min. Negative controls, containing no template cDNA, were run with every batch. The amount of PCR products was calibrated by using the relative expression levels of human ß-actin. Complementary DNA samples were initially amplified with ß-actin specific primers (5' sense, 5'-CAGCCATGTACGTTGCTATCCAG-3', and 3' anti-sense, 3'-ACTTCACACTGCACCTGTAGG-5'). These samples were electrophoresed on 1.2% agarose gel and transferred to Hybond N^+ nylon membranes (Amersham, UK) for hybridization with a [^{32}P]-labeled internal oligonucleotide probe, GAGCAAGAGATGGCCAC. After densitometric scanning of the actin products, the intensity balancing of all the samples was no higher than 3-fold.

Table I. Characteristics of lymphoma cases.

Characteristics	No. of cases
CD5	0/28
CD10	23/28
CD19	28/28
CD20	28/28
CD21	16/28
CD23	9/28
Bcl-2	26/28
bcl-2 rearrangement	15/28

The same cDNA samples were amplified with CD44 primers. We devised the probes using information from the sequence of human CD44 DNA (23). The primers were P1 (458, 5'-GACACATATTGCTTCAATGCTTCAGC-3') and P2 (939, 3'-TAAGGTCTTACCGACGACTAGTAGAACCGTAG-5'), and a probe (S1 designed to anneal to the common portion of the CD44 molecules) was 478, 5'-CCTGAAGAAATTGTACATCAGTCACAGAC-3' according to the sequence of CD44S.16. Exon-specific probes were the following: for exon 8, 5'-ACCACACCACGGGCCTTTGA-3', for exon 9, 5'-CACCCTCCCCTCATTCACCA-3', for exon 10, 5'-GGCAACAGATGGCATGAGGG-3', and for exon 11, 5'-CATGGGACGAGGTCATCAAGC-3' as in the genomic structure of CD44 described by Screaton *et al* (5). Twenty-three cycles of PCR were chosen because this number had been determined to be in the linear range of amplification for both ß-actin and CD44s. The blots were exposed for 5 h (standard CD44) and 24 h or longer (variant CD44), and then scanned with a bioimaging analyzer BAS-2000II (Fujix, Tokyo). For rehybridization, the blots were boiled in 0.5% SDS.

Results

Cases. The 28 FL cases showed marked variations in growth patterns. Six of them exhibited discrete follicular growth with lymphoid mantles and minimal infiltration of the interfollicular areas (intrafollicular FL). In 8 cases of infiltrative FL, the follicular structure was often destroyed, and the follicular center cells infiltrated to the interfollicular area. In 8 cases, areas of diffuse growth were observed (partial FL). In 2 cases, only a few follicles remained and a diffuse growth pattern was prominent (minimal FL). All cases of FL were positive for CD19 and CD20 as a B-cell marker, and negative for CD5 as a T-cell marker. The Bcl-2 protein was over-expressed in 22 cases of FL. Rearrangement of the bcl-2 gene was detected in 15 cases using a PCR method (Table I).

Immunohistochemistry of CD44. In the reactive lymph nodes, B lymphoid cells in the germinal center were CD44C negative, whereas the ones in the mantle zone were positive. T cells and macrophages both in the paracortex and follicles were positive for CD44C in the plasma membrane. Twenty-two of 28 cases of FLs showed positive reaction for CD44C (Table II).

