Chronic Lymphoproliferative Disorders

The relevance of sequence insertions in the Mcl-1 promoter in chronic lymphocytic leukemia and in normal cells

We measured the frequency of insertions in the Mcl-1 promoter in chronic lymphocytic leukemia (CLL) and in normal individuals. Insertions were found in 37/54 (69%) of the CLL samples. However, insertions were not associated with prognostic markers and were also detected in 38/66 (58%) of normal controls and in normal cells isolated from CLL patients. Thus, Mcl-1 insertions are not acquired during leukemogenesis and are unlikely to play an important role in this disease.

haematologica 2005; 90:1285-1286
(*http://www.haematologica.org/journal/2005/9/1285.html*)

Mcl-1 is an anti-apoptotic Bcl-2 family protein, implicated in inappropriate cell survival and poor response to therapy in chronic lymphocytic leukemia (CLL).[1,2] Moshynska *et al.*[3] recently described short insertions (6 and/or 18 base pairs) in the Mcl-1 promoter in 29% (17/58) of CLL samples. Insertions were associated with increased Mcl-1 RNA and protein expression, poor response to therapy and overall survival. Insertions were not detected in healthy controls or in normal tissue from CLL patients suggesting that insertions were acquired during leukemogenesis and contributed to resistance to apoptosis. It was important, therefore, to investigate Mcl-1 promoter insertions in other cohorts of CLL patients and normal individuals.

Following ethical approval, we isolated genomic DNA from peripheral mononuclear cells of 54 CLL patients (Table 1). We developed a fluorescent fragment (GeneScan) assay to detect Mcl-1 promoter insertions. Part of the Mcl-1 promoter was amplified by polymerase chain reaction (PCR) using primers 5'-AAAAACCTCTGGCGAAAACCA-3' and 5'-FAM-TTATCAGGGTTTTAGGGCGG-3' and the size of the products was determined using an ABI 3100 Genetic analyzer and GeneScan software 3.7 (Applied Biosystems) (Figure 1A). We detected promoter insertions in 37/54 (69%) CLL samples (Table 1). Additional assays were performed on 6 samples using a second set of Mcl-1-specific primers and gave equivalent results. Sequencing of cloned PCR products derived from patients 1 and 21 demonstrated that the position and sequence of the insertions were identical to those described previously.[3] The presence of insertions (all cases with insertions considered as a single group) was not statistically significantly associated with key prognostic markers for CLL,[4] CD38 expression, V_H status or Zap70 expression (Pearson's χ^2 analysis, all $p>0.05$). We were unable to study the relationship with survival, disease progression or response to chemotherapy because of the short follow-up available for our group of patients.

We investigated whether Mcl-1 insertions were also present in the DNA from normal individuals. Following ethical approval, blood was obtained from two groups of individuals; 20 elderly women who had provided a routine blood sample at a hospital out-patient's clinic and 46 healthy adults of all ages. Six and 18-nucleotide insertions were detected in 38/66 (58%) of normal individuals. To determine whether the insertions detected in CLL cells were also present in normal cells of CLL patients, we used immunomagnetic isolation (Miltenyi Biotec) to separate the normal T cells and malignant B cells from two individuals with insertions (patients 16 and 40). The resulting B-cell preparations contained ~99% B cells,

Table 1. Mcl-1 promoter genotype in CLL patients.

