Two hits revisited again

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Abstract

Introduction and methods—Since the concept of the “two hit hypothesis” was introduced over 20 years ago, a wealth of genetic data has accumulated on the mutations found at tumour suppressor loci. Perhaps surprisingly, these data conceal large gaps in our knowledge which genetic and functional studies are beginning to uncover. The “two hit hypothesis” must be updated to take account of this new information.

Results and discussion—Here, we discuss both the results of recent studies and some of the questions that they highlight. In particular, how valid are conclusions from inherited Mendelian syndromes when applied to sporadic cancers? Why is allelic loss so common and how does it occur? Are the “two hits” random or interdependent? Is abolition of protein function always optimal for tumorigenesis? Can “third hits” occur and, if so, why? How can mismatch repair deficiency and the methylator phenotype be incorporated into the “two hit” hypothesis? We suggest that the “two hit hypothesis” is not fixed but is evolving as our knowledge expands.

Keywords: two hit model; tumour suppressor; carcinogenesis

The “two hit” model of gene dysfunction in carcinogenesis advanced by Knudson and others has always been something of a “curate’s egg”. Neither a “two stage” model of carcinogenesis, nor the notion that genes can act as recessive in the normal cell; and (3) smaller deletions of
several kilobases may be selected against in gametes, or may occur rarely as spontaneous events compared with point mutations or small frame shifts. In contrast, allelic loss is more common than a truncating mutation as the “second hit” (even allowing for the ease of detecting allelic loss compared with truncating mutations). In part, these findings are expected if one considers the cells involved. Individual cells are more likely to tolerate allelic loss than is the whole organism and once one allele is mutant loss of the other allele can occur by several mechanisms (that is, allelic loss is a much more common spontaneous event than a truncating mutation). It is also conceivable for some tumour suppressor genes that allelic loss is preferentially selected over truncating mutations.

It might be expected that selection would favour one mechanism for allelic loss over another, depending on the individual gene involved. For example, chromosomal non-disjunction or large deletions would not be tolerated if hemizygosity of another locus on that chromosome were highly disadvantageous in that cell, or if mechanisms to maintain genomic integrity were sufficiently active to cause apoptosis. In contrast, a small deletion involving not only the primary tumour suppressor target, but also a nearby gene might be advantageous, whether immediately (if haploinsufficiency were selected), or later once the nearby gene had acquired a “second hit” (if it too were a tumour suppressor). Arguably, however, the most attractive mechanism of allelic loss in Mendelian tumour syndromes is mitotic recombination, closely followed by interstitial deletion, because these minimise the side effects on other loci.

There is not a large body of evidence concerning the mechanism of allelic loss in Mendelian tumour syndromes. In familial adenomatous polyposis (FAP), interstitial deletions appear to be common.\(^5\) In hereditary cylindromatosis, allelic loss seems to involve a remarkably invariant region on chromosome 16q, with mitotic recombination the most plausible mechanism.\(^5\) For familial gastric-breast cancer caused by germline E-cadherin mutations, deletions of variable size occur;\(^5\) a similar picture is seen with the deletions in NF1 in neurofibroma, NF2 derived schwannomas, \textit{MEN1} associated tumours, and \textit{SMAD4/DPC4} in juvenile polyposis.

The above discussion has concentrated on Mendelian tumour syndromes, where constitutional DNA can be used to determine the germline genotype and tumour DNA the somatic genotype. The study of allelic loss in sporadic tumours is generally more problematical, simply because there is no means of determining which “hit” occurred first and because detecting both “hits” in tumours is inherently more problematical than in Mendelian cancer syndromes. One feature of sporadic tumours is the relative deficiency of homozygous deletions in tumour suppressor genes, compared with that expected from the frequency of allelic loss (with some exceptions, such as \textit{p16} and \textit{SMAD4}). Two possible causes for this observation are, firstly, that mitotic recombination and gene conversion do not by themselves lead to bi-allelic gene inactivation and, secondly, as for Mendelian syndromes, that homozygous deletions must be small if they are not to be deleterious, and such forms of allelic loss may well have a low spontaneous frequency. Taking into account the difficulties often inherent in studying tumour samples, especially early lesions, and the understandable reluctance of many investigators to screen all exons of large genes such as \textit{BRCA1}, \textit{BRCA2}, and \textit{APC} for mutations, current data support the notion that the most common combination of “hits” in sporadic cancer is inactivation of one allele by a truncating mutation and loss of the other allele. In general, this situation parallels that seen in the Mendelian tumour syndromes, the only caveats being that this conclusion is based on a paucity of data on bi-allelic inactivation of tumour suppressor genes, and may take insufficient notice of possible confounding effects, such as genomic instability in cancers.

