

The role of alternative splicing of the adhesion molecule, CD44, in lymphoid malignancy

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Abstract

Aim—To investigate the expression of CD44 isoforms containing variant exon 6 (v6) in a well characterised cohort of patients with non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukaemia (CLL), and to correlate this with phenotype and disease course.

Methods—Cryostat sections of OCT embedded diagnostic nodal material from NHL patients and cryopreserved mononuclear preparations from CLL patients were used as sources of RNA. After reverse transcription, PCR was carried out with amplimers positioned at either side of the variant exon insertion site to amplify all possible CD44 isoforms. Those isoforms containing v6 were identified after Southern blotting and hybridisation with a radiolabelled oligonucleotide.

Results—Of 32 NHL samples analysed, 16 did not express CD44 isoforms containing v6, six expressed an isoform containing exon v6 alone, and 10 expressed v6 long isoforms which contained exon v6 in addition to other variant exons. These data did not correlate with lymphoma classification, disease staging, or the presence or absence of extranodal disease. However, those patients expressing v6 long CD44 isoforms had a worse overall survival than those that did not. The plateau of the survival curves was 50% compared with 82%. No v6 long isoforms were detected in the 21 CLL samples investigated.

Conclusions—The expression of v6 long CD44 isoforms is associated with aggressive disease in NHL, independent of grade, stage, or presence of extranodal disease.

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Cell-cell and cell-extracellular matrix interactions are fundamental to many biological processes including cell growth, differentiation, and motility. These adhesive interactions are governed by several families of cell surface receptors, for example cadherins, selectins, integrins, members of the Ig superfamily, and CD44.

CD44 is a highly glycosylated cell surface protein present on B and T lymphocytes, granulocytes, monocytes, erythrocytes, and a variety of non-haemopoietic lineages. It appears to have a role in lymphocyte homing, lymphocyte activation, and tumour metastasis. This wide array of

functions may be mediated by a family of CD44 isoforms generated by alternative RNA splicing and post-transcriptional modifications (for review see Zoller¹).

The CD44 gene has at least 20 exons; the standard or haemopoietic form spans a region of seven extracellular exons, a transmembrane region, and a cytoplasmic exon, which may be short or long. The remaining variant exons are subject to alternative splicing and are inserted between exons 5 and 6 of the standard form.² So far, 17 different variant exon combinations have been identified. The insertion of these variant exons into the standard form yields proteins that all differ at the membrane proximal extracellular domain. Further diversity is brought about by glycation and the addition of chondroitin sulphate side chains.

In a rat tumour model, transfer of cDNA of a CD44 isoform containing variant exons 4-7 initiated metastasis formation of a locally growing adenocarcinoma. An antibody recognising an epitope of variant exon 6 (v6) inhibited this metastatic spread.³ The same CD44 molecule containing v6 is transiently expressed on lymphocytes after antigen stimulation.⁴ Thus CD44v6 appears to be a common factor in lymphocyte activation and metastasis, and has led to speculation that tumour cells may metastasise by mimicking migrating developing lymphocytes.

The role of CD44 variant expression in human malignancies was first addressed by Matsumura and Tarin. They used a qualitative reverse transcription polymerase chain reaction (RT-PCR) and blot hybridisation technique to show changes in CD44 variant expression in neoplasia and tumour progression in man. Although data are not yet available for all types of human malignancies, it is evident that expression of CD44 variants is associated with tumour progression in some malignancies, for example breast⁶ and bladder carcinomas.⁷ However, variant isoforms other than v6 have also been implicated.

