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.

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 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...
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 Cdkn1a (p27Kip1) 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 microenvi-

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