Up to this point, allelic loss has been considered as a locus specific event. There is evidence that this is the case in the crucial early stages of tumorigenesis.\(^4\) Although there are some data showing individual variation in the rate of spontaneous mitotic recombination,\(^4\) there is no evidence for a generalised tendency to allelic loss outside the inherited chromosome breakage syndromes. Later in tumorigenesis, however, allelic loss can occur as a result of karyotypic instability. There is increasing evidence that aneuploidy/polyploidy is the result of defects in the mitotic machinery, caused, for example, by mutations in mitotic checkpoint genes such as \textit{hBUB1} and \textit{hBUBR1},\(^6\) and amplification of \textit{STK15}.

It is conceivable that cells can tolerate aneuploidy only if the normal apoptotic processes are severely disrupted. Some of the genetic changes in tumours displaying aneuploidy may be selected if they target inactivation of tumour suppressors. Other changes may simply act via gene dosage effects or have little selective advantage. In late cancers with complex karyotypic changes, it may be particularly difficult to distinguish allelic loss resulting from gain and deletion of material, even on the same chromosome arm.

The implications of polyploidy for allele loss are often not discussed. If a tumour has undergone polyploidisation, it can be argued that tumour suppressor inactivation is much less likely than oncogene activation (although gene dosage and dominant negative effects may still occur and be selected). The simple reason for this hypothesis is that a pentaploid tumour, for example, must inactivate all five copies of a tumour suppressor, an extremely unlikely event. By contrast, only one copy of an oncogene needs to be activated. (Perhaps this is one reason why a substantial minority of cancers seem to follow a near diploid pathway.) Supporting evidence for this hypothesis is hard to come by, especially given the confounding effects of karyotypic instability in aneuploid tumours. Nevertheless, there is some evidence...
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Polyplody also affects the sensitivity and specificity of the scoring of allele loss. To illustrate this point, assume that constitutional DNA is heterozygous at a microsatellite locus, with allele intensities \( N_1 \) and \( N_2 \) respectively and ratio \( N_1/N_2 \). If allele 1 is simply deleted in the tumour, the ratio of alleles becomes \( (0\alpha N_1+(1−\alpha)N_1)/(\alpha PN_2+(1−\alpha)N_2) = (1−\alpha)N_1/(1−\alpha+2\alpha)N_2 \), where \( \alpha \) is the proportion of tumour tissue in the sample studied and P is the level of ploidy for \( N_2 \) subsequent to allele loss (that is, the number of copies of \( N_2 \) in the tumour as sampled). Usually, the ratio of alleles in the tumour is divided by that in normal tissue to give the allele loss ratio, in this case \( (1−\alpha)/(1−\alpha+2\alpha) \).

If allele loss occurs by mitotic recombination, the allelic ratio in the tumour becomes \( (0\alpha N_1+(1−\alpha)N_1)/(2\alpha PN_2+(1−\alpha)N_2) \). Thus, if a tumour sample contains 50% normal tissue (therefore the often used allele loss threshold of 50% reduction in the relative intensity of one allele). If loss occurs by mitotic recombination or regain, however, the allele loss ratio is 0.333. If the tumour sample contains 20% normal tissue \( (\alpha=0.80) \) and is diploid, the allele loss ratio with deletion is 0.5 (hence the often used allele loss threshold of 50% reduction in the relative intensity of one allele). If loss occurs by mitotic recombination or regain, however, the allele loss ratio is 0.667.

The germline wild type is almost never lost in these patients. (An alternative, gain of the germline wild type/somatic mutant, has not been excluded but seems very unlikely.) Occasional loss of the germline mutant as a “third hit” has also been reported in the \( p53 \) gene. 

Germline mutations were divided into types A and B. Type A included missense mutations within the core DNA binding domain (codons 100–293), resulting in full length but functionally compromised proteins and including putative dominant negative and gain of function changes. Type B mutations comprised missense changes outside the core DNA binding region.
domain and were all protein truncating mutations. With the exception of breast cancers in the families studied, type B mutations were associated with a higher frequency of allelic loss in tumours; it is not known whether or not tumours from patients with type A germline mutations carried a “second hit” at p53. These data are consistent with a model in which some p53 mutations (type A) are gain of function (no selection for allelic loss), others (type A or type B) are dominant negatives (an effect as the mutant heterozygote, but with selection for subsequent allelic loss in some tumours), and yet others (truncating type B) result in loss of function and require allelic loss as a “second hit” to have an effect.