One model of lymphocyte malignancies regards them as the neoplastic counterparts of normal lymphoid cells arrested at a particular stage of maturation/activation and as such sharing many phenotypic and functional characteristics of normal lymphoid cells. This seems to hold true for standard CD44 expression which is high in malignancies where the "normal lymphocyte" counterpart is early in the differentiation process (null and common types of acute lymphoblastic leukaemia (ALL)), is reduced in subsequent stages of development (pre-B ALL, B and T ALL), and is then again prominently expressed by so called "mature" lymphoid

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Table 1 Clinical details of 32 non-Hodgkin's lymphoma patients at diagnosis and expression of CD44 v6 long isoforms

Patient	Lymphoma classification			Stage	Treatment	END	V6 long
	Working	Kiel	REAL				
1	Intermediate	High	Diffuse large B	I	1	N	Y
2	Intermediate	Low	Mantle	IV	2	Y	N
3	Low	Low	Follicle centre	II	2	N	N
4	Intermediate	High	Diffuse large B	I	3	N	N
5	Intermediate	Low	Follicle centre	II	2	N	N
6	High	High	Diffuse large B	II	2	N	Y
7	Low	Low	Follicle centre	I	3	N	Y
8	High	High	Diffuse large B	IV	2	Y	N
9	Intermediate	High	Diffuse large B	IV	1	Y	N
10	Low	Low	Follicle centre	III	2	N	N
11	Intermediate	High	Diffuse large B	I	2	N	N
12	Low	High	Follicle centre	IV	1	Y	N
13	Intermediate	High	Follicle centre	III	2A	N	Y
14	High	High	Peripheral T	II	2	N	N
15	Low	Low	Lymphocytic	IV	2	Y	N
16	Intermediate	Low	Follicle centre	IV	2	Y	N
17	Low	Low	Follicle centre	III	2	N	N
18	High	High	Diffuse large B	IV	2	Y	N
19	Intermediate	High	Diffuse large B	II	1	N	Y
20	High	High	Peripheral T	IV	5	Y	Y
21	High	High	Precursor	III	1	N	N
22	High	High	Peripheral T	I	3	N	N
23	Low	Low	Follicle centre	IV	2	Y	Y
24	Intermediate	High	Diffuse large B	IV	1	Y	N
25	Intermediate	Low	Follicle centre	III	1	N	Y
26	Intermediate	High	Diffuse large B	IV	4	Y	N
27	Low	Low	Follicle centre	III	2	N	N
28	Low	Low	Follicle centre	IV	2	Y	N
29	Intermediate	High	Diffuse large B	I	1	N	N
30	Intermediate	Low	Follicle centre	III	1A	N	Y
31	Intermediate	Low	Follicle centre	IV	5	Y	Y
32	Low	Low	Follicle centre	I	2	N	N

Treatment: 1, chemotherapy and radiotherapy; 2, chemotherapy alone; 3, radiotherapy alone; 4, none; 5, not known; A, autologous bone marrow transplant. END, extranodal disease; REAL, revised European-American classification of lymphoid neoplasms.

tumours (chronic lymphocytic leukaemia (CLL), myeloma). The germinal centres and lymphomas originating from them—for example, follicular lymphomas, centroblastic and centrocytic lymphomas—show a heterogeneous expression.⁸

Non-Hodgkin's lymphoma (NHL) is a heterogeneous malignancy with variable histology and clinical course. It may be limited to lymphoid tissue or spread extranodally, but rarely becomes overtly leukaemic. Chronic lymphocytic leukaemia, often classified as a low grade lymphoma, primarily involves the peripheral blood and bone marrow but may be associated with tissue infiltration. Thus these lymphoid malignancies share some characteristics but differ in their normal pattern of dissemination.

Preliminary investigations have suggested a possible role for CD44 variant expression in lymphoid disorders. Immunocytochemical investigations found that CD44v6 expression was associated with high grade NHL.⁹ In addition, extranodal or disseminated diffuse large cell lymphomas had increased levels of CD44 and CD44v6 transcripts, as assessed by RT-PCR,¹⁰ though few studies have correlated this information with clinical data. These observations have prompted us to investigate the expression of CD44v6 in a well characterised cohort of NHL and CLL patients and to correlate this with phenotype and disease course.

