Review article

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.

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The “two hit” model of gene dysfunction in carcinogenesis advanced by Knudson¹ 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 tumours was an entirely new concept. However, the critical insight Knudson and contemporaries provided was that a disease with a recessive cellular basis could be inherited as a high penetrance dominant, as long as the probability of somatic mutation in the wild type allele in a susceptible cell was high. The importance of this, undoubtedly profound, insight is, however, limited by the fact that the Mendelian tumour syndromes are rare. Moreover, most tumour suppressors contributing to tumorigenesis acquire both “hits” somatically in classical recessive fashion. Nevertheless, the model has been hugely influential and central in stimulating a flurry of studies into tumour suppressor genes and their functions.

There is now a wealth of genetic data on specific tumour suppressor genes, such as *APC*, *BRCA1*, *BRCA2*, *RB1*, *p53*, and *PTEN*, although it should be noted that much of this information has been obtained through studies of the germline rather than the soma. While an apparently detailed knowledge of these genes exists, there remain many unanswered questions. One major area of comparative ignorance is the nature of the “two hits”, in terms of the spectrum of genetic changes that occur and their effects on protein function. Of the types of mutation which can abolish protein function, those most commonly observed at tumour suppressor loci are (1) small scale “truncating” mutations (nonsense or frameshift changes) and (2) a group of larger scale changes, which can be considered together under the category of “allelic loss”. Allelic loss, which for the purposes of discussion will be used synonymously with loss of heterozygosity (LOH) and allelic imbalance, is central to the “two hit” hypothesis. LOH leads either to deletion of the tumour suppressor locus (and possibly of flanking loci) or to “reduction to homozygosity” (a rather clumsy but accurate description of a process in which two alleles come to be identical without net loss of genetic material). Allelic loss can result from chromosomal non-disjunction (with or without regain), from deletion of part of a chromosome arm, from mitotic recombination, and from gene conversion. Allelic loss should not, therefore, be equated with deletion, as has occasionally been asserted; indeed, it should always be borne in mind that apparent allelic loss might actually result from gain of genetic material.

Another area where there is a paucity of knowledge is the specific nature of the “two hits”, both in terms of the spectrum of genetic changes that occur and their effects on protein function. Considering firstly the spectrum of two hits in Mendelian tumour syndromes, the “first hit” at a tumour suppressor locus is usually a truncating mutation; it is rarely the equivalent of allelic loss. Deletions are very rare at loci such as *PTCH* and *p53* and occur uncommonly at other loci such as *VHL*.² Why is allelic loss so rarely seen as a first hit? We believe that there are several possible reasons for this: (1) large deletions and aneusomies confer a selective disadvantage on many cells; (2) mitotic recombination and gene conversion probably have little or no functional effect in the normal cell; and (3) smaller deletions of

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several kilobases may be selected against in gametes, or may occur rarely as spontaneous events compared with point mutations or small frameshifts. 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 hemizyosity 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.³ In hereditary cylindromatosis, allelic loss seems to involve a remarkably invariant region on chromosome 16q, with mitotic recombination the most plausible mechanism.⁴ For familial gastric-breast cancer caused by germline E-cadherin mutations, deletions of variable size occur⁵; a similar picture is seen with the deletions in *NF1* in neurofibroma, *NF2* derived schwannomas, *MEN1* associated tumours, and *SMAD4/DPC4* in juvenile polyps.

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 *p16* and *SMAD4*). Two possible causes

for this observation are, firstly, that mitotic recombination and gene conversion do not by themselves lead to biallelic 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 *BRCA1*, *BRCA2*, and *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 biallelic 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.⁴⁻⁶ Although there are some data showing individual variation in the rate of spontaneous mitotic recombination,⁷ 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 *hBUB1* and *hBUBR1*,⁸ and amplification of *STK15*.⁹ 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

that gain or amplification of genetic material predominates over loss in grossly aneuploid cancers (for example, Roylance *et al.*¹⁰).

Polyploidy 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 N1 and N2 respectively and ratio N1/N2. If allele 1 is simply deleted in the tumour, the ratio of alleles becomes $(0\alpha N1 + (1-\alpha)N1)/(0\alpha N2 + (1-\alpha)N2) = (1-\alpha)N1/((1-\alpha+\alpha P)N2)$, where α is the proportion of tumour tissue in the sample studied and P is the level of ploidy for N2 subsequent to allele loss (that is, the number of copies of N2 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 $(1-\alpha)/(1-\alpha+\alpha P)$. If allele loss occurs by mitotic recombination, the allelic ratio in the tumour becomes $(0\alpha N1 + (1-\alpha)N1)/(2\alpha P N2 + (1-\alpha)N2) = (1-\alpha)N1/((1-\alpha+2\alpha P)N2)$, giving an allele loss ratio of $(1-\alpha)/(1-\alpha+2\alpha P)$. Thus, if a tumour sample contains 50% clonal tumour tissue ($\alpha=0.50$) 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 0.333. If the tumour sample contains 20% normal tissue ($\alpha=0.80$) and is diploid, the allele loss ratio with deletion is 0.2, but with mitotic recombination or regain, it is 0.17 ($0.2/1.2$). In a polyploid cell, the ratios are slightly more extreme. For a tetraploid, for example, with 80% tumour tissue, which has undergone allele loss as a diploid by deletion, the allele loss ratio will be $0.2/(0.2+(2 \times 0.8)) = 0.11$. With mitotic recombination, the ratio becomes $0.2/(0.2+(2 \times 0.8 \times 2)) = 0.06$. We believe that imposing ratios greater than 0.5 (or less than 2.0) for scoring allele loss is too conservative, risks false positive results, and presupposes such a large proportion of contaminating normal tissue that any analysis must be subject to error. Evidently, if reduction (or gain) in allele copy number occurs after polyploidisation, allelic ratios may change more subtly. Such small changes in allelic ratios may be difficult to distinguish from background (such as random variation in the PCR), have doubtful functional effects through changes in gene dosage, and presumably cannot be said to "inactivate" tumour suppressor genes.

