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Progress paper

Using robots to find needles

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The somatic mutation theory of ageing posits that one of the contributory factors in the ageing process is the accumulation of mutations in the soma (see Kirkwood, 1988; Morley, 1998; Vijg, 2000). An elegant proposition indeed, but one for which it has proven problematic to provide comprehensive data from across the genome to show that somatic mutations do indeed accumulate with age. The trouble is that specific mutations at a given locus, even in a 'disposable soma' (Kirkwood, 1987), are rare and investigators are left struggling to find the proverbial needle in a haystack. Approaches to measuring mutations that have arisen in vivo have therefore been restricted to measuring mutations that may be efficiently screened for in vitro. This has been largely limited to the detection of chromosomal abnormalities that can be easily visualised, the analysis of mitochondrial DNA mutations that occur at high frequency and can be assayed using PCR, telomere shortening and the detection of mutations for which cell selection techniques can be applied (see Vijg, 2000). The quantification of age-dependent changes in chromosomal abnormalities, mitochondrial mutations and telomere shortening has proved useful, but it is unclear how these mutations relate to more subtle mutations in nuclear genes. The problem of measuring somatic mutation frequencies in nuclear genes has been, at least partially, addressed by measuring mutations at the X-linked *HPRT* locus using the T-lymphocyte cloning assay (Hou et al., 1999). By culturing T-lymphocytes from peripheral blood samples it is possible to select for rare cells that have acquired mutations in their *HPRT* gene and become resistant to 6-thioguanine. *HPRT* mutation frequencies measured thus increase with age, but even in old individuals are still very low, occurring in approximately 1 in 10^5 cells (Jones et al., 1995). The T-cell cloning assay is also technically demanding and very labour intensive, requires fresh peripheral blood samples and is prone to significant inter-laboratory variation (Hou et al., 1999). Moreover, measuring *HPRT* mutations is most practically performed using T-cells and is complicated by deletion mutations that may arise as a by-product of V(D)J recombination processes in these cells. By its very nature, measuring mutation frequencies at the *HPRT* gene is limited to a single X-linked locus that may itself be under in vivo selection and may not therefore be representative of the genome. This assay is nonetheless one of the gold standard methodologies in ageing research, and has been used to quantify the negative effects of environmental exposure, such as smoking (Vrieling et al., 1992), and germline mutations in DNA repair genes (e.g. in xeroderma pigmentosum patients (Cole et al., 1992)). Whilst there are a few other endogenous genes to which a similar cell selection paradigm can be applied (e.g. the *HLA-A* (Morley et al., 1990) and *glycophorin A* (Langlois et al., 1986) loci), such a strategy cannot be used for the vast majority of genes. In mice, the use of transgenic reporters such as lacZ/lacI has also proven very useful, revealing age and tissue-specific mutation frequencies from 1 in 10^5 up to 1 in 10^4 in old animals (Dolle et al., 2000; Stuart et al., 2000). Obviously though, genetic modification cannot be used to measure in vivo mutation frequencies in humans.

Thus, finding one needle is hard enough, and quantifying the number of needles per haystack and how it differs between individual haystacks has proven harder still. Alternative approaches though face the same problem; how to quantify low mutation frequencies at a particular locus? One potential solution is to identify loci with high basal mutation frequencies for which it may be technically simpler to quantify mutations and hence the role of age/genetic/environmental modifiers may be more easily assessed. One class of loci for which this has already proven, at least partially, feasible are tandem repeats such as microsatellites. Microsatellites are short tandem repeats typically comprised of monomers 1–6 bp in length, and arrays of 5–30 repeats (Tautz et al., 1986; Litt and Luty, 1989; Weber, 1990). Germline length change mutation rates are much higher

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than the rate of point mutations, in the order of 1 in 10^3 to 1 in 10^4 per generation (Weber and Wong, 1993) compared to base substitution rates of 1 in 10^8 per base per generation (Giannelli et al., 1999). These high germline mutation rates lead to high levels of variability in the general population and microsatellite typing now forms the basis of the DNA profiling technologies used in individual identification and criminal forensics (Gill, 2002). Their high levels of informativeness and their location scattered throughout the genome have also made microsatellites of extreme value as markers in genetic linkage analyses (Dib et al., 1996).

The utility of microsatellites as reporters of genomic instability has been well demonstrated in the field of cancer biology. It is now established that the mutation frequency of microsatellites in many tumours may be so high that mutant fragments may be simply detected in the PCR amplification products of a bulk sample of tumour DNA, a phenomenon referred to as microsatellite instability (MSI) (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993) (see Fig. 1). The MSI phenotype is most common in the tumours of individuals with the cancer predisposition syndrome hereditary non-polyposis colon cancer (HNPCC) (Peltomaki et al., 1993). These individuals inherit germline mutations in a single allele of one of the major DNA mismatch repair genes (Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Nicolaides et al., 1994; Papadopoulos et al., 1994). Rare cells in these individuals can acquire a 'second hit' in the remaining wild-type allele that leads to mismatch repair deficiency within the cell, and a mutator phenotype that can lead to the accumulation of the multiple mutations necessary to initiate cancer (Loeb, 1994). Microsatellite length change mutations are assumed to arise by a process of DNA replication slippage whereby polymerase stalling within the repeat tract can lead to misalignment of the free 3′-end and the incorporation of either more or less repeats on the daughter strand than present on the template strand (Schlotterer and Tautz, 1992). Normally, such replication errors would be corrected by the DNA mismatch repair pathway. However, in the absence of DNA mismatch repair, such mutations can accumulate to very high frequency, as in HNPCC tumours.