Methods

PATIENTS

CD44 expression was investigated in retrospective samples from 60 patients with primary

NHL or CLL who presented at the Royal Victoria Infirmary between 1986 and 1996. Samples were collected before treatment. Diagnostic nodal tissue was stored as frozen pathological specimens embedded in OCT and maintained at -80°C . Mononuclear preparations of CLL samples were cryopreserved as viable cells using standard procedures. Specimens were stored prospectively from all diagnostic nodal and CLL material, where possible; thus this patient panel represents, as far as possible, an unbiased selection of cases.

NHL classification was performed according to the working formulation,¹¹ the Kiel classification,¹² and the REAL classification,¹³ after applying conventional histological and immunohistochemical techniques. Staging of the lymphomas was undertaken in accordance with the Ann Arbor classification after clinical status, chest x ray, computed tomography, and bone marrow biopsies were assessed. Details of disease stage, presence of extranodal disease, and modes of treatment are shown in table 1. Survival data were available for all NHL cases. In NHL, CD44 expression was correlated with histological grade, staging of disease, nodal versus extranodal disease, and overall survival.

CLL cases were identified according to clinical presentation, peripheral blood appearance, and immunophenotyping studies. These included 16 B-CLL, five T-CLL, four prolymphocytic, two hairy cell, and one B lymphoblastic NHL. For B-CLL patients, CD44 expression was correlated with overall survival.

CELL LINES

A cell line derived from adenocarcinoma of the breast (ZR75-1)—donated by Dr F May, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne—served as a positive control for RT-PCR and Southern blotting experiments. These cells express standard CD44 and a range of exon v6 containing CD44 molecules.

RNA ISOLATION

A method was developed to extract high quality RNA from stored OCT embedded pathological nodal material.¹⁴ Five pooled cryostat sections ($20\ \mu\text{m}$) were stored frozen at -20°C in a guanidine isothiocyanate (GITC) solution. After thawing, samples were homogenised using a battery operated disposable mortar and pestle (Anachem). The method of Chirgwin *et al* was used to isolate RNA after caesium chloride ultracentrifugation, phenol/chloroform extraction, and ethanol/salt precipitation.¹⁵ Denaturing formaldehyde gel electrophoresis was used to assess RNA quality.

Qiagen RNeasy kits were used to prepare RNA from viable cell stores of CLL samples, according to the manufacturer's instructions. Approximately 1×10^7 cells were used per preparation.

CDNA PREPARATION AND POLYMERASE CHAIN REACTION

The method of Cross *et al* was used to reverse transcribe approximately $5\ \mu\text{g}$ RNA, using random hexamers and M-MLV (Life

