Microsatellite Instability within the CD30 Gene Promoter Distinguishes between Normal and Neoplastic Cells in Lymphoma

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AJR and MF carried out the experimental work performed the typing and wrote the first draft of the manuscript. Author DVS provided the clinical specimens and clinical evaluation and authors DU and LJA managed the analyses of the study and completed the final version of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: As differences in promoter activity and CD30 surface expression between CD30+ lymphoid cell-lines and peripheral blood leukocytes have been shown previously to be independent of the size of the CD30 promoter microsatellite, in this study we investigate the instability within the region in a range of neoplasms including malignant lymphoma, breast and colon carcinoma and lung adenocarcinoma.
Study Design:A representative sample of CD30+ and CD30- lymphomas and cell lines as well as non-haemopoietic malignancies and normal tissues were typed for CD30 microsatellite length and compared.
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Methodology: DNA was prepared from archived biopsy specimens and used to PCR ampify the CD30 microsatellite region prior to size determination using an Genescan instrument (Applied Biosystems).

Results: This study has identified instability within the CD30 promoter microsatellite region in DNA from the tumour tissue of cases of malignant lymphoma, colon carcinoma and lung adenocarcinoma but not in DNA from benign lymphoid cells or normal tissues.

Conclusion: These findings indicate that variability in the length of the CD30 microsatellite may be a general characteristic of the neoplastic phenotype and may reflect defects in the mismatch repair system in these malignancies rather than a specific feature of CD30-positive neoplasms. Our results suggest that the CD30-MS may be a useful marker to distinguish between normal lymphoid tissue and lymphoid malignancy.

1.INTRODUCTION

The human CD30 antigen is a member of the tumour necrosis factor (TNF) receptor family and is characterised as a 120kDa type I transmembrane glycosylated protein [1,2]. Stimulation of CD30 by its corresponding ligand (CD30L) results in various pleiotropicbiological effects including proliferation, activation, differentiation and cell death[3-5]. Expression of CD30 in healthy individuals occurs only weakly on the surface of activated lymphoid cells, in particular, activated peripheral blood B and T cells and small numbers of cells in the mantles of hyperplastic lymph nodes and tonsils [6,7]. CD30 is overexpressed in malignant lymphomas such as Hodgkin lymphoma (HL) [8,9] and anaplastic large cell lymphoma (ALCL)[7], and embryonal carcinoma of the testis [10-12].

Expression of CD30 is partly regulated at the transcriptional level by the specific activity of upstream promoter elements. We and others have identified important transcriptional elements upstream of the transcriptional start-site, including Sp1, AP1 and ETS transcription factor binding sites as well as a microsatellite sequence (CD30-MS) of the type $((CCAT)_{2-12}CCACTTATGCAT)_n$ [13-15] in the promoter that binds transcription factor YY1 and acts to repress CD30 expression[16]. Further studies indicate a marked degree of polymorphism in the CD30-MS[13,15-19]. We have tested whether microsatellite deletion was correlated with CD30 expression level, using CD30-expressing cell lines derived from HL and non-Hodgkin lymphoma (NHL) and in lymph node biopsies[13,16]. Laser capture microdissections of CD30+ and CD30− cells from ALCL lymph nodes were used to assess correlations between CD30-MS lengths and CD30 expression levels in individual cells. In both cases, there did not appear to be any direct relationship between microsatellite length and CD30 expression level.

As differences in promoter activity and CD30 surface expression between CD30+ lymphoid cell-lines and peripheral blood leukocytes are independent of the size of the CD30-MS[16,17], in this study we investigate the instability within the CD30-MS in a range of benign and neoplastic tissues.

2. MATERIALS AND METHODS

2.1 Patient Material

DNA was obtained from archival material (PathWest Laboratory Medicine, Perth, Australia) of a range of normal tissues, normal peripheral blood lymphocytes from nine members of a healthy Australian Caucasian family, 12 non-Hodgkin lymphomas of various types and 4 non-haemopoietic malignances (Table 1). DNA was isolated from these tissues using a standard phenol/chloroform extraction and ethanol precipitation protocol. All samples were obtained and used under Australian National Health and Medical Research Council Human Ethics guidelines.

