The two-hit theory hits 50

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ABSTRACT **Few ideas in cancer genetics have been as influential as the "two-hit" theory of tumor suppressors. This idea was introduced in 1971 by Al Knudson in a paper in the Proceedings of the National Academy of Science and forms the basis for our current understanding of the role of mutations in cancer. In this theoretical discussion proposing a genetic basis for retinoblastoma, a childhood cancer of the retina, Knudson posited that these tumors arise from two inactivating mutations, targeting both alleles of a putative tumor suppressor gene. While this work built on earlier proposals that cancers are the result of mutations in more than one gene, it was the first to propose a plausible mechanism by which single genes that are affected by germ-line mutations in heritable cancers could also cause spontaneous, nonheritable tumors when mutated in somatic tissues. Remarkably, Knudson described the existence and properties of a retinoblastoma tumor suppressor gene a full 15 years before the gene was cloned.**

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Let's put ourselves, if we may, into the mindset of cancer researchers a half century ago. By the early 1970s, we have come to accept the idea that cancer is a genetic disease resulting from mutations in particular genes. We also know that chromosomal aberrations occur in many cancers, including loss or gain of genetic material, but the identity, and even the existence, of cancer driver genes and how they might operate is entirely unknown to us. Recently reported somatic cell fusion experiments, in which normal human cells have been induced to fuse with malignant rodent cells, suggest that normal cells possess dominant tumor-suppressive properties and that these properties are associated with particular chromosomes (Harris *et al.*, 1969), but the genes that confer such suppression, and how they might do so, are obscure. In addition, there is considerable evidence that that most cancers involve more than a single mutational event (Nordling, 1953; Ashley, 1969). On the other hand, some of our fellow cancer researchers have also shown that acutely transforming retroviruses can rapidly induce cancers in their hosts, suggesting that, at least in avian and mammalian species, single "oncogenes" can transform cells and cause tumors (Huebner and Todaro, 1969). Finally, we know that certain cancer predispositions can be passed down from parent to child, even if the genetic basis for this phenomenon remains uncharacterized.

Among the many questions that puzzled our midcentury scientist were: how can these various findings—some of which suggest a multigene cause for cancer and others that suggest a single event—be reconciled? What is the relationship between dominant oncogenes and recessive "anti-oncogenes?" Also, are the genetic mechanisms underlying relatively rare inherited forms of cancer related to the more common forms of this disease, which seem to occur spontaneously?

The pediatric cancer retinoblastoma represented a particularly compelling model in which to address this last question. This type of cancer affects retinoblast cells in the developing eye and typically presents in childhood. Curiously, some affected patients were known to develop an early, aggressive, often bilateral form of the disease, and, if they survived, could pass susceptibility to retinoblastoma to their children. Other children developed such tumors later in childhood, never presented with bilateral disease, and did not impart additional risk to their offspring. This cancer drew the attention of Alfred Knudson, at that time a 49-year-old physician studying heritable metabolic disorders. Following a study of patient charts that dated back decades, he suggested in his seminal 1971 National Academy of Science paper that, in both familial and nonfamilial cases of retinoblastoma, the number of mutations required to initiate this tumor was two, that they must occur before retinal cells differentiate, and that the gene or genes affected likely act in a recessive manner (Knudson, 1971). The ideas presented in this paper

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Abbreviations used: APC, adenomatous polyposis coli; ATM, ataxia telangiectasia; BRCA, breast cancer susceptibility gene; CDK, cyclin-dependent kinase; FANCA, Fanconi's anemia complementation group A; LKB1, liver kinase B1; mTORC1, mechanistic target of rapamycin complex; NF, neurofibromatosis; PALB, partner and localizer of the BRCA2; PARP, Poly (ADP-ribose) polymerase; PTH, patched; RB, retinoblastoma; PTEN, phosphatase and tensin homolog; SMAD4, SMAD family member 4, Mothers against decapentaplegic homolog 4; TSC, tuberous sclerosis; VHL, von Hippel Lindau; WT, Wilm's tumor.