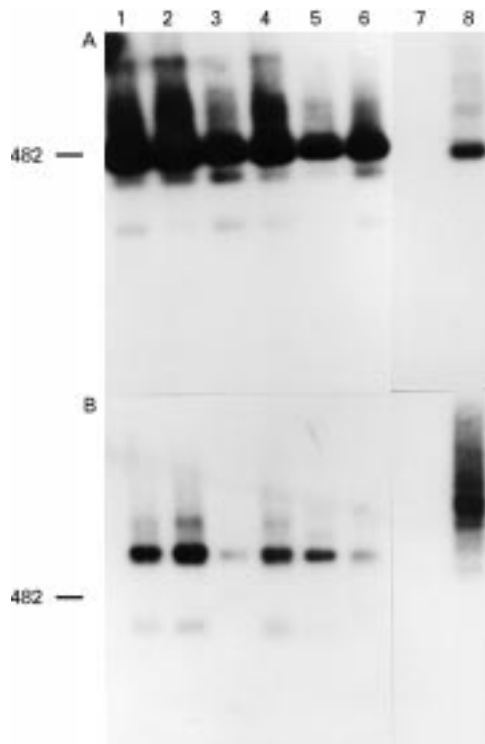


Figure 1 Analysis of CD44 isoforms in non-Hodgkin's lymphoma samples after reverse transcription polymerase chain reaction (RT-PCR), Southern blotting, and hybridisation with a radiolabelled oligoprobe to variant exon 6 (panel B). Filters were then stripped and rehybridised with a radiolabelled oligoprobe to a non-variant part of CD44 (exon 4) (panel A). Lanes 1–6 refer to samples from patients 30, 6, 16, 13, 1, and 5, respectively. Lane 7 refers to the negative control (water blank), and lane 8 to the positive control (ZR75 cell line). The position of 482 base pairs is marked in both panels. This is the PCR product size which was amplified from CD44 transcripts containing no variant exon insertions. Several larger bands are apparent in both panels and represent CD44 molecules with any variant exon insertions (panel A) and those specifically containing v6 (panel B). The signal intensity from the standard form served as an internal control for loading.

Technologies).¹⁶ PCR primers and reaction conditions were as described by Matsumura and Tarin.⁵ PCR products were analysed by gel electrophoresis using 3% Nusieve agarose (FMC Bioproducts) and photographed on a transilluminator, after ethidium bromide staining.

BLOTTING AND PROBING

After Southern blotting onto Genescreen (NEN), filters were hybridised with end labelled internal oligonucleotide probes specific to exon 4 (S1),⁵ to identify all possible CD44 isoforms and specific to variant exon 6 (GTAGTACAACGGAAGAAACAGCTACCAGA) to identify all CD44 isoforms containing this exon. Filters were then washed and developed as autoradiographs using standard techniques.

POSITIVE AND NEGATIVE CONTROLS

Positive controls were an aliquot of ZR75 cells which were included during the processing of each patient panel at the RNA preparation stage. A reagent blank consisting of water, rather than RNA, was included in the cDNA reaction.

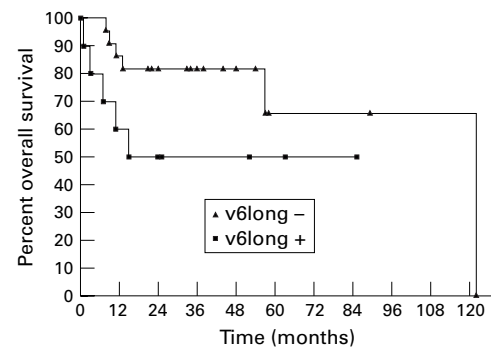


Figure 2 Actuarial survival curves according to expression of CD44 v6 long isoforms in non-Hodgkin's lymphoma.

STATISTICAL ANALYSES

Statistical analyses were carried out using a Prism computer program package (Graphpad Software). Contingency tables were analysed by Fisher's exact or χ^2 test. p Values were two tailed. Survival curves were created using the method of Kaplan and Meier and were compared using the log-rank test.

Results

Analysis of CD44v6 isoform expression was performed in a panel of NHL and CLL samples. During the processing of every panel of samples, ZR75 cells, and a water blank were included as positive and negative controls, respectively. Autoradiographic signals were never detected in water controls. ZR75 positive controls routinely produced distinctive ladders of CD44 variants, detectable with probes against the standard part of the CD44 molecule and exon v6.

All patient samples expressed the standard form of CD44. For NHL, samples could be classified in three ways: those expressing standard CD44 alone (16/32), those expressing the directly spliced v6 isoform—that is, variant 6 exon within the standard CD44 sequence without further variant exons (6/32), and those which expressed isoforms containing v6 plus other variant exons (10/32) (v6 long). For all 28 CLL and 11 of the NHL patients, there was sufficient stored specimen to repeat the analyses and we found no examples of discrepant results. Details of the patients and v6 long isoform detection are summarised in table 1. A representative autoradiograph from a lymphoma panel showing v6 long isoforms is shown in fig 1.

No significant association was found between the expression of v6 long isoforms compared with standard alone or standard plus v6 short, with respect to lymphoma histological classification, disease staging, or the presence or absence of extranodal disease (Fisher's exact test and χ^2 test). However, lymphomas that expressed v6 long isoforms were associated with a worse overall survival than those that did not (fig 2). There was a trend for the survival curves to plateau at 50% compared to 82% for patients not expressing v6 long isoforms. However, this did not reach statistical significance ($p = 0.09$). Modes of treatment were not

significantly different within each subgroup. The median follow up was 26 months.

