Gut

Leading article

Microsatellite instability

See paper by Ponz de Leon et al, p32

Biology

GENETIC INSTABILITY IN TUMOURS

Genetic perturbation has been implicated in the development of tumours since the turn of the century. Indeed, genetic instability of one sort or another may be considered to be a hallmark of cancer itself, and the discovery of microsatellite instability (MSI) made it evident that there was more than one mechanism underlying this process. [1 2] As with most new discoveries, there was an initial flurry of excitement with raised hopes and exaggerated claims, followed by the realisation that MSI was not as simple, easy, or likely to give results as had first been thought. The understanding of MSI and its potential clinical utility has continued to develop, but it is only now that its real usefulness is becoming apparent.

MICROSATELLITE INSTABILITY: DID IT FALL OR WAS IT PUSHED?

DNA synthesis is liable to errors, either as part of DNA replication before cell division, or as part of DNA repair (unscheduled DNA synthesis). All mutations result from permanent changes in covalent bonds and are never spontaneous, although hydrolysis and oxidation inter alia may masquerade as spontaneous agents. When somatic cells divide it is important to keep genetic variation to a minimum (except in special cases—for example, antibody diversity in B lymphocytes) as these cells may become malignant through the accrual of mutations in cancer related genes. DNA repair is a major mechanism by which cells diminish the action and effects of DNA damaging agents in causing mutations. It is not perfect and the resulting genetic variation between cells causes tumours to evolve, which are consequent upon selection pressures within the host. However, a rise in mutation rate may not be a selective advantage to a tumour cell.[3 4]

DNA mismatch repair (MMR) is the form of DNA repair responsible for the correction of mismatched and/or unmatched bases, as reviewed by Jiricny.[5] Mismatched or unmatched bases can be secondary to DNA damage, but DNA replication seems to be prone to a process known as slippage, where one DNA strand rides up over the other, in a manner reminiscent of a zip fastener that becomes snagged because its links are not taken alternately from left and right. This slippage is more likely to happen in regions of repetitive sequence and is thought to occur because DNA polymerases are less able to process through these regions. In prokaryotes, repetitive sequences of any length are rare and may have been selected out for this reason. However, it is strange why four billion years of evolution have not resulted in a DNA polymerase that is not prone to slippage, though DNA structure may have imposed evolutionary constraints.[6] It is not clear to what extent slippage is because of an inherent limitation of DNA polymerases or is due, at least in part, to either more prevalent DNA damage, or less active repair, in repetitive sequences. However, it has been known for some time that DNA damage and repair do not occur uniformly along a stretch of DNA, even between strands.[7]

MUTATIONS IN MICROSATELLITES

In higher organisms there are long stretches of apparently redundant DNA between genes, within which repetitive sequences (termed microsatellites) may be found. However, microsatellites or repetitive sequences can also be found in the protein encoding regions of many eukaryotic genes. Repetition in a microsatellite may be based on a single base $(A)_n$ (also known as polyA), two bases $(CA)_n$, or three or more bases. Two or more repeats qualify as a repetitive sequence—for example, CACA. Microsatellites may be either monomorphic and show no variation in size between cells or individuals, or polymorphic (a variety of sizes). Very occasionally, perhaps once in ten thousand times, the length of a microsatellite can be seen to change when it is passed on from one individual to another—for example, $(CA)_7$ becomes $(CA)_8$ in an offspring. Classically, this has been ascribed to slippage in DNA replication, presumably at meiosis. When this alteration in microsatellite size was found somatically in tumour cells, compared with normal tissues, it was termed replication error (RER).[1 2 8] However, it was not a simple pattern where one microsatellite had changed size (although this is sometimes observed, and is properly called microsatellite alteration); multiple microsatellites were seen to be affected, and many different extra alleles (sizes) were found at any one microsatellite.[1 2] Thus, this processs has been termed microsatellite instability (MSI).