A generalised tendency to replication errors (RERs), often a consequence of defective mismatch repair, represents an alternative to karyotypic instability in some tumours. Before such tumours become RER positive, tumour suppressor genes are as likely as RER negative tumours to acquire one of the "two hits" by allelic loss. Thus, allelic loss at *hMSH2*¹¹ and *hMLH1*¹² are common "second hits" in hereditary non-polyposis colorectal cancer and promoter methylation at *hMLH1* is common in sporadic RER+ tumours¹³ (although rare at *hMSH2*). After a tumour becomes RER positive, there is, however, likely to be a bias against allelic loss as the "second hit" in favour of a frameshift mutation.¹⁴

There has been evidence accumulating that the simple "two hit" model, one protein inactivating (usually truncating) mutation, plus another similar mutation or allelic loss, does not hold for all tumour suppressor genes. This initially came from data which showed that some mutations at loci such as *p53* could be oncogenic, whereas other mutations could be of a tumour suppressor type.¹⁵ More recently, three particular discoveries have suggested that the basic "two hit" model requires updating: (1) identification of "third hits" at tumour suppressor loci; (2) dependency in Mendelian and sporadic cancers that the site and type of "second hits" depend on the site and type of the "first hit"; and (3) the role of promoter methylation as one or both of the "two hits". It is currently unclear as to whether these phenomena are locus specific or generally applicable.

"Third hits", mutations in, or loss of, either tumour suppressor allele after the first "two hits" have occurred, with probable pathogenic effects, have been found at the *APC* locus in early colorectal adenomas from FAP patients with "attenuated" disease. If these adenomas have acquired their "second hit" at *APC* through a truncating mutation, usually in the so called mutation cluster region (MCR, codons 1286-1513), then they sometimes subsequently show loss of the germline mutant allele.¹⁶⁻¹⁸ 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.¹⁹ Further studies have found not only "third hits", but fourth, fifth, and even sixth mutations in tumours from Li-Fraumeni syndrome patients,²⁰ although the possibility that these occur in different subclones of the tumour must be borne in mind. Overall, the data suggest that, while the "two hits" at *APC* or *p53* are sufficient for tumorigenesis, the selective advantage which they confer is suboptimal and thus further mutations are selected. For *p53*, this may simply involve progressive ability of mutant protein to impede function of the wild type allele. This model of tumorigenesis is difficult to reconcile with selection for simple loss of *APC* or *p53* function in tumours.

In addition to these "third hits", *p53* and *APC* also provide examples of the dependence of the "second hit" on the "first hit" at tumour suppressor genes. Furthermore, both Li-Fraumeni syndrome and FAP appear to be exceptions to the rule that truncating germline mutation, followed by allelic loss, are the usual "two hits" in Mendelian cancer syndromes. Studies have shown non-random allelic loss at *p53* in the tumours of Li-Fraumeni patients.^{19, 21} 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

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 1300¹⁸; 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 functions.

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 FAP¹⁸ and Li-Fraumeni^{19, 21} patients. Other tumour suppressors, such as *DPC4/SMAD4*,²⁷ also form oligomers, but have allelic loss as the usual "second hit". For multifunctional pro-

teins, 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 harbours 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 al.*²⁹), although it has been reported in von Hippel-Lindau syndrome³⁰ and hereditary diffuse gastric cancer.³¹ Evidence from genes such as *hMLH1* in colorectal cancer¹³ 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.¹² 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.³² The mechanisms by which this occurs and how it targets specific loci are under investigation.³³ Thus, hypermethylation is not seen in FAP²⁹ and observed uncommonly as an independent event at *APC* in sporadic cancers,^{29, 34} since *APC* mutations probably initiate the growth of most sporadic colorectal cancers.³⁵ 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

evolve, with recent findings such as “third hits” at *APC*¹⁶⁻¹⁸ and *TP53*²⁰ and identification of the “methylator phenotype”.³² Progress has undoubtedly been hampered by methodological problems, such as contaminating normal tissue in tumour samples, and by the sheer tedium of performing comprehensive mutation detection and allelic loss screens. Intrinsic features of tumours, such as karyotypic instability, have made studies even more difficult. The future holds the prospect of continued evolution for the two hits model: identifying increasing complexity of tumour suppressor action; studying the functional effects of mutant genotypes rather than individual alleles; using rapid throughput technologies to identify the targets of allelic loss; characterising genes which cause karyotypic instability in tumours; and identifying the points at which the genetic pathways of replication errors, karyotypic instability, and methylator phenotype diverge.

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