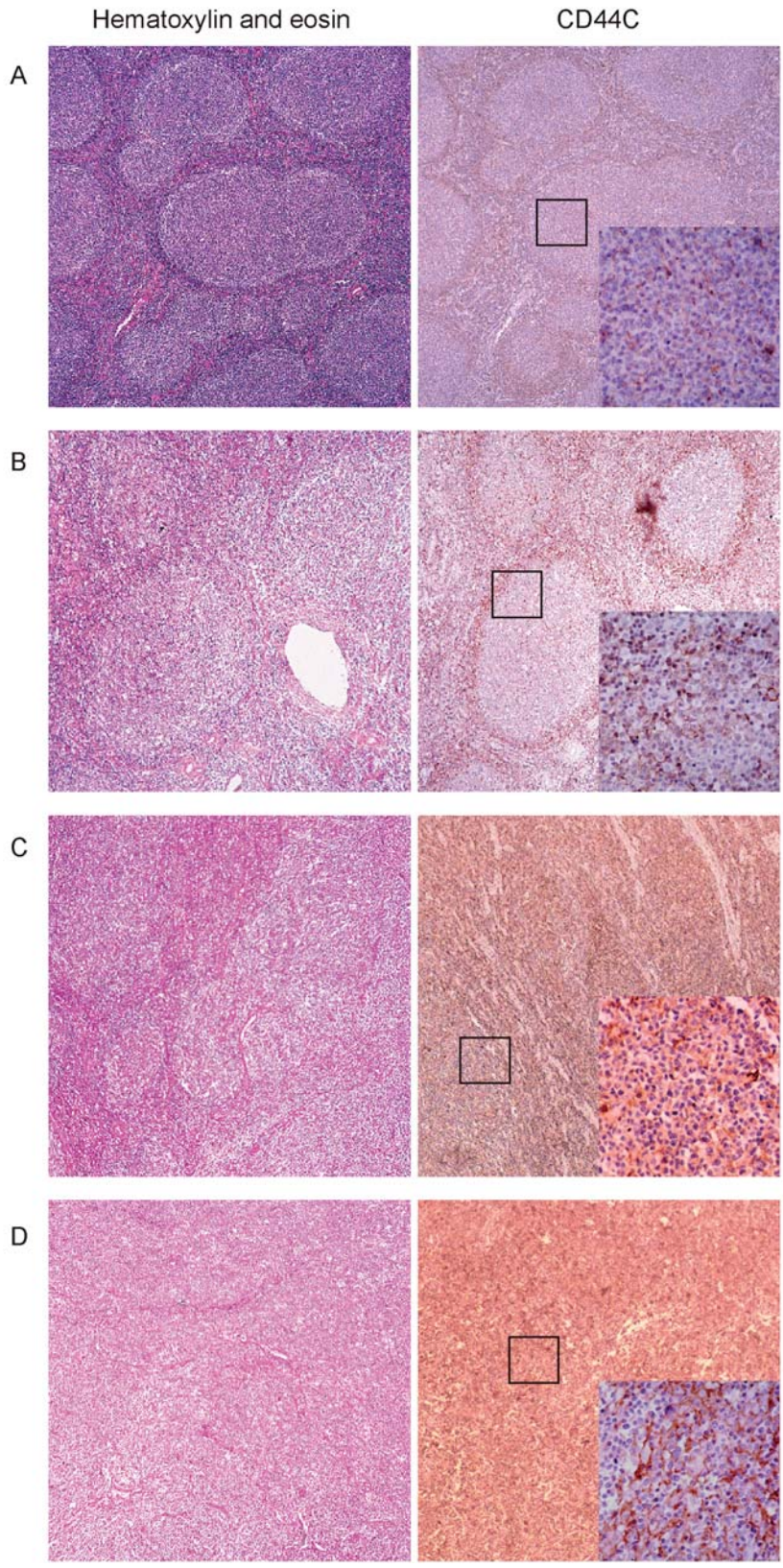

Figure 1. CD44 expression in follicular lymphoma. The lymphoma cells in the intrafollicular area were negative for CD44C (A). Infiltrative cells of interfollicular FL (B), partially follicular type (C) and minimally follicular type (D) were positive for CD44C. Inset, magnification of immunostaining of the CD44C.

In the three cases of intrafollicular FLs, the small cleaved cells were negative for CD44C (Fig. 2). In one case of intrafollicular FL, it was converted to infiltrative FL a year later without any treatment. The lymphoma cells in the converted case were CD44C positive (Fig. 1B). All cases of FLs with a diffuse pattern were positive for CD44C (Fig. 1C and D).