Pt[a]	Sex[b]	Age	Stage[c]	Mcl-1 genotype[d]	V_H status[e]	V_H gene[f]	CD38 (%)[g]	Zap70 (%)[h]
1	M	51	nk[i]	0/18	U	1-69	57	56
2	F	72	B	0/18	U	3-09	76	nd
3	M	nk	B	0/18	U	1-69	49	16
4	M	56	A	0/6	U	1-69	25	88
5	F	50	A0	0/6	U	1-08	3	13
6	M	76	A	0/0	U	2-05	38	84
7	M	66	C	0/18	U	3-66	80	50
8	F	68	A	0/0	U	2-70	4	1
9	M	57	A0	0/0	U	5-51	3	27
10	F	67	A	0/0	U	2-05	1	85
11	M	60	B	0/6	U	7-04.1	33	64
12	M	87	B	0/6	U	4-34	61	40
13	M	64	A0	0/18	U	1-18	38	36
14	M	48	nk	0/6	U	1-69	45	33
15	M	59	A0	0/0	U	7-04.1	42	90
16	M	47	A	6/18	U	1-69	3	35
17	M	77	A	0/0	U	1-69	100	24
18	F	73	A	0/6	U	4-34	2	5
19	M	64	nk	6/18	U	3-48	60	40
20	F	81	A0	0/6	M	4-34	3	14
21	M	70	A	0/6	M	1-69	5	12
22	F	73	A	0/0	M	4-34	3	1
23	F	83	A	0/18	M	3-21	19	28
24	F	84	A	0/0	M	4-34	1	3
25	M	60	A	0/0	M	3-15	3	22
26	M	81	A	0/0	M	6-01	5	42
27	F	nk	A0	0/6	M[j]	1-02/6-01	4	8
28	F	70	A	6/18	M	3-23	4	2
29	M	88	A	0/0	M	3-74	26	14
30	M	80	A	0/0	M	3-21	85	0
31	M	75	A	0/6	M	1-02	2	1
32	F	73	A	0/18	M	3-21	4	6
33	M	60	nk	6/18	M	4-34	1	3
34	F	66	nk	0/0	M	4-59	5	2
35	M	74	A	0/0	M	4-34	3	4
36	F	81	A	0/18	M	4-34	1	23
37	M	55	A	6/6	M	5-51	0	28
38	M	65	A0	0/18	M	3-23	2	14
39	M	67	A	0/18	M	2-05	0	7
40	F	63	nk	0/6	M	4-34	1	11
41	F	76	A	6/18	M	3-72	1	nd
42	M	74	A	0/6	M	3-15	0	0
43	F	80	C	0/0	M	4-61	0	39
44	M	58	C	6/6	M	4-30.4	10	7
45	M	71	A	0/6	M	1-69	3	2
46	M	83	A	0/18	M	3-30.3	1	5
47	M	60	A	0/0	M	3-23	7	0
48	F	88	A0	0/18	M	3-23	66	10
49	F	80	A	0/6	M	4b	5	3
50	F	76	A0	0/0	M	315	97	4
51	M	82	A	0/6	M	3-23	1	1
52	F	84	A	0/18	nd[k]	–	2	2
53	M	64	nk	18/18	M (IgG)	3-23	94	4
54	M	55	nk	0/18	M (IgG)	5-51	26	0

[a]54 CLL samples were studied, obtained between 04/02/03 and 18/02/05. All patients were untreated and expressed IgM, except patients 53 and 54 who were IgG positive. [b]M: male; F: female; [c]disease stage is according to the Binet scale; [d]Mcl-1 genotype (0, wildtype; 6, 6-base insertion; 18, 18-base insertion); [e]V_H mutation status, greater than 2% deviation from germline V_H sequence was considered as mutated; U: unmutated; M: mutated; [f]V_H gene expression; [g]CD38 (>30% considered positive); [h]Zap70 expression (>20% considered positive); [i]nk: not known; [j]patient 27 showed two populations of cells, both with mutated V_H genes but different gene segments; [k]nd: not determined.

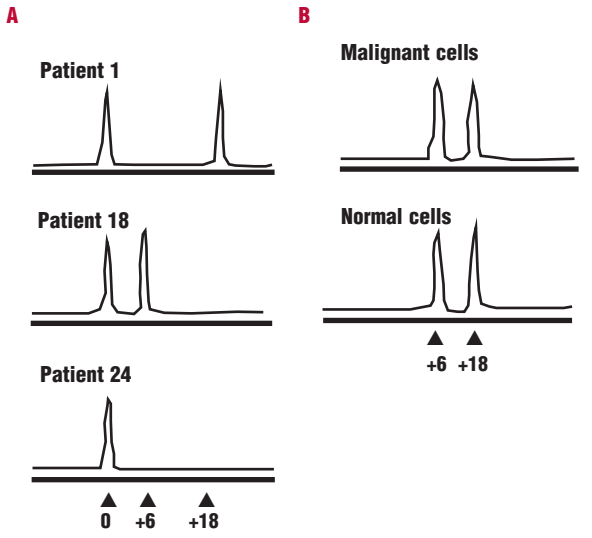

Figure 1. Mcl-1 promoter insertions. Mcl-1 promoter insertions were detected using a GeneScan assay. A. Representative results obtained with DNA from 3 CLL patients. B. Analysis of Mcl-1 insertions in B- and T-cells isolated from patient 16. The position of wild type (0) and +6 and +18 PCR products are indicated.

whereas the normal cell fractions contained 17% or 36% contaminating B cells, respectively. Mixing experiments demonstrated that the fragment assay could quantitatively detect the various alleles, and the detection of identical insertions in the normal and malignant cells therefore demonstrated that insertions are not restricted to malignant cells (Figure 1B).