CD44 v6 long transcripts were not detected in our panel of CLL patients (data not shown). Short v6 isoforms were expressed in 18 of 28 patients, while the remaining patients expressed the standard form alone. Analysis of B-CLL patients showed no difference in survival between these two groups.

Discussion

Alternative splicing and post-translational modifications enable CD44 to exist in many forms and may explain its pivotal role in a wide variety of diverse physiological and pathological processes. Specific isoforms may play distinct roles in these processes but as yet their structure and function have not been characterised. The standard form of CD44 is known to bind hyaluronan, fibronectin, collagen types I and IV, osteopontin, and a mucosal vascular endothelial surface, but as yet ligands for the variant isoforms have not been identified. However, it is known that insertion of variant exons into the extracellular domain alters CD44 ligand specificity and function. For example, cells expressing the epithelial variant which has exons 12, 13, and 14 inserted into the standard molecule have a much lower affinity for hyaluronate.¹⁷ In addition, human Burkitt's lymphoma cells expressing the standard and epithelial forms of CD44 were shown to have different rates of local growth and metastasis in a nude mouse model.¹⁸

In our panel of NHL analysed by RT-PCR and Southern blotting, we have identified a subgroup of patients (31%) in which expression of CD44 v6 long isoforms in diagnostic material is associated with poorer survival. The overall survival at five years was 50% for those lymphoma patients who expressed CD44 v6 long transcripts, compared with 82% for those who did not. Immunocytochemical studies have also shown that CD44v6 expression is associated with an unfavourable survival in NHL.^{19,20} The CD44 long isoforms we have described in aggressive lymphomas were also detected by Stauder *et al.*,²⁰ almost exclusively in their high grade lymphomas. This same group positively stained with antiCD44v6 antibody. However, they used immunocytochemical data rather than PCR/Southern data to perform clinical correlations. Interestingly, low grade lymphomas which expressed short v6 isoforms did not stain with the same antibody. Thus it is possible that the CD44 protein containing the products of v6 exons plus others inserted into the extracellular domain is in some way more antigenic than that containing the product of v6 alone.

We did not detect v6 long expression in any of the CLL samples tested, and v6 short expression did not appear to correlate with survival in our B-CLL population. However, there are reports that quantitative expression of CD44 standard molecule influences the clinical course of CLL, although results between groups are discrepant.^{21,22}

CD44 isoforms containing v6 appear to have a role in lymphocyte activation: there is a tran-

sient expression of CD44v6 after activation of both B and T lymphocytes,^{9,23} and in the rat, anti-CD44v6 antibodies inhibited lymphocyte activation both in vitro and in vivo.⁴ Interestingly, the CD44 long isoforms we have described in aggressive lymphomas are also expressed in lymphocytes at the stage of lymphocyte activation.²⁰

Variant exon insertion of the standard CD44 molecule may alter interactions between cell-cell and cell-extracellular matrix, resulting in functional changes in cell adhesion, migration, infiltration, and response to growth or apoptotic stimuli. Aggressive lymphomas and activated lymphocytes certainly share physical properties—both have invading and migratory abilities which may be mediated by CD44v6 long isoforms. Less aggressive NHL and CLL resemble the non-activated lymphocyte in terms of CD44v6 long expression and this may explain their more indolent clinical course. However, while activated lymphocytes express CD44v6 long isoforms only transiently, aggressive lymphomas do so constitutively. The constitutive expression of CD44 v6 long isoforms in malignant lymphocytes may equip them with novel cell-cell and cell-ECM interactions conducive to an aggressive clinical course.

Whether CD44v6 expression directly influences lymphoma biology or is a result of some other factor associated with aggressive behaviour cannot be deduced from this study. However, we have shown that it is associated with a considerably worse prognosis and thus may identify those patients who require more aggressive treatment.

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