Table II. Expression of CD44 in follicular lymphoma.

Subtypes	Immunohistochemistry		PCR-Southern	
	Localization	CD44C	CD44s	CD44 variant[a]
Reactive lymph nodes	GC	0/5	3/3	0/3
Follicular lymphoma				
Intrafollicular	IF	0/6	nd	nd
Infiltrative	IF	0/8	nd	nd
	IA	8/8	2/2	2/2
Partially follicular	DA	12/12	1/1	1/1
Minimally follicular	DA	2/2	2/2	2/2
Total		28	5	5

[a]Molecular weight bands higher than 482 bp with the exon 10 or 11 probes. GC, germinal center; IF, intrafollicular area; IA, infiltrative area; DA, diffuse area; nd, not done.

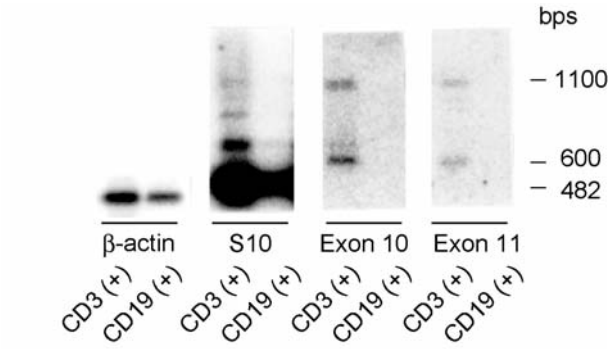

Figure 2. Expression of CD44 transcripts in reactive lymph nodes. The amplified ß-actin PCR products are found at 567 for 23 cycles using oligonucleotide primers. CD3 positive cells from a reactive lymph node (lane 1) showed four bands at 482, 700, 900 and 1100 bp with the S1 probe, two bands at 609 and 1100 bp with the exon 10 probe, and two bands at 612 and 1100 bp with the exon 11 probe. CD19 positive cells from reactive lymph nodes (lane 2) showed only a 482 bp band with the S1 probe and no variant transcripts with the exon 10 and 11 probes.

CD44 transcripts. Amplification of the region spanning the primers P1 and P2 is predicted to generate a product of ~482 bp from cDNAs encoding CD44s, and ~878 bp from cDNAs encoding the epithelial variant of CD44 (CD44E). With up to 34 cycles of PCR, no CD44 transcripts were detected in cDNAs prepared from the pre-B cell line NALM-6, and thus it was used as a negative control in the following experiments.

The cDNAs amplified from CD19 cells selected from reactive lymph nodes yielded a 482 bp band with the S1 probe for detection of the CD44 common region, but no transcripts containing exons 10 and 11 were amplified (Fig. 2, lane 2). The transcripts amplified from the cDNA of CD3-positive lymphocytes from the reactive lymph nodes included four different sizes of transcripts with the common probe S1 (482, 700, 900, and 1100 bp bands). Two different sizes of transcripts were detected (609 and 1100 bp) with the exon 10 probe, and 612 and 1100 bp bands with the exon 11 probe