In conclusion, we have confirmed the presence of insertions in the Mcl-1 promoter in CLL. However, these insertions are not associated with prognostic markers and are also present in unaffected individuals. Similar findings were presented by Tobin *et al.*[5] while this manuscript was under preparation. However, we have additionally shown that identical insertions are present in the normal cells of CLL patients clearly demonstrating that they are not CLL specific. It is not clear why Moshynska *et al.* failed to detect insertions in non-malignant cells derived from two CLL patients in their study.[3] However, they used direct sequencing which may not be as reliable as GeneScan analysis for allele discrimination in heterozygotes. This may also explain why the frequency of CLL patients with insertions was significantly lower in their study (29%) than in ours (69%) or that by Tobin *et al.* (61%), who also used GeneScan analysis. Consistent with this, almost 50% of the insertions detected by Moshynska *et al.* were homozygous, compared to ~10% in our study and the study by Tobin *et al.*, suggesting technical difficulties in identifying patients with heterozygous insertions.

Taken together, these findings demonstrate that Mcl-1 insertions are polymorphic variants, present in the normal population, and are not acquired during leukemogenesis. It remains possible that insertions are associated with elevated Mcl-1 promoter activity and suppression of apoptosis, and may therefore contribute to the development of CLL. However, the lack of association with prognostic markers and the similar frequency of insertions in normal individuals and CLL patients (64% and 63%, respectively, when our results are combined with those of Tobin *et al.*) do not support this hypothesis. It is possible that environmentally derived signals, rather than genetic variations, may be a major determinant of Mcl-1 expression in CLL.[6]

Silvia Coenen, Becky Pickering,* Kathleen N. Potter,° Peter W.M. Johnson,* Freda K. Stevenson,° Graham Packham**

**Cancer Research UK Clinical Centre and °Molecular Immunology Group, Cancer Sciences Division, University of Southampton School of Medicine, Southampton General Hospital, UK*

Acknowledgments: we thank Christian Ottensmeier, C. Ian Mockridge, Myra Armstrong, Andrew Duncombe, Debbie Richardson, Terry Hamblin and Helen McCarthy for assistance. We thank Anna Murray and Andrew Sharp (Salisbury District Hospital) for providing access to normal DNA samples and Sue Harris for performing the GeneScan analysis.

Funding: this work was supported by Cancer Research UK and the Leukaemia Research Fund.

Key words: chronic lymphocytic leukemia, apoptosis, Mcl-1, promoter, mutation.

Correspondence: Graham Packham, Cancer Research UK Clinical Centre, Somers Cancer Research Building (MP824), Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK. Phone: international +44.023.80796184. Fax: international +44.023.8079 5152. E-mail: G.K.Packham@soton.ac.uk

References

1. Michels J, Johnson PW, Packham G. Mcl-1. Int J Biochem Cell Biol 2005;37:267-71.
2. Packham G, Stevenson FK. Bodyguards and assassins: Bcl-2 family proteins and apoptosis control in chronic lymphocytic leukaemia. Immunology 2005;114:441-9.
3. Moshynska O, Sankaran K, Pahwa P, Saxena A. Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia. J Natl Cancer Inst 2004; 96:673-82.
4. Shanafelt TD, Geyer SM, Kay NE. Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL. Blood 2004;103:1202-10.
5. Tobin G, Skogsberg A, Thunberg U, Laurell A, Aleskog A, Merup M, et al. Mcl-1 gene promoter insertions do not correlate with disease outcome, stage or V(H) gene mutation status in chronic lymphocytic leukaemia. Leukemia 2005;Epub ahead of print].
6. Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood 2002; 100:1795-801.