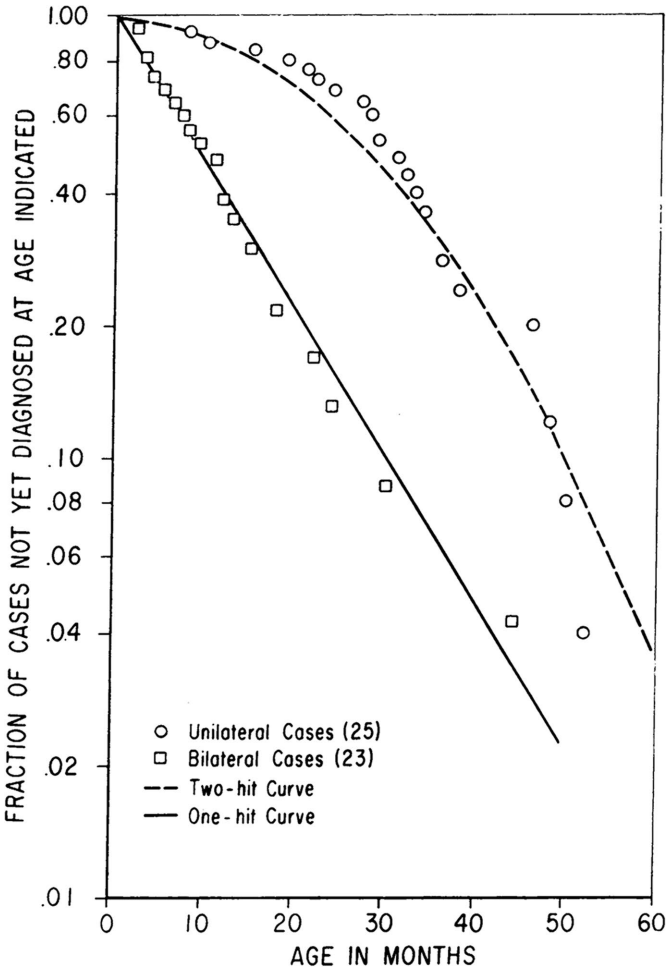

FIGURE 1: The plot from which Knudson proposed the two-hit hypothesis (Knudson, 1971, with the permission of the National Academy of Sciences, USA).

continue to guide our thinking about cancer genetics to the present day.

While the impact and implications of the 1971 paper were profound, the paper itself was profoundly simple. It was four pages long. It had a single author. It neither listed nor required any grant support. It showed no blots (Southern published his eponymous technique in 1975, PCR was more than a decade away, and restriction enzymes were not yet discovered), no sequences (DNA sequencing methods were introduced in 1975), and but one simple figure, showing a straight line and a hyperbolic line on a log scale (Figure 1). In a way, its analytic methods represented a style of science that, while not too uncommon at that time, later fell into relative disfavor as experimental molecular techniques allowed for genes to be isolated, sequenced, mutated, and introduced into cells and animals. By the mid-1970s, gene jockeys were in, and theoretical biologists were out.

Interestingly, Knudson's statistical approach anticipated much of today's cancer research literature; that is to say, the work consisted entirely of dataset analysis and mathematical modeling. The numbers being crunched were rather simple by today's standards: the age of onset of retinoblastoma in pediatric cases, whether these children developed unilateral or bilateral disease, how many tumors were present, and whether these tumors were hereditary or not. The key facts were that the familial cases tended to present at a younger age, were often bilateral, and, in a related point, could arise as multiple independent tumors. He also noted that not everyone who inherited the mutation(s) actually developed tumors; some retinoblastomas skipped a generation. This feature, plus a knowledge of how many cells comprise the retina, suggested that the affected gene(s) was recessive and allowed Knudson to infer a mutational frequency rate per cell division that closely matched previous predictions and was consistent with the observed tumor burden in familial cases.

From these data, and unassisted by any form of computer, Knudson used curve-fitting and Poisson statistics to derive an important conclusion: the incidence curve for heritable cases fit a model in which the development of retinoblastoma required not one but two mutational events, or two "hits." Whether these events were disabling mutations in each of the two alleles of a hypothetical retinoblastoma gene (as indeed proved to be the case) or instead were activating mutations in one allele each of two separate genes, could not be ascertained at that time, though the observation that retinoblastoma cells sometimes lost part of chromosome 13 favored the first interpretation. A decade later, the case for the two-hit theory received crucial experimental support when Cavenee and colleagues applied restriction site polymorphism analysis to retinoblastomas (Cavenee *et al.*, 1983). These studies showed that retinoblastomas commonly display loss of polymorphic restriction sites, consistent with the idea that these tumors involve damage to one allele of an *RB* gene and subsequent loss of the second copy. The two-hit theory provided an appealing genetic model that could be used to explain both heritable and spontaneous cases of retinoblastoma: the former had one hit in a tumor suppressor gene in the germline and only required one more hit in a somatic retinal cell, whereas the latter required that the first and second mutation to occur in a somatic cell. This model explained why spontaneous cases of retinoblastoma occurred later in life and were never bilateral, as the number of stem cells, the mutation rate, and the amount of time for retinoblasts to terminally differentiate was insufficient for more than one tumor to initiate. The result of these analyses led to a clear prediction regarding the existence and properties of tumor suppressor genes, predictions that have largely withstood the test of time.