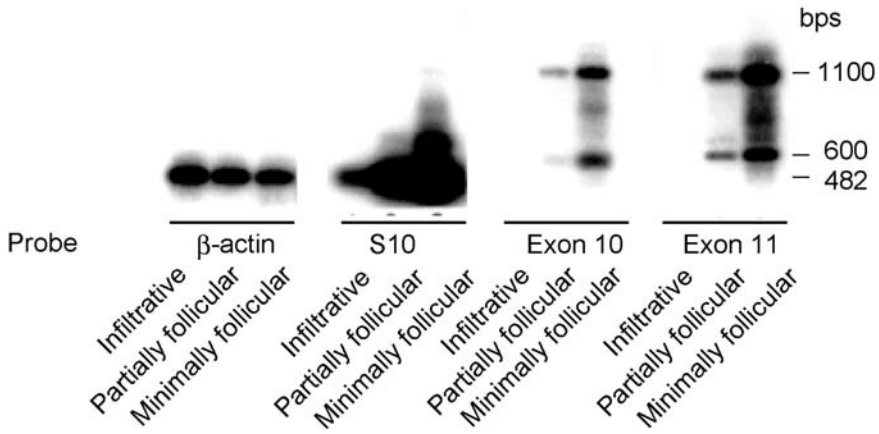

Figure 3. Expression of CD44 transcripts in lymphomas. The amplified ß-actin PCR products are found at 567 for 23 cycles using oligonucleotide primers. Lane 1, CD19 selected cells from infiltrative FL; lane 2, CD19 selected cells from partially FL; lane 3, CD19 selected cells from minimally FL. With the S1 probe, lane 1 showed a major 482 bp band. Variant transcripts are found at 609, 900, 1100 bp with the exon 10 probe and at 612, 900 and 1250 bp with the exon 11 probe in lane 2 and 3.

ane 1). With the exon 8 and 9 probes, signals were ved.

The cDNAs amplified from CD19 positive cells selected from two cases of infiltrative FL yielded only a 482 bp band without variant transcripts (Fig. 3, lane 1). CD19 selected cells from partially FL showed a single 482 bp band with the S1 probe and both a 609 and 1100 bp band with the exon 10 probe or the exon 11 probe (Fig. 3, lane 2). In two cases of minimally FL, the amplification of the cDNAs using the same combinations of oligonucleotides resulted in a 482 bp band with the S1 probe, 609, 900 and 1000 bp bands with the exon 10 probe, 612, 900 1100, and 1250 bp bands with the exon 11 probe (Fig. 3, lane 3). There were also a few faint cDNA products between the two major variant transcripts. No signals were found using the exon 8 or 9 probes in the materials examined (data not shown).

A summary of the expression patterns of the CD44 proteins and mRNAs is given in Table II. The results demonstrate that the expression of CD44s and CD44 variants containing exon 10 and 11 were upregulated according to the diffuse evolution of the follicular lymphoma.

Discussion

In most follicular lymphomas, the constitutive expression of Bcl-2 confers longevity to follicular B-cells, resulting in their accumulation through the lymphoid system (24,25). Secondary genetic changes, such as the mutation of p53 or the accumulation of the c-myc protein are required for malignant transformation and histological progression from a follicular pattern to a diffuse pattern (24-26).

Adhesion proteins including CD44 play pivotal roles in tumor progression and metastasis. CD44 is expressed as several different isoforms, achieved by the alternative mRNA splicing of eleven variant exons (27). It has been demonstrated that CD44 is expressed in diffuse large B-cell lymphomas and the expression of CD44 is correlated with their aggressive behavior. The expression of exon 10 containing transcripts has been found in the intermediate and high grade categories of diffuse B-cell lymphomas (28,29). In the present study, lymphoma cells from the cases of intrafollicular type cancers did not express CD44. Cells from the infiltrative lymphoma showed the expression of CD44s. The results suggest that CD44s is involved in the invasion of lymphoma cells as in the other epithelial neoplasm.

Tumor progression could be acquired step by step through mutation and selection induced by pleiotropic control proteins for various genes (30). The present study suggests that genetic alterations during tumor progression of the germinal center cell tumor induce the expression of CD44s which are later accompanied by high molecular weight variant forms. Our findings are quite consistent with the reported observation that the activated oncogene c-Ha-ras induces CD44 promoter activity and leads to increased levels of CD44s accompanied by the appearance of alternatively spliced RNAs (31). Therefore, the elucidation of the regulatory mechanism of the serial genetic changes of CD44 expression in lymphoma cells may lead toward approaches for suppressing tumor progression and developing novel cancer treatments.

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