In FAP, most colorectal tumours do not show allelic loss, but have a second truncating mutation. This is illustrated by a description of a single family with attenuated FAP who showed no loss of the germline “wild type” allele in their tumours.17 Truncating mutations in APC seen in FAP and sporadic colorectal tumours have been reported to occur between codons 1250 and 1450,22 although, because of selective screening of the gene, many studies have probably exaggerated the true frequency of clustering within this region. It appears that allelic loss at a high frequency in FAP is a feature of patients harbouring germline mutations in regions of the gene near to codon 1300; these patients also tend to have more severe disease. In contrast, the tumours of most patients, who possess germline mutations away from codon 1300, tend to harbour truncating “second hits” in the MCR. These phenomena occur in both colorectal adenomas and desmoid tumours from FAP patients, although the regions associated with allelic loss are different in the two types of tumour.18 Non-independence of the “two hits” at APC is also seen in sporadic colorectal cancers.23 Again, it appears that different combinations of APC mutations confer tumours with different selective advantages. Loss of the germline mutant in some FAP adenomas can therefore be explained, because these tumours end up with a genotype similar to the strongly selected combination of protein truncation near residue 1300 and loss of the other allele. It is likely that APC protein truncated near codon 1300 either has optimal stability and some dominant negative effect,24 or that such a protein has optimal loss of C-terminal functions and retention of N-terminal function alone.

The identification of “third hits” and dependency of the “second hit” on the “first hit” can provide clues about mutant gene function. It is noteworthy, for example, that the APC and TP53 proteins form dimers25 and tetramers26 respectively, with concomitant possibilities of dominant negative protein interactions and sequential selection of mutants with increasing degrees of loss of function. Oligomerisation appears not, however, to be the sole cause of non-random “second hits” in the tumours of FAP19 and Li-Fraumeni20 patients. Other tumour suppressors, such as DPC4/SMAD4,27 also form oligomers, but have allelic loss as the usual “second hit”. For multifunctional proteins, it may be that one or more critical functions must be lost for tumorigenesis to occur, whereas loss of other functions provides a smaller selective advantage and loss of yet other functions provides no advantage or is disadvantageous. Suitable in vitro assays to determine the effects of mutants on protein function must consider both alleles of genes like p53 and APC together (that is, as genotype rather than allele).

Turning off gene expression by promoter methylation has been well described for several years. It is also known that methylation can occur not as a primary event, but secondary to mutation. Therefore, the third amendment to the “two hits” hypothesis is not simply the fact of methylation in tumours, but the finding that one allele of a tumour suppressor can be inactivated by methylation when the other harbourues a mutation or allelic loss. The role of promoter methylation in cancer has been reviewed in detail elsewhere.28 However, there are certain points to highlight. Promoter methylation is rarely the “second hit” in the Mendelian syndromes (for example, Esteller et al29), although it has been reported in von Hippel-Lindau syndrome30 and hereditary diffuse gastric cancer.31 Evidence from genes such as hMLH1 in colorectal cancer32 shows that methylation may be a common way of inactivating certain genes in sporadic tumours, even though allelic loss is the most common “second hit” at hMLH1 in hereditary non-polyposis colon cancer.33 One explanation for this disparity between the inherited and sporadic forms of the same cancer is that methylation of tumour suppressors does not initiate tumorigenesis, probably because the methylator phenotype itself requires mutations in genes controlling gene expression and/or methylation.34 The mechanisms by which this occurs and how it targets specific loci are under investigation.35 Thus, hypermethylation is not seen in FAP36 and observed uncommonly as an independent event at APC in sporadic cancers.37 38 since APC mutations probably initiate the growth of most sporadic colorectal cancers.39 Under this hypothesis, the observed methylation of BRCA1 in sporadic breast cancers suggests that changes at this locus do not usually initiate sporadic breast tumorigenesis, consistent with the absence of BRCA1 mutation in sporadic breast cancers. A similar situation may hold for hMLH1 in colorectal cancer. One aspect of methylation in cancer remains very puzzling. How can methylation, which is generally supposed to be a global tendency or targeted to a specific gene, specifically inactivate one copy of a gene, while leaving the other (mutant) copy unmethylated? For methylation plus allelic loss, this can be explained if the gene involved is hemizygous. For methylation plus truncating mutation, however, possibilities include the methylated allele actually harbouring a germline variant or somatic mutation (in the promoter or elsewhere) which predisposes in cis to methylation. Evidence for these possibilities is currently lacking.

The “two hits” model has progressed a long way in the past 25 years and it continues to
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