Table 1. CD30 Microsatellite Allele sizes in different tissue types

Category	Number of samples	Number of alleles	Size range (bp)
Non-neoplastic tissues	**9**		
Peripheral blood leukocytes	6	2	373-377
Kidney	1	2	358/373
Normal Lymph node	2	1/2	343-377
Lymphomatoid papulosis	1	2	348/373
Neoplastic tissues			
Lymphoid neoplasms	**12**		
Anaplastic large cell lymphoma*	4	7	187-377
Mediastinal large B-cell lymphoma	1	8	187-377
Small lymphocytic lymphoma	1	12	187-377
Follicular lymphoma	2	4/3	187-380
Mantle cell lymphoma	2	2/7	187-370
Diffuse large B-cell lymphoma	2	7/9	182-377
Small B-cell lymphoma, unspecified, lung	1	7	183-373
Extranodal marginal zone lymphoma (MALT lymphoma)	1	12	183-377
Non-lymphoid neoplasms	**4**		
Lung adenocarcinoma	1	3	273-374
Breast invasive ductal carcinoma	1	1	357
Colon carcinoma	1	6	187-374
Malignant mesothelioma	1	2	358/377

* Includes previously typed ALCL samples [16].

2.2 Cell Culture

Lymphoid cell lines L-540, L-428, KARPAS-299, HDMY-Z, and SU-DHL-1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The Jurkat E6-1 cell-line was obtained from the American Tissue Culture Collection (Manassas, Virginia, USA). All cell lines were cultured according to the recommended protocols. Total DNA was isolated from cell lines using the QIAamp DNA Blood Mini kit, (Qiagen Pty Ltd, Chadstone, Australia) according to the manufacturers' instructions.

2.3 CD30 Microsatellite Typing

Approximately 100 ng of DNA was used in 50 µl PCR reactions with 25 pmol each of the CD30-MS primers 30MSF (5'- ACCCATTTACCCACTCACCTGC-3') and 30MSR (5'-CAACTGGCCTAGGGAGACTGC-3')[13].PCR was performed using either the FastStartTaq DNA Polymerase kit (Roche Applied Science, Basel, Switzerland) or the Platinum Taq DNA Polymerase kit (Life Technologies Australia, Mulgrave, Australia) according to the manufacturer's instructions. The constituents for each reaction included 1x buffer, $MgCl_2$, 0.2 mMdNTPs, 1x GC-rich Solution and 2 Units DNA Polymerase. PCR conditions comprised of denaturation at 95ºC for 4 minutes; 40 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds; and a final extension at 72ºC for 7 minutes. PCR products were analysed on 2% agarose to check PCR efficiency prior to size determination. To establish accurate sizing of the CD30-MS, the PCR products were prepared for analysis on an ABI PRISM 310 Genetic Analyzer. The data were analysed using the program GeneScan Analysis 3.1.2 (Applied Biosystems Life Technologies Foster City, CA).
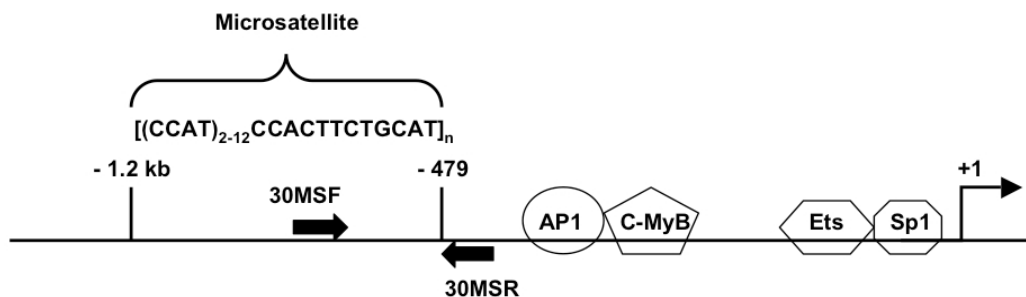

Fig. 1. Map detailing the microsatellite region of the CD30 promoter and the position of PRC primers used for amplification (30MSF and 30MSR)
The transcriptional start site, indicated by an arrow and designated +1, is that described previously[16].
Also shown are previously identified binding sites for transcription factors AP1, cMYB, ETS and Sp1.