Confusingly, MSI is sometimes abbreviated to MI or MIN; the latter is also the abbreviation for a mouse strain with the murine equivalent of familial adenomatous polyposis. The converse of MSI has been called chromosomal instability (CIN), or preferably, microsatellite

Abbreviations used in this paper: C, cytosine; HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; LOH, allelic loss; *O*6meG, O6-methylguanine, MGMT, *O*6meG-methyltransferase; MSI, microsatellite instability; MSS, microsatellite stability; MMR, mismatch repair; PPV, positive predictive value; RER, replication error; T, thymine.

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stability (MSS). Tumours with MSI have also been described as having a mutator phenotype, because in prokaryotes particular mutator phenotypes are seen in association with the loss of particular DNA repair genes. However, this is another confusing term because a mutator phenotype (for instance, aneuploidy or MSI) can be found in all cancers. It is ironic that a multiplicity of terms for this one process has developed in a fashion reminiscent of the process itself. Nevertheless, it is important to realise that point mutations occur all over the genome in all cancers, but more so in tumours which have lost MMR, and within these, usually in repetitive sequences. Thus, it is expected that there will be a different spectrum of mutations, even mutated genes, in MSI positive tumours compared with MSI negative ones.[4 5 9]

MICROSATELLITE INSTABILITY AS A MECHANISM IN CARCINOGENESIS

The importance of MSI as a mechanism in human carcinogenesis first became apparent with the study of a rare dominant cancer predisposition syndrome (hereditary non-polyposis colorectal cancer (HNPCC)).[1 2] Allelic loss (LOH) in tumours, and dominant cancer syndromes, usually indicates the action of tumour suppressor genes. Thus, when linkage studies narrowed down the location of an HNPCC gene to a region of chromosome 2, it was to be expected that LOH of the same region would be seen in HNPCC tumours. However, when LOH of candidate regions was sought by a study of polymorphic microsatellites in HNPCC tumours, MSI was found instead.[1 2 5] Yeast biologists recognised that this pattern of mutation was characteristic of cells which had lost MMR, and thus the first human MMR gene involved (*MSH2*) was identified. It was called *MSH2* (*MutS Homologue 2*) after its closest MMR homologue in *Escherichia coli* (*mutS*). Soon afterwards, the second major HNPCC locus was found on chromosome 3p and was identified as *MLH1* (*MutL Homologue 1*). Since then, other related MMR genes have been identified: *PMS1*, *PMS2*, and *GTBP* (G:T mismatch binding protein, or *MSH6*). The protein products of these genes act in concert as part of complexes to enable the identification and excision of mis- or unmatched bases.[5] MSH2 and MLH1 proteins are vital, whereas the functions of PMS1, PMS2, and GTBP are, to a degree, redundant, which may explain why the majority of HNPCC mutations occur in either *MSH2* or *MLH1*.

PMS1 and *PMS2* genes are so called because mutations in their yeast gene homologues were originally identified because they caused aberrations in the post-meiotic segregation of chromosomes. Mice with genetically engineered knockouts in MMR genes show a similar process, and MMR is implicated in chromosomal recombination at both meiosis and mitosis.[10 11] Early studies of MSI positive tumours from both HNPCC patients and those apparently occurring sporadically, showed that these were either diploid or more usually near diploid in chromosomal constitution, in contrast to the majority (approximately 85%) of colorectal carcinomas which are aneuploid, with grossly abnormal chromosome complements.[1 8 9 12] This may be evidence that tumours start out along a neutral, or possibly aneuploidy based, pathway of genetic instability. Later, if MMR is lost, the tumour may become committed to an MSI based pathway and will be inherently unlikely or even unable to become further aneuploid because of abrogation of mitotic recombination or a related mechanism, due to loss of MMR. Thus, there is a question as to whether tumour cell commitment to aneuploidy, at some or any stage, is incompatible with subsequent loss of MMR.