It would be more than a decade before the first "two-hit" gene, *RB*, was mapped, isolated, and sequenced (Friend *et al.*, 1986; Lee *et al.*, 1987) and even longer before its biochemical role in regulating cell proliferation was understood in any detail. However, in the meanwhile, dozens of other tumor suppressor genes were characterized, most governed by the rules laid out by Knudson in his 1971 paper.

Looking back from a space of 50 years, the 1971 work profoundly reoriented our thinking about cancer genetics in a way that few other single works have done. Importantly, it led to testable predictions that were later—in some cases, much later—proved true. That is not to say, however, that the two-hit theory itself has not evolved. For example, Knudson himself was the one of the first to recognize the possibility that haploinsufficiency (i.e., a one-hit scenario) could alter cellular behavior in ways that contributed to tumorigenesis even in the absence of a second hit. In fact, he spent the last decade of his career studying such effects in cells derived from cancer-prone families (Berger *et al.*, 2011; Peri *et al.*, 2017). Haploinsufficiency was first experimentally verified in mouse models of the *Cdkn2a* ($p27^{kip1}$) tumor suppressor. Mice lacking one allele of *Cdkn1b* were larger than their littermates, but smaller than those lacking both alleles. Crucially, the heterozygous mice were more prone to tumorigenesis when treated with various mutagens or when bred to oncogene expressing mice (Fero *et al.*, 1998). Many other examples of

haploinsufficiency were subsequently described. In this respect, Knudson's initial two-hit theory was perhaps too parsimonious in its division of tumor suppressor genes into recessive and dominant categories. Most of the proteins encoded by tumor suppressor genes might more aptly be considered as rheostats than as on/off switches: gene dosage matters, and rigid threshold effects are not always seen. To add to the complexity, over the past decades it has become clear that mutations in tumor suppressor genes can also result in dominant-negative or even neomorphic functions, in which the mutant protein carries out functions that are different than those performed the wild-type form (Takiar *et al.*, 2017). To extend our light-switch analogy, the key feature of neomorphic tumor suppressor proteins isn't whether they act as rheostats or on/off switches, but whether they turn on the stereo instead of the lights. To make matters even more interesting, certain tumor suppressors are suppressors only in particular contexts; that is, depending, as it were, on the time of day and the particulars of the room they're in, they can act either as on or as off switches. For example, *Notch*, a central mediator of cell-to-cell signaling, is endowed with both tumor suppressor and tumor-promoting activities that are highly cell and context dependent (Dotto, 2008). Several other tumor suppressor genes display a similar duality (Datta *et al.*, 2020).

Another key modification of the two-hit theory is that, despite the simplicity and enduring appeal of the number "two" in its title, the theory applies best to tumor initiation, not necessarily to tumor growth and development. In fact, even in retinoblastoma, it quickly became apparent that two hits are not enough to cause full-blown cancer, and additional "third" hits are required. That is to say, *RB1* inactivation is necessary for retinoblastoma tumor initiation but not sufficient for full malignant transformation (Wang *et al.*, 1994; Sellers and Kaelin, 1997).

The mapping, cloning, and characterization of additional tumor suppressor genes enabled Kinzler and Vogelstein to propose that these genes fell into at least two general classes: gatekeepers and caretakers (Kinzler and Vogelstein, 1997). The former represented most of the classical tumor suppressor genes, including *APC*, *NF1*, *NF2*, *RB1*, *TSC1/2*, *VHL*, and *WT1*. These gatekeepers regulate cell division and/or survival through their interaction with elements of signal transduction pathways, and their loss directly initiates growth of the incipient tumor. In contrast, the caretakers, such as *ATM*, *BRCA1* and *BRCA2*, and *FANCA*, are involved in maintaining genome integrity through their actions in various aspects of DNA unwinding and repair. In this model, mutational inactivation of such caretaker genes leads to genetic instabilities, increasing the number of mutations of all genes, inactivating gatekeepers and activating oncogenes.

