OPINION

The significance of unstable chromosomes in colorectal cancer

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A very large fraction of cancers have an abnormal genetic content, called aneuploidy, which is characterized by changes in chromosome structure and number. One explanation for this aneuploidy is chromosomal instability, in which cancer cells gain or lose whole chromosomes or large fractions of chromosomes at a greatly increased rate compared with normal cells. Here, we explore experimental and theoretical evidence for the initiation of chromosomal instability in very early colorectal cancers, and reflect on the role that chromosomal instability could have in colorectal tumorigenesis.

The advent of multicellular life presented a problem not faced by single-celled organisms: how to ensure that cells cooperate with each other and do not choose to grow autonomously. Genes and regulatory pathways evolved to guarantee that cells would only divide when needed. Problems associated with the failure to maintain such tight restrictions on growth can lead to cancer, and of the 30,000 or so genes in the human genome, a significant fraction can be considered anti-cancer genes in a general sense — for example, genes that let cells know what they are doing at various points in time and space, those that control the cell cycle and those that ensure the stability of genetic material. This makes the somatic evolution of cancer different from all other evolutionary scenarios: as cells progress towards cancer, they can, theoretically, destroy or mutate a large fraction of their genes to gain a selective growth advantage. By contrast, advantageous mutations — that is, those that increase reproductive fitness (acquiring a growth advantage) — are normally so rare in most evolutionary contexts that they can almost be neglected.

Intuitively, the tremendous benefit that is derived from extensive changes in the genome is the rationale for believing that cancers are likely to exploit genetic instability to increase their fitness. This has generated significant interest in understanding the causes and effects of various forms of genetic instability in many kinds of cancer. Our own interests lie in experimental and theoretical approaches to study such instabilities in colorectal cancers. In this perspective, we discuss our view of one form of genetic instability — chromosomal instability (CIN) — and comment on its importance in tumorigenesis and its timing relative to the initiation and progression of the disease.

Initiation of colorectal tumorigenesis

The colon consists of ~10^7 crypts, each of which contains several thousand differentiated cells and a small number (between 1 and 10) of stem cells. Stem cells reside at the bottom of the crypts and divide slowly and asymmetrically, whereas differentiated cells divide rapidly and travel to the top of the crypt, where they undergo apoptosis[1]. Each day, a total of approximately 10^{10} cells are shed by the colon and have to be replaced, and each cell division represents a risk for cancer because of the mutational events that can occur during normal DNA replication and chromosome segregation[2].

Colorectal cancer is thought to be initiated by inactivation of the adenomatous polyposis coli (*APC*) tumour-suppressor pathway in a cell somewhere within the colon. In ~85% of cases, the *APC* gene is mutated, whereas the *β-catenin* gene is mutated in aproximately half the remaining cases[3,4]. The β-catenin protein is regulated by APC, and mutations in either gene have the same physiological effects[5,6]. In the few colorectal cancers without known mutations in *APC* or *β-catenin*, it is likely that other genes in the same pathway, or unusual mutations of *APC* or *β-catenin*, are to blame.

The crypt in which the *APC*-mutant cell resides becomes dysplastic as abnormal cells accumulate to slowly produce a polyp. The development of a large polyp probably requires the acquisition of further mutations — for example, in the *KRAS* or *BRAF* oncogenes. Subsequently, 10–20% of these large polyps will progress to cancer by acquiring additional mutations in genes of the TGF-β pathway, the p53 pathway and other pathways that are still being actively researched[7,8]. Individual cells within the bulk population that acquire such mutations are clonally selected on the basis of their improved fitness. This clonal selection creates a bottleneck in the development of the cancer, as mutations within this individual cell — advantageous and random — become fixed in future generations as its daughters overtake the rest of the cells in the tumour[9,10]. Despite clonal selection, tumours are heterogeneous because of the continuing accrual of genetic changes[11]. The whole process — from the occurrence of the first *APC* mutation to the development of a metastatic cancer — generally takes 20–40 years, and genetic instability develops at some point during this time[9–16].