3. RESULTS AND DISCUSSION

3.1 CD30 Promoter Microsatellite Instability in CD30-Positive and CD30-Negative Lymphoid Cell Lines

Previous studies using a DNA sequencing approach have suggested that deletions within the CD30-MS repressive element were not associated with upregulation of this gene in the lymphoid cell lines HDLM-2, KMH-2, L-428 and L-540[17]. The present study determined CD30-MS length in CD30-overexpressing HL lines L-428 and L-540, and ALCL lines SU-DHL-1, KARPAS-299. Also the CD30-negative HL line, HD-MyZ, as well as the acute T cell leukemia cell line Jurkat E6-1 (which expresses very low levels of CD30) were characterised. PCR amplification of the CD30-MS from each cell line was performed to examine 350-400 bp at the 3'-end of the microsatellite region (Fig. 1). Accurate sizing of the microsatellite region was performed and the results are summarised in Fig. 2. Instability of the microsatellite was identified in all cell types examined, as indicated by the detection of greater than the one or two expected alleles. This result is consistent with our previous results indicating instability in ALCL cell lines.
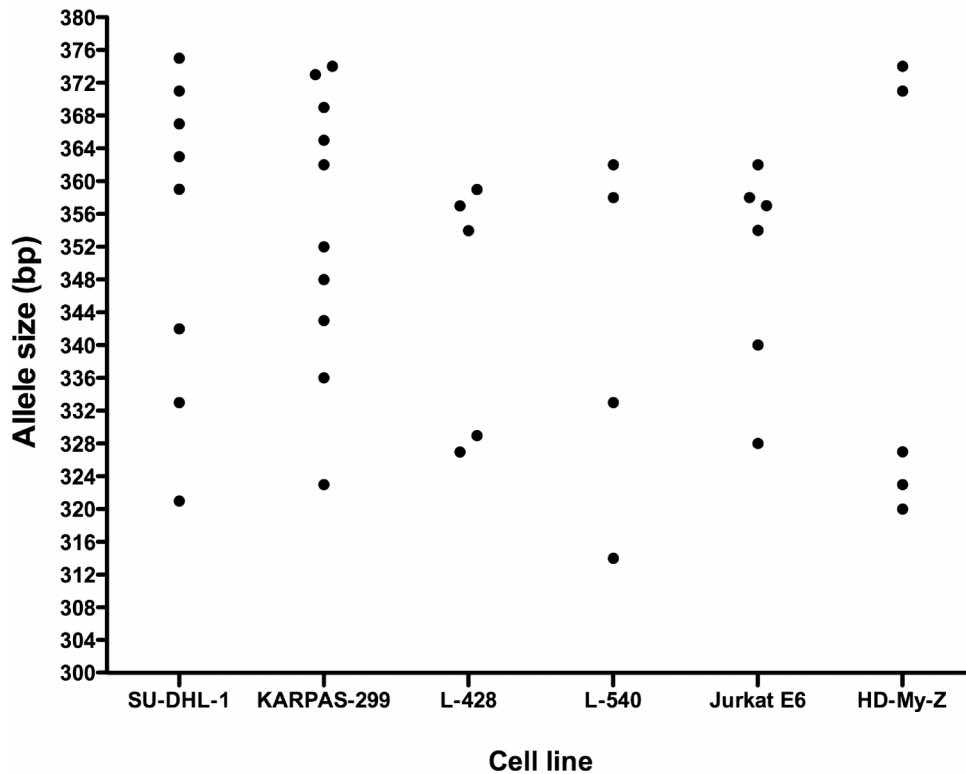
Fig. 2. Multiple CD30 microsatellite alleles in ALCL, HL and NHL cell-lines. Cell lines are as indicated. The CD30 microsatellite allele sizes are indicated in basepairs (bp)