MICROSATELLITE INSTABILITY: ITS RELATION TO DNA DAMAGE

Intact MMR is also related to cellular responses to DNA damaging agents—for example, *O*6-methylguanine (*O*6meG) is a common lesion in DNA after attack by alkylating agents and is able to base pair with either cytosine (C) or thymine (T). If it pairs with thymine, a G6A mutation will result. There is a dedicated DNA repair mechanism for the correction of *O*6meG residues (*O*6meG-methyltransferase (MGMT)); if lost, cells become exquisitely sensitive to the toxic effects of alkylating agents. However, if MGMT lacking cell lines are cultured in vitro in the presence of subtoxic levels of alkylating agents, clones which have regained resistance to alkylating agents are found to grow out, but not by regain of MGMT.[13] This process was puzzling until it became apparent that intact MMR was necessary for a cytotoxic response to alkylating agents to occur. Normally, *O*6meG residues would be repaired largely by MGMT, and any missed, which caused *O*6meG:T mismatches, would be flagged by the MMR machinery; thus, the affected stretch of DNA (perhaps up to 1000 bases long) on the newly synthesised strand would be removed and resynthesised, including the mismatched T. This process would continue until the resynthesised DNA polymerase did not miscode when it got to the *O*6meG residue—that is, until it correctly matched *O*6meG:C, and the *O*6meG residue could be repaired by MGMT. If, however, a cell loses MGMT, its MMR capacity is liable to be swamped if the cell is exposed to even low levels of exogenous or endogenous alkylating agents. If this occurs, so much DNA is being actively repaired at replication that it precipitates apoptosis via p53 inter alia. However, a cell that has lost MMR as well as MGMT is not subject to this limitation; when it is exposed to an alkylating agent it cannot repair the damage. Furthermore, it cannot activate MMR and thereby induce its own death and thus, has escaped the cytotoxic effect of a mutagen. A corollary of this is that the cell has an increased mutation rate, but this is secondary though it might affect subsequent events. Cells that have lost MMR, and therefore have MSI, may be either resistant or highly sensitive to the effects of particular alkylating agents, and this has clear implications for the prediction of response to chemotherapy.[13 14] Additionally, it may also affect response to radiotherapy, given the inter-relation between alkylation and radiation induced DNA damage and repair mechanisms.

MICROSATELLITE INSTABILITY: A LINK BETWEEN CARCINOGENESIS AND TUMOUR SELECTION?

The connection between selection pressure because of an environmental agent and development of cellular resistance, will be familiar to those who use antibiotics. However, it may help us understand why loss of MMR is advantageous to a tumour cell, although there is an indication as to why the loss of one MMR allele might be an advantage.[15] There is a wealth of evidence that exogenous and endogenous environmental DNA damaging agents can have both mutagenic and cytotoxic effects. It would not be unreasonable to suppose that a small proportion of cells (perhaps no more than 1%) are killed by DNA damage induced cytotoxicity, especially in rapidly dividing tissues such as the intestinal mucosa; at least one prevalent human mutagen implicated in colorectal tumorigenesis can cause MSI, albeit in an animal model.[16 17] Therefore, it may be advantageous for a tumour cell to lose cytotoxic sensitivity to DNA damage and, as others have shown, this need not be either a huge advantage[3] or by a mechanism involving *O*6meG. Tissue specific differences in DNA repair capacity (other than MMR) and/or mutagen metabolism may explain the variation between tissues, and regions of the

colorectum, in the development of MSI positive tumours. It is possible that this is why a raised mutation rate is not a selective advantage but a paraphenomenon, and why DNA damaging agents may be important in carcinogenesis not only by acting, necessarily, as mutagens but also by providing a form of selection pressure.[4]

MICROSATELLITE INSTABILITY: BLACK AND WHITE, OR SHADES OF GREY?