Defining CIN

Approximately 15% of colorectal cancers show a form of genetic instability that is characterized by mismatch repair (MMR) deficiency. As this instability was first found in stretches of repetitive DNA, it was named microsatellite instability (MIN, also known as MSI). The remaining 85% of colorectal cancers, and an even larger proportion of other solid tumour types, do not show this deficiency, but contain an abnormal chromosomal content — that is, they are aneuploid (BOX 1). Experimental evidence indicates that aneuploidy arises in these cancers because of CIN — an accelerated rate of gains or losses of whole or large portions of chromosomes. CIN, like MIN, was postulated to allow cells to rapidly acquire genetic changes that are required for tumorigenesis.

One should recognize that CIN refers to the rate with which whole chromosomes or large portions of chromosomes are gained or lost in cancers. It is not synonymous with the state of aneuploidy that is observed in a static image of the chromosomal content of a cancer cell[12]. In other words, although we believe that CIN is a process that drives most cancers to aneuploidy, the presence of aneuploidy *per se* does not imply the existence of CIN. There are several ways in which a cancer cell could become aneuploid in the absence of CIN. First, the cell could have gone through many more cell divisions than normal cells within a tissue, without a difference in the rate of chromosomal change per division. It is known that gross chromosomal changes occur in normal cells, so this possibility is a real one. Second, it is possible that the cancer cell was exposed to an endogenous or exogenous agent that induced aneuploidy, perhaps by interfering with proper spindle formation. The resultant daughter cells would be aneuploid, but not chromosomally unstable, in future generations.

It is also possible that cancer cells develop chromosomal changes at the same rate as normal cells, but that gross chromosomal changes are lethal to the latter, but not the former. This possibility is consistent with the idea that oncogene and tumour-suppressor-gene mutations often seem to reduce apoptosis in the cancer cell. Although the ability to survive chromosomal changes might be scored as CIN in some assays, the mechanisms underlying this process would be very different. For example, mutations in genes that control the G2 checkpoint might result in CIN by stimulating chromosomal changes, whereas mutations in genes that control apoptosis would have no effect on the rate at which such changes occur if the rate in all cells was measured, rather than simply in surviving cells.

CIN has only been formally shown for the gain or loss of whole or large portions of chromosomes in cancers. There is no assay at present that can reliably measure the rate of other chromosomal changes, such as rearrangements, deletions, insertions, inversions and amplifications (TABLE 1). These latter changes are at least as common as losses or gains of whole chromosomes[13,14]. Whether such changes reflect an underlying CIN rather than one of the other mechanisms noted above is an essential question for future research.

Box 1 | Genetic instabilities in colorectal cancers

Microsatellite instability can be found in approximately 15% of colorectal cancers. It is generally due to a loss of mismatch repair (MMR) function in cancer cells, secondary to inactivation of MMR genes such as *MLH1* or *MSH2*. Loss of MMR function renders tumour cells susceptible to the acquisition of somatic mutations throughout the genome. Simple repeat sequences are particularly susceptible to mutations in the absence of MMR, and the instability in these tumours is therefore often referred to as microsatellite instability (MIN, also known as MSI)[53–55]. Cancer cells that possess MIN have a diploid or near-diploid chromosomal content and have a mutation rate at the nucleotide level that is two to three orders of magnitude greater than that observed in normal cells[56].

Cancer cells that do not possess MIN are generally aneuploid — that is, they have an abnormal chromosome content. To understand the processes responsible for aneuploidy, the rate at which colon cancer cell lines gain and lose chromosomes was measured[16]. Clones were generated and expanded through a defined number of generations before they were examined by fluorescence *in situ* hybridization with centromeric probes. In cell lines that did not show MIN, the probability of losing or gaining a chromosome was ~0.01 per chromosome per cell division. The corresponding rate in MIN-cell lines was much lower and could not be accurately determined. The accelerated rate of chromosomal gains and losses was termed chromosomal instability (CIN). Many cancers also possess structural abnormalities, including interstitial deletions, inversions and translocations, but it remains unclear whether these changes occur at a higher rate in cancers than in normal cells. These structural changes are not measured by the CIN assay described above.

