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BIO-TECHNICAL METHODS SECTION (BTS)

Fluorescent BAT-25 and BAT-26 analysis of T cell prolymphocytic leukaemia

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T cell prolymphocytic leukaemia (T-PLL) is a chronic mature T cell malignancy with many random cytogenetic abnormalities. These imply that maintenance of genomic integrity is impaired. This is supported by the recent finding that the ataxia telangiectasia gene, *ATM*, which contributes to maintaining genomic integrity, is frequently mutated in this disease. To evaluate in T-PLL the role of other genes with comparable function, a fluorescence-based semi-automated assay was developed for BAT-25 and BAT-26. These markers contain sequences that are particularly unstable in cells with DNA mismatch repair defects. Application of the assay to 20 T-PLL cases found no evidence for such defects.
Keywords: T cell prolymphocytic leukaemia; microsatellite instability; BAT-25; BAT-26

Introduction

Maintenance of genome integrity is commonly subverted during tumorigenesis and can be detected, for example, when mutation of the *hMSH2* mismatch repair gene[1] leads to the phenotype of microsatellite instability (this phenotype is by convention written as MIN+). Mismatch repair defects are also associated with cell cycle checkpoint defects.[2] However, simultaneous with mismatch repair gene defects, other genes that contribute both to genome integrity and to cell cycle control are also mutant in some tumors. For example, in acute myeloid leukaemia, *p53* mutations have been reported in a third of cases that do not express *hMSH2* protein.[3]

T cell prolymphocytic leukemia (T-PLL) is a chronic mature T cell malignancy whose karyotype displays not only certain recurrent abnormalities but also large numbers of apparently random abnormalities. These random abnormalities imply that maintenance of genome integrity is frequently subverted in T-PLL. This subversion is probably due, at least in part, to mutation of the ataxia telangiectasia gene, *ATM*, a gene that contributes to maintenance of genome integrity and acts at cell cycle checkpoints.[4] In T-PLL, homozygous missense mutations cluster at the 3′ end of *ATM* and rearrangements of one or both alleles are frequent.[5,6]

We have sought to evaluate the role of other genes that might contribute to impaired maintenance of genomic integrity in T-PLL: we detected no sequence changes in *p53*.[5] To evaluate the role of DNA mismatch repair genes, we have now examined poly(A)$_n$ tracts BAT-25 and BAT-26. The MIN + phenotype is very strongly associated with shortened tracts due to deletion of some bases while constitutional DNA is generally quasi-monomorphic.[7]

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Methods and results

We developed and exploited a rapid non-radioactive semi-automated assay for BAT-25 and BAT-26 using primer sequences that have been described.[8] The BAT-25 forward primer was synthesized with its 5′ nucleotide bearing a HEX moiety and with the BAT-26 forward primer 5′ nucleotide bearing a FAM moiety (PE Applied Biosystems, Warrington, UK). After PCR amplification, products were mixed with TAMRA fluorescent size marker (Applied Biosystems), denatured and fractionated in 4.5% 29:1 acrylamide: bisacrylamide 8 M urea on a 36-Well-to-Read plate in an ABI 377 Automated Fluorescent DNA Sequencer (Applied Biosystems). Fluorescence was detected using filter set C. Data was collected and stored using the GeneScan Collection Software 2.0, quantitated by GeneScan Analysis Software and analysed and interpolated with Genotyper Software v.1.1.1 (Applied Biosystems).

Samples taken at diagnosis prior to treatment from 20 T-PLL cases seen at or referred to the Royal Marsden Hospital NHS Trust were tested along with 24 normal healthy controls and the MIN+ cell lines JURKAT and HCT116. Each PCR gave rise to a Genescan trace containing a set of peaks. Each peak is presumed to correspond to a dye-labelled single-stranded DNA molecule if it was identified in two assays at the same position. The position of a peak indicated the length of the corresponding DNA molecule as interpolated by Genescan software. The number of peaks in a set was the spread value. The height of a peak measured the amount of PCR product present in units related to fluorescence. Thus the highest peak of a set identified the most abundant species.

In all T-PLL and normal samples, the main BAT-26 peak ranged between 119 and 121 bases with a spread value of 5 to 8. This contrasted with the MIN+ controls JURKAT (main peak: 109 bases; spread value: 10 bases) and HCT116 (main peak: 108 bases; spread value: 4 bases) (Figure 1 and Tables 1 and 2). In T-PLL and normal samples, the main BAT-25 peak ranged between 124 and 127 bases with a spread value of 4 to 8. One T-PLL sample had a main peak at 121 bases with a spread value of 4. JURKAT and HCT116 had main peaks of 119 bases and a spread values of 6 bases (Tables 1 and 2). Thus both BAT-26 and BAT-25 identified the wild type MIN-