In the intervening half century since the initial Knudson paper appeared, the range and variety of mechanisms for tumor suppressor gene inactivation has been more completely defined, incorporating epigenetic as well as genetic events. *RB1* itself provides a good example, as silencing of expression of this gene by methylation of CpG islands in its promoter has been noted in sporadic cases (Sakai *et al.*, 1991; Ohtani-Fujita *et al.*, 1993; Greger *et al.*, 1994). In these cases, an epigenetic mechanism of gene inactivation was supported by the lack of mutations in the *RB* gene sequence. A similar phenomenon has been reported for the *VHL* gene in spontaneous clear-cell renal carcinoma (Herman *et al.*, 1994) as well as other tumor suppressor genes.

Interestingly, at about the same time these ideas were being formulated, Knudson's colleague at the Institute for Cancer Research (now the Fox Chase Cancer Center), Beatrice Mintz, was busy demonstrating that the cells comprising the tumor cell microenvironment exerted a suppressive effect on cancer cells (Mintz and Illmensee, 1975). In this scenario, loss of a single allele of a tumor suppressor gene in a fibroblast or an immune cell might well impact the growth of an adjacent cancer cell with single or biallelic loss of the same tumor suppressor. A good example of this phenomenon can be seen in one of Knudson's enduring interests, neurofibromatosis (NF) type 1 syndrome, associated with the tumor suppressor *NF1*. Here, malignant Schwann cells show biallelic loss of the *NF1* gene, just as predicted by proper "Knudsonian" two-hit mechanics, and the surrounding immune cells are hemizygous for *NF1* (i.e., have one hit) due to germline mutation. Importantly, these microenvironment cells *have* to be hemizygous for tumors to develop, as demonstrated by transplantation studies in conditional mouse models (Yang *et al.*, 2008). Such stromal effects have led to the idea that there is a third category of tumor suppressor genes—the landscapers—that predispose to cancer by contributing to a more tumor-conducive stroma (Kinzler and Vogelstein, 1998).

Knudson lived to see many of the proteins encoded by tumor suppressor genes functionally linked by virtue of their effects on common signaling pathways that regulate the cell cycle, apoptosis, and protein synthesis. He was particularly interested in determining whether some or all of the tumor suppressor genes that are mutated in phakomatoses—heritable neurocutaneous cancer syndromes that include Cowden's disease (*PTEN*), Gorlin's disease (*PTH*), juvenile polyposis (*SMAD4*), Peutz-Jeghers (*LKB1*), neurofibromatosis type 1 (*NF1)* and *-2 (NF2),* tuberous sclerosis (*TSC1* and *-2*), and Von Hippel Lindau (*VHL*)—could somehow be shown to act in a single pathway, as in fact we now know many of them do. He used to refer to this idea as his "grand unification theory" for tumor suppressor genes.

Regarding therapeutics, I think Knudson, whom I knew well as a friend, colleague, and mentor at Fox Chase, would have been disappointed at our relative lack of progress in devising effective treatments for many types of cancers driven by tumor suppressor gene mutations. For example, despite the fact that *TP53* is the single most commonly mutated gene in cancer, knowledge of *TP53* status has not readily translated into targeted therapies. Part of the reason for this relative lack of progress is obvious: it is much easier to disrupt the action of an oncoprotein than to fix a broken tumor suppressor protein. Direct targeting is not possible if a protein isn't expressed, and that is the scenario in many tumor cells driven by tumor suppressor gene mutations. Instead, the dominant strategy in this situation has been to target downstream signaling elements, for example, by impeding mitogen-activated protein kinase signaling in *NF1*-mutant tumors or mTORC1 in *TSC*-mutant tumors. On the other hand, we have been able to exploit vulnerabilities of cancers with certain caretaker gene mutations, such as *PALB2*, *BRCA1*, and *BRCA2*, as these cells become solely dependent on PARP for DNA repair, rendering them susceptible to small molecule inhibitors of this enzyme. Other "synthetic lethal" strategies have been proposed for various additional tumor suppressor genes (Nijman and Friend, 2013). Finally, given recent advances in gene editing and gene replacement methodologies, it is not unreasonable to think that long before the next 50 years have passed, we will be able to repair damaged tumor suppressor genes in tumor cells and/or replace them with undamaged alleles. If so, we will have come full circle, using a genetic cure for a genetic disease.

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