The origin of CIN

Given the pervasiveness of aneuploidy in cancers and our limited knowledge of its origins, it is not surprising that numerous ideas have been invoked to explain it. Because most late-stage cancer cells contain between 60 and 90 chromosomes, many individuals proposed that these cells are near-triploid in chromosome number[15] because of tetraploidization and subsequent chromosome loss. There are a number of ways in which both aspects to this process (tetraploidization and chromosome loss) can occur in cancer cells.

One unlikely possibility in this scenario is that the chromosomal complement following a tetraploidization event is inherently unstable and that CIN is a trivial consequence of the near-doubling of the genetic content. Two lines of evidence serve to refute this hypothesis. First, the CIN phenotype seems to be dominant, as it can be conferred on a chromosomally stable, diploid cell when it is fused with a CIN cell. These experiments indicate that the mere presence of an abnormal number of chromosomes is not sufficient to lead to CIN, as the fusion of two non-CIN cells, resulting in tetraploidization, does not result in CIN[16]. Second, a fraction of cancers contain mutations or abnormal expression patterns of spindle-checkpoint genes such as *BUB1* and *MAD2* (REF. 17–21). Recreation of these mutations in non-CIN lines can convert them to CIN with resultant aneuploidy, presumably because of a disruption of the cell's normal error-checking mechanisms, which prevent mis-segregation of DNA during mitosis. Such results indicate that there might be a genetic mechanism underlying CIN in at least a subset of cases.

Many other genetic mechanisms that could potentially lead to CIN have been suggested and discussed[12,22], but there can be no guarantee that the basis of CIN is genetic. Epigenetic events that do not involve mutational changes in nucleotides could certainly have a significant role[23,24]. It is also possible that CIN is related to the abnormal architectural features of the cancer cell. Indeed, many reports of abnormal centrosomes and spindles have been identified in various types of cancer[25,26]. Definitive evidence that such changes are the cause rather than the consequence of CIN, however, will require a much deeper understanding of the mechanisms that normally safeguard the integrity of the chromosome complement. In yeast, more than 100 different genes have been shown to cause a CIN phenotype[27]. Identification of the key human genes responsible for CIN, either through abnormal expression or mutation, is only in its infancy.

Table 1 | **Genetic alterations in cancer cells**

Event	Cause	Disease with increased rate	Detection	LOH
Mutation	Nucleotide-excision repair defect, mismatch-repair defect	XP, HNPCC	Sequencing, microsatellite analysis	No
Conversion	Somatic recombination	Bloom's syndrome	SCE	Yes
Whole chromosome loss	Mitotic error (such as non-disjunction)	Colorectal cancer	FISH	Yes
Structural chromosomal changes, including deletions, translocations and inversions	Double-strand break(s), telomerase defects, recombination	?	M-FISH SKY	Sometimes
Amplification	?	?	CGH, DK	No

This table outlines the types of mutations that are normally found in cancers of all types and their causes. Mutation, chromosome loss, or structural changes can all occur as the first step of a tumour-suppressor-gene inactivation. All of these mechanisms plus mitotic recombination can occur as the second step. Note, however, that homozygous deletions of larger parts of chromosomes are almost always lethal for a cell; so, it is unlikely that large deletions could be both the first and the second hit in one cell. Some of these mutational events can contribute to the loss of heterozygosity (LOH), which is often the cause of the second hit of a tumour suppressor (see text for details). Many disease states are known to have increased rates of specific types of these various genetic events, and are indicated as such above. The experimental techniques used to detect somatic genetic events are a diverse array of molecular and cytological techniques[57–59]. CGH, comparative genomic hybridization; DK, digital karyotyping; FISH, fluorescence *in situ* hybridization; HNPCC, hereditary non-polyposis colorectal cancer; M-FISH, multiplex-FISH; SCE, sister-chromatid exchange; SKY, spectral karyotyping; XP, xeroderma pigmentosum.

The benefits of CIN

Underlying our discussion of the genetic instability of cancers is the implicit assumption that this hypermutability confers a growth advantage on the cell. Just as cancers with MIN have a marked predisposition towards acquiring point mutations in genes that can affect the balance between cell birth and cell death, CIN must also provide leverage towards the unregulated growth of a cancer cell.