MSI in tumours is typically assessed by comparing the fragment length profiles of microsatellites amplified from bulk DNA samples of tumour and normal tissue from the same individual (Boland et al., 1998). PCR amplifications are performed with 10-100 ng input DNA, equivalent to more than 1000 cells worth of DNA. Amplification products are resolved using denaturing polyacrylamide gel electrophoresis and detected using either radioactively, or fluorescently, labeled PCR primers. Unfortunately, *Taq* polymerase slippage that occurs during PCR results in a set of characteristic PCR stutter

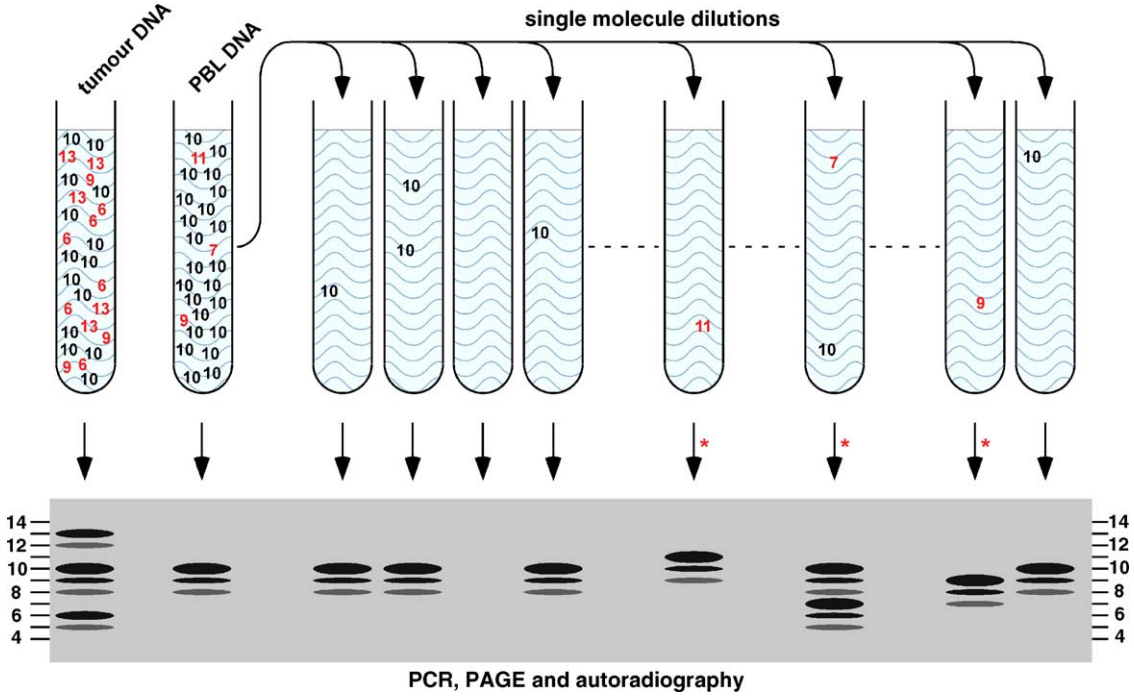

Fig. 1. Detecting microsatellite instability using small pool PCR. DNA isolated from a mismatch repair deficient tumour may contain a high frequency of mutant microsatellite alleles (6, 9 and 13 repeats, in red), different in length from the progenitor allele (10 repeats, in black). Following PCR with a radioactively labelled primer, denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography, the tumour sample presents a complex banding pattern composed of amplification products from the progenitor allele, the mutant alleles and PCR stutter derivatives. This is clearly distinguishable from the normal peripheral blood leukocyte (PBL) DNA from the same individual in which the frequency of mutant alleles is very low. Using standard PCR procedures, the low frequency mutants present in the PBL DNA are not detected and only the progenitor allele and its PCR stutter derivates are observed. If the DNA is diluted to the single molecule level before PCR amplification, most reactions will contain zero, one, or occasionally two or more copies of the progenitor allele. However, a few reactions will contain rare mutant alleles (red asterisk), which can be easily identified following autoradiography. The frequency of reactions in which mutant molecules are observed can be used to calculate absolute mutation frequencies. Although the format of the output data is different, qualitatively similar fragment length profiles are generated using fluorescently labelled PCR primers and automated DNA sequencing type apparatus.