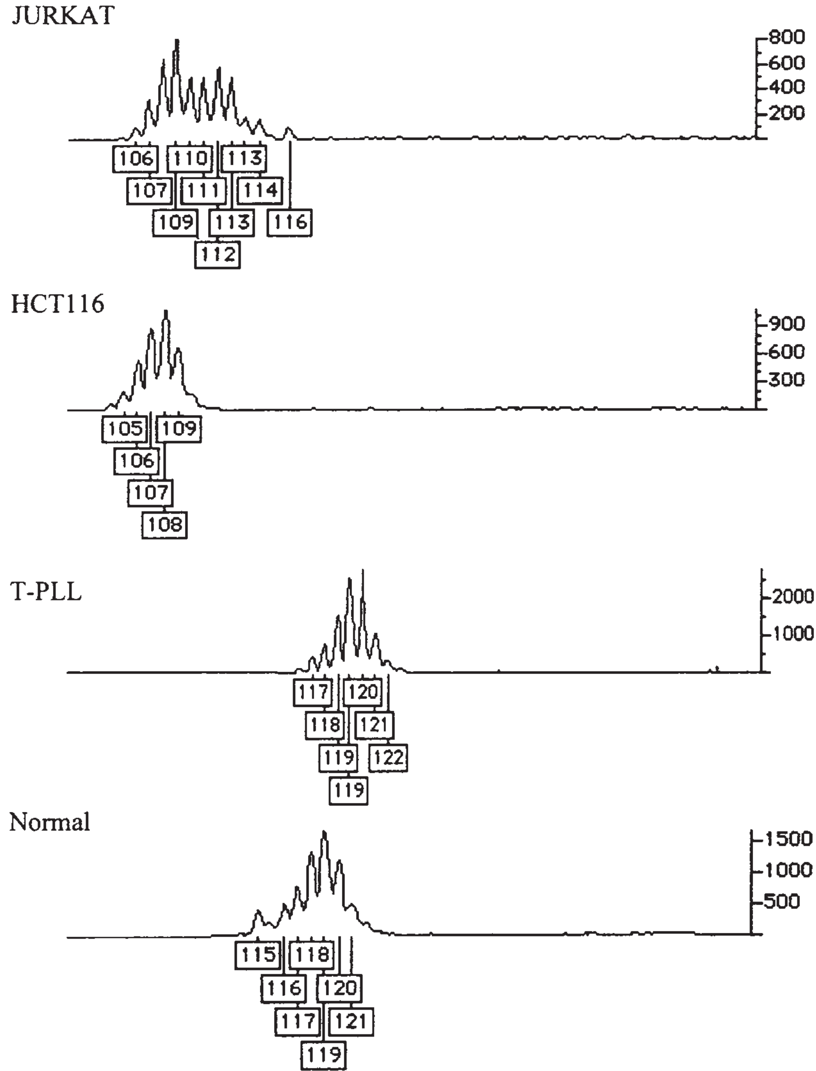

Figure 1 Genescan traces for BAT-26. The relative fluorescence and absolute length are shown of BAT-26 amplification products from the DNAs indicated. Boxed numerals are the lengths in bases of peaks indicated by a vertical line from each box. The right scale is in arbitrary fluorescence units.

Table 1 Spread of BAT-26 and BAT-25 product lengths

Spread value	*20 T-PLL*	*24 Normals*
BAT-26		
5	2	6
6	4	8
7	12	8
8	2	2
BAT-25		
4	3	3
5	12	10
6	4	7
7	1	3
8	0	1

Table 2 Length of BAT-26 and BAT-25 products

Main peak length	*20 T-PLL*	*24 Normals*
BAT-26		
119	1	0
120	4	8
121	15	16
BAT-25		
124	3	1
125	6	7
126	9	13
127	1	3

phenotype in all T-PLL cases. This corresponds to 95% confidence that more than 87% of T-PLL cases are MIN–. Efficiency of detection of MIN+ status by BAT-26 is 99.5%.[9]

Discussion

Whereas previous methods of detection of BAT alleles have relied on Southern blotting and thus have identified both strands of each DNA molecule and may lack precision in determining the absolute length and the spread value for a sample, our modification of the technique uses a single dye-labelled primer. In conjunction with the ABI 377 sequencer and software, this permits specification of the size and quantity of each peak. Each peak corresponds to one strand (the 'forward' strand) of one of the products of amplification. The set of peaks then identifies all these products by size to the nearest base. This precision is of value to the study of poly$(A)_n$ tract variability.

More than two peaks were observed in all cases. Hence a single allele gave rise to >1 product. These extra products may arise from heterogeneity in the DNA sample, from non-templated base addition during PCR and from 'slippage' due to an absence of proof-reading. The fact that both BAT-26 and BAT-25 show multiple peaks points to this heterogeneity being a property of the poly$(A)_n$ tract, rather than of some other sequence in the amplicon. Use of the proofreading *Pfu* DNA polymerase reduced, but did not eliminate, spread (data not shown). Hence slippage within the poly$(A)_n$ tract is one of the sources of multiple peaks. *Pfu* DNA polymerase was not employed regularly since it gave poor amplification.

HCT116 and JURKAT had spread values for BAT-26 of 4 and 11, respectively. HCT116 has two copies of the gene (*hMSH2*) at chromosome 2p22 in which lies BAT-26 (personal communication, B Vogelstein, Baltimore, MD, USA). Cytogenetic analysis showed that up to three copies of 2p22 were present in the hypotetraploid line JURKAT (data not shown). This suggests that variation in spread values arises from differences in the value of n in the BAT-26 poly$(A)_n$ tract in different DNAs although experimental factors may also contribute to variable spread.

Since submission, a similar fluorescent technique has been described.[10]

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