3.2 Stability of the CD30 Microsatellite in CD30-Negative Malignant Lymphoma and Benign Lymphoid Tissues

Studies were undertaken to determine whether instability of CD30-MS was a feature of cells isolated from tumour tissue biopsies obtained from patients with a range of CD30-negative malignant lymphomas. To determine whether instability was specifically associated with the neoplastic phenotype, DNA also was analysed from tissue biopsies of normal tissue as well as from normal peripheral blood leukocytes.

The extent of CD30-MS polymorphism was determined following analysis of the peripheral blood leukocytes, benign lymphoid, and malignant lymphoma samples (Fig. 3). The results indicated that a high degree of variability exists in the length and the number of variants for all malignant lymphoma samples (F - O). These sizes range from 182 bp, as seen in diffuse large B-cell lymphoma (sample F), to 377 bp contained in follicular lymphoma (sample L).

The non-neoplastic lymphoid tissue samples (Fig. 3; samples A, B, C and D) were investigated in order to determine whether CD30-MSinstability was a characteristic of all lymphoid tissues or restricted to neoplasms. The results indicated that the individual genotypes for two benign lymphoid tissue samples and one normal peripheral blood leukocyte sample were stable (Fig. 3). Both normal peripheral blood leukocyte A and benign lymphoid tissue B are heterozygous, with two alleles at 373 bp and 377 bp for the peripheral

blood leukocyte A and two alleles at 342 bp and 377 bp for benign lymphoid tissue B. Benign lymphoid tissue C is homozygous, with allele sizes of 373 bp. The lymphomatoidpapulosis sample, also considered benign, likewise displayed a stable diploid genotype. Stable genotypes were also identified from DNA isolated from the peripheral blood of all members of an Australian Caucasoid family, in which Mendelian inheritance of the alleles was confirmed (Fig. 4). Although only small sample numbers were screened, these results suggest that although the CD30-MS sequence is very polymorphic, instability is restricted to malignant lymphoma tissues. Taken together, in 12/12 cases of NHL, instability was seen, whereas 0/9 cases of non-neoplastic tissue showed instability.
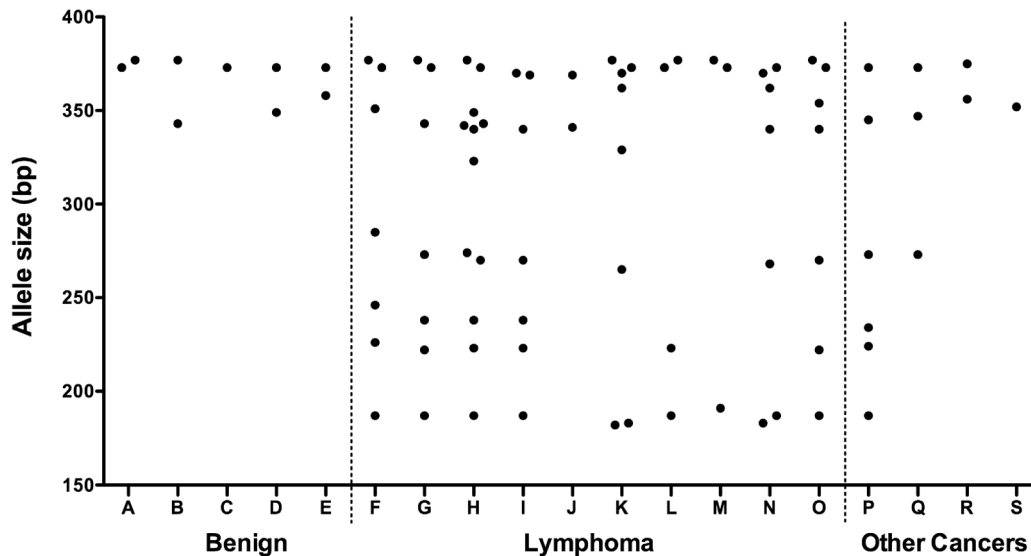