Early testing for MSI involved analysing a dozen or more different microsatellites, and it was difficult to classify tumours as MSI positive or MSI negative—namely, how many and which markers were needed to exhibit extra alleles to define MSI?[2] Inevitably, a proportion of tumours fell into a grey area, showing instability at only 10% of the microsatellites tested; this was subsequently termed low level MSI (MSI-L). More recently, polyA based microsatellites—such as *BAT-25* and *BAT-26*, have been studied, which reinforces earlier work that suggested that MSI-L positive tumours could be classified with MSI negative tumours, and that high level MSI (MSI-H) is associated with HNPCC (though, as discussed later, not because of HNPCC).[2 8 12 18 19] However, intriguing evidence has been presented that the fine distinction between MSI negativity and MSI-L positivity may reflect real biological differences and novel morphogenetic pathways.[20]

Utility

MICROSATELLITE INSTABILITY AS A PREDICTIVE TEST FOR HNPCC

The finding that MSI characterised HNPCC tumours led to suggestions that HNPCC could be diagnosed by testing tumours for MSI, even though approximately 15% of apparently sporadic tumours have MSI as well. These suggestion may have been due, at least in part, to an over estimation of the prevalence of HNPCC as a cause of MSI positive colorectal tumours; early figures suggested that 5–10% of all colorectal cancers are attributable to HNPCC. Significantly, two recent papers give substantially lower figures (approximately 1%), in line with other independent estimates, both molecular and epidemiological.[21–24] It has also been shown that not all HNPCC associated tumours are MSI positive and thus, for reasons which include the limitations of the methods used to determine MSI and/or the occurrence of sporadic MSI negative tumours in HNPCC gene carriers, there is a proportion of tumours which are false negatives.[25] If the rate of MSI in colorectal cancers is 15%, the proportion of all colorectal cancers due to HNPCC is 1%, and the maximum proportion of MSI positive HNPCC colorectal cancers is 95%, there is a positive predictive value (PPV) of 6% for HNPCC upon finding MSI in a randomly selected colorectal cancer.[12 21–25] As there is a lower prevalence of approximately 2–3% of MSI in adenomas (at least the MSI-H characteristic of HNPCC), finding MSI in one would give, theoretically, a higher PPV for HNPCC (32%); however, the data are not as extensive as for cancers.[8 26]

There is a poor PPV for randomly analysing colorectal cancers for MSI as a screening test for HNPCC, although the negative predictive value is good (99.9%).[27] However, currently, most MSI testing of tumours is performed as part of a family study, or in individuals with a young age of onset—a highly selected group who are more likely to harbour HNPCC mutations, thus raising the PPV. Therefore, the question for a diagnostic laboratory becomes whether an individual who qualifies for HNPCC gene testing, on the basis of family history or age of onset, should not be tested for an HNPCC mutation if their tumour is MSI negative. Most laboratories would probably not stop testing on finding an MSI negative tumour, given an approximate 5% chance of such a finding in an HNPCC gene carrier.[25] Conversely, finding a single MSI positive tumour in an individual who had neither an early age of onset nor family history would not carry a high enough PPV for most service laboratories to instigate mutation screening; furthermore, it would add little extra in the context of family history or age of onset to justify testing for an HNPCC mutation.[27] However, in the case of an isolated individual with two tumours, even without a family history or early age of onset, a test for MSI would be worthwhile as the odds of finding two MSI positive tumours at random are low, especially with adenomas (table 1).[8 12 21–26] Finally, apart from which microsatellites to test, practical problems with MSI testing include obtaining consent for access to tissue (especially next of kin issues for deceased patients), locating and obtaining the tissue itself, contamination (some histopathology laboratories will only send sections which may not have been cut under satisfactory circumstances—for example, clean microtome), confirming the histology, and most importantly, extracting satisfactory DNA from the tissue. With a low PPV, the time and expense involved in MSI testing might be better spent on mutation screening.