We imagine that one advantage that chromosomal instability confers on a cell is an improved likelihood of inactivating tumour-suppressor genes. Mutational inactivation of both alleles of a tumour suppressor must occur for a cell to acquire a growth advantage[28]. Inactivation of the second allele of a tumour-suppressor gene, via mitotic recombination, chromosome loss or other structural change, has historically been defined as 'loss of heterozygosity' (LOH). Accelerated LOH is an obvious mechanism by which CIN can contribute significantly to the process of tumour-suppressor-gene inactivation. LOH observed in colorectal and pancreatic cancers is much more commonly caused by losses of chromosome material than by mitotic recombination[13,29]. It will be of interest to investigate this issue in other common tumour types, as mitotic recombination has been proposed to be significant in the chromosomal abnormalities that are found in retinoblastomas[30].

In addition to inactivation of tumour-suppressor genes, CIN can provide growth advantages to the cancer cell by causing extensive changes in gene expression. When a chromosome is lost (gained), all genes on that chromosome might be expected to be expressed at half (twice) the normal level. At least two processes counter such changes. First, dosage compensation occurs as a result of the numerous feedback and feedforward loops that regulate most cellular pathways. Second, losses of whole chromosomes are usually accompanied by reduplication of the remaining chromosome, leaving a normal chromosome copy number in a cell even when LOH is present[13,29]. Such reduplication might be necessary to protect the cell from death due to haploinsufficiency, in which half-normal levels of a gene product lead to phenotypic effects. Despite these counter-measures, many gross chromosomal changes are likely to induce changes in the expression of at least some of the numerous genes that are present on the affected region of the chromosome. These changes could easily result in growth advantages by increasing cell proliferation or decreasing cell death. It is important to note in this context that even a tiny growth advantage can prove to be a significant force for clonal dominance in a rapidly dividing cell population. A 1% increase in the ratio between cell birth and cell death, for example, will result in a marked clonal overgrowth over time (the affected cell will increase from 0.001% of the population to 99.9% of the population in slightly more than 5 years when the cell divides once every 24 hours).

CIN also provides a mechanism by which the cancer cell can fine-tune its growth characteristics to meet changing environments. Such environments include the stromal and hormonal milieu surrounding metastatic lesions and the chemicals introduced during chemotherapy in an attempt to eradicate the lesion. The chromosomal instabilities found in virtually all solid tumours might, in the future, turn out to be the single largest cause of therapeutic failures.

The timing of CIN

The transformation of a colorectal epithelial cell to a metastatic tumour cell is not a rapid process. Numerous studies have shown that it generally requires 20–40 years for this transition to occur. The process is driven by sequential mutations in oncogenes and tumour-suppressor genes, providing incremental growth advantages to waves of clonal expansions. When during this long process does CIN occur?

To determine whether CIN could be identified in the earliest stages of tumorigenesis, we evaluated 32 tiny adenomas with an average diameter of 2 mm[31]. Using highly quantitative methods, we found that more than 90% of these lesions had allelic imbalances of one or more of the five chromosomes tested. Even when chromosome 5q — where *APC* resides — was excluded because of the expectation that alterations at this locus initiated colorectal tumours, more than two thirds of the tiny tumours showed at least one allelic imbalance of the other chromosomes tested. In many tumours, the allelic imbalances did not occur in the entire population of neoplastic cells, but were evident in only a fraction. By contrast, the chromosome 5q allelic imbalances, when they occurred, generally seemed to affect most neoplastic cells. The presence of allelic imbalances, the molecular equivalent of aneuploidy, in these early tumours supports the idea that CIN occurs very early during tumorigenesis (FIG. 1). As noted above, however, the measurement of a state of imbalance cannot provide conclusive evidence of an increased rate of instability.

This timing for CIN is consistent with the early development of MIN. There are two independent and compelling arguments that support the hypothesis that MIN occurs very early in the neoplastic process. As reviewed

earlier in this article, inactivation of the APC pathway represents the first genetic alteration that directly affects growth. At least a third of MIN cancers contain mutations in *β-catenin* instead of *APC*, whereas