Fig. 3. Comparison of CD30 microsatellite alleles in healthy and malignant tissues of lymphoid and non-lymphoid origin
A. normal peripheral blood leukocytes; B. benign lymphoid tissue sample 1; C. benign lymphoid tissue sample 2; D. lymphomatoidpapulosis; E. normal kidney; F. diffuse large B-cell lymphoma 1; G. diffuse large B-cell lymphoma 2; H. extra-nodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT) type; I. mantle cell lymphoma 1; J. mantle cell lymphoma 2; K. mediastinal large B-cell lymphoma; L. follicular lymphoma 1; M. follicular lymphoma 2; N. pulmonary low-grade B-cell lymphoma; O. small lymphocytic lymphoma; P. colon carcinoma; Q. lung adenocarcinoma; R. malignant mesothelioma; S. ductal carcinoma of the breast.Data points within the same sample that are offset are to aid resolution.

3.3 CD30 Microsatellite Instability in Neoplasms of Non-Lymphoid Origin

Having established that CD30-MS instability is a feature of malignant lymphoid tissue, studies were undertaken to determine whether this instability was also evident in other non-haemopoietic malignancies. The neoplastic samples selected for analysis were from colon carcinoma, lung adenocarcinoma, malignant mesothelioma and invasive ductal carcinoma of the breast (Fig. 3, samples P-S). Normal kidney tissue was also included as an example of normal tissue of non-lymphoid origin (sample E). There was no repetition of any particular allele size throughout all samples and microsatellite lengths ranged from 187 bp for colon carcinoma to 377 bp for mesothelioma. The normal kidney sample had a stable, defined genotype as expected, appearing heterozygous 358/373 bp. Although only single samples

were assessed, the results showed that only the colon carcinoma and lung adenocarcinoma samples had unstable genomes. The colon carcinoma biopsy was found to have six alleles, whilst the lung adenocarcinoma had three alleles. In contrast the malignant mesothelioma and breast carcinoma samples were stable and were heterozygous (358/377 bp) and homozygous (357/357 bp) respectively. The results indicated that CD30-MS instability is not unique to neoplasms of lymphoid origin but are also present in at least colon carcinoma and lung adenocarcinoma. A comparison of CD30-MS allele number and size for all samples tested is presented in Table 1.