Table 1 MSI test result parameters for diagnosis of HNPCC in individuals unselected by age of onset or family history[a]

MSI detected in:	*One carcinoma*	*One adenoma*	*Two carcinomas*	*Two adenomas*
Sensitivity	95%	95%	90%	81%
Specificity	86%	98%	98%	>99%
Positive predictive value	6%	32%	29%	95%
Negative predictive value[b]	>99%	>99%	98%	>99%

[a] based on 15% of colorectal cancers and 3% of adenomas having MSI, 1% of colorectal tumours being due to HNPCC, and 95% of HNPCC tumours having MSI, see text for details.[8 12 21–26] [b] based on not finding MSI in a single tumour, or in both of two tumours.

In summary, if the decision to test for a germline mutation in an HNPCC gene can be made on the grounds of family history or age of onset, there may be little point in testing for MSI if only a single cancer is available. Testing of an adenoma may to be more useful, but not finding MSI when a family history or age of onset would suggest HNPCC, is probably not sufficient evidence against mutation testing. However, if an individual has had more than one tumour, or tumour tissue is available from more than one related individual, MSI testing is recommended. In particular, if two such tumours are found to be MSI negative it strongly suggests that the family does not have HNPCC, and therefore does not warrant mutation testing. Brown *et al* have found that a greater proportion of small families are MSI negative, implying that the criteria for HNPCC should be more stringent and related to family size to avoid overdiagnosis.[23]

WHICH MICROSATELLITES TO TEST?

MSI testing is problematic because of the variation between studies of the microsatellites. Early studies used a bank of up to a dozen different microsatellites, mostly $(CA)_n$ repeats, which were often chosen semi-randomly and carefully to avoid issues of bias due to allelic loss in tumours.[1 2 8 12] More recently, attention has centered on mononucleotide repeat microsatellites, such as *BAT-25* and *BAT-26*.[28–30] These are polyA based and have the advantage of being quasimonomorphic (the vast majority of individuals are homozygous for the same sized allele); this removes the need for DNA from normal tissue for comparison.[30] However, most importantly, polyA based microsatellites exhibit excellent sensitivity (reportedly 99%) at detecting MSI-H.[30] It is hoped therefore that MSI testing will eventually be performed with a few standardised markers,

including one of the polyA based microsatellites, enabling direct comparison of findings between both research and service laboratories, although there will still be a problem with comparing earlier studies which did not use a polyA based microsatellite.[2 8 12 28 31]

IS THERE AN ALTERNATIVE TO MICROSATELLITE INSTABILITY TESTING?

Recently, there have been some promising developments using immunohistochemistry (IHC) for MSH2 or MLH1 protein which offer a better test for loss of MMR in tumours than MSI. Advantages include the fact that IHC is a standard technique in histopathology laboratories as it uses paraffin wax sections, even of small tissue biopsy samples, and provides direct evidence of involvement of one gene or another, thus potentially halving the workload in mutation screening.[32] Like MSI testing, IHC should have good specificity and sensitivity and similar predictive values, but this needs formal confirmation.

MSI TESTING FOR ALL COLORECTAL TUMOURS?

Testing for MSI may be problematical as an indicator of germline HNPCC gene mutation, however it, or perhaps IHC, may be more useful as a general predictor of tumour behaviour. A number of studies have shown that patients with MSI positive colorectal tumours (either sporadic or HNPCC associated) exhibit better survival than those with MSI negative tumours, even when matched for stage and grade.[8 12 33] Additionally, MSI status may predict response to chemotherapy which could be useful in future treatment strategies.[13] Importantly, finding MSI in colorectal cancers seems to be a good predictor of those individuals who will go on to develop a metachronous tumour.[34 35]

Conclusion

In summary, MSI testing of tumours may prove to be of greater utility in predicting individual outcome, such as prognosis and risk of a metachronous tumour, than in predicting HNPCC gene carriers. Techniques such as IHC may be more suitable for detecting HNPCC gene carriers, but assay of tumour MMR status by MSI analysis, especially using polyA based markers, will have a role to play. If testing of colorectal and other tumours for loss of MMR becomes routine then the issues of how and by whom it should be done, and who is going to pay, will have to be resolved.[36] However, until clinicians and service laboratories can be convinced of the value of MSI testing, it will languish as a research method.

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