A number of studies have indicated that microsatellite instability may be a useful prognostic indicator. For instance, while detection of microsatellite instability in sporadic colon carcinoma can be correlated with a favorable prognosis[20], microsatellite instability in breast carcinoma has been correlated with reduced survival[21]. For most other tumors, the diagnostic value of microsatellite instability remains unknown. Screening for microsatellite instability occurring within the CD30 gene may therefore be a useful method to detect defects in DNA mismatch repair that may predispose to the development of neoplasia and hence provide additional prognostic information.
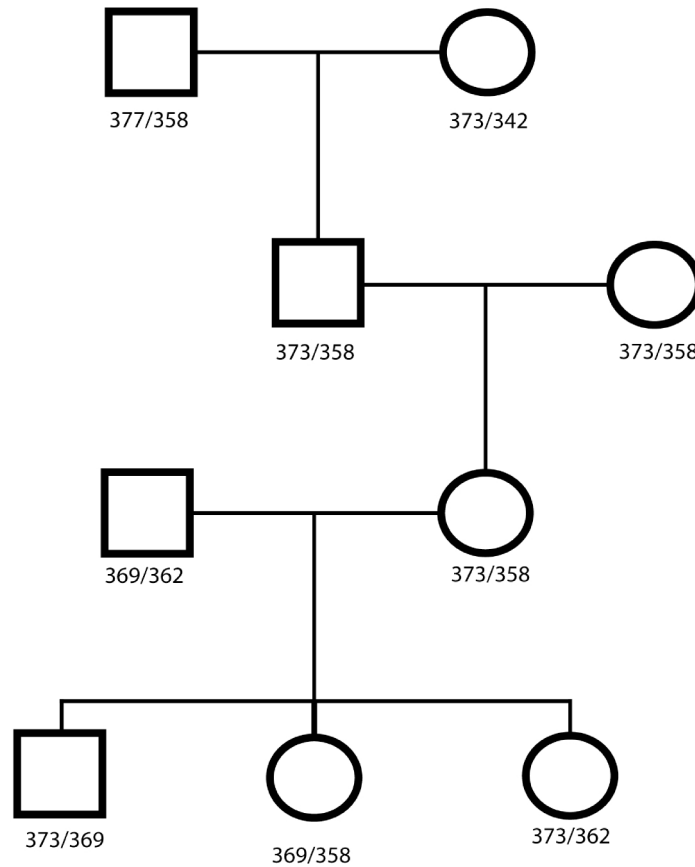

Fig. 4. CD30 microsatellite allele pedigree of a healthy Caucasian Australian family.DNA was obtained from normal peripheral blood lymphocytes

4. CONCLUSION

Our previous in vitro studies indicate that the CD30-MS acts to transcriptionally repress CD30 expression and that deletion of part or all of the CD30-MS sequence increased expression [13,17]. However, in ALCL biopsies there appears to be no correlation between CD30 expression and CD30-MS length [16]. In the current study we have confirmed that there appears to be no correlation between the level of CD30 expression and the length of the CD30-MS. All of the CD30-positve HL and ALCL cell lines tested display truncated CD30-MS alleles and yet express CD30 at extremely high levels. Conversely, the Jurkat cell line expresses a low level of CD30 and yet has a CD30-MS size profile similar to the ALCL lines.

Our results suggest that the CD30-MS is hypermutable. Microsatellite instability is a phenotype caused by the loss of DNA mismatch repair activity and forms an alternative pathway in cancer development [22]. The instability of multiple microsatellite-repeat sequences in cancer has been associated with defects in the DNA mismatch repair enzymes MSH2 and MLH1 [23-26]. Microsatellite instability has been documented in many lymphoid and non-lymphoid malignancies. For instance microsatellite instability can be detected in about 15% of all colorectal cancers including hereditary nonpolyposis colon carcinoma and sporadic carcinomas of the colon [22,27]. Other malignancies displaying microsatellite instability include cancers of the stomach, pancreas [28], esophagus [29] and endometrium [30] as well as a number of NHL subtypes[31].

Microsatellite instability has been examined recently for a number of NHL subtypes [31] indicating that microsatellite instability is present, but at low frequency for any particular locus. For instance in assaying DLBCL cases using 5 microsatellite loci, only 3 cases out of 25 displayed instability at one of the 5 loci. Similar low levels of instability were seen in ALCL (1 out of 4 cases) and FL (1 out of 17 cases) [31]. Although we have assessed few cases, overall our results suggest that the CD30-MS may be extremely unstable in NHL as we see multiple alleles in all cases studied, except for one of two mantle cell lymphomas (J). In support of the notion that the CD30-MS shows an unusual degree of instability, similar hypermutability was observed for 2 of the non-lymphoid malignant neoplasms studied. Although further work is required, our results suggest that in view of the high degree of instability seen in a range of tumour types, the CD30-MS region could be a useful marker to distinguish neoplastic cells from their normal counterparts.

CONSENT

Patient consent was not required for this study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the UWA Human Ethics Committee and have therefore been performed in accordance with the ethical standards laid down by the Australian Government National Health and Medical Research Council, a signatory of the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors declare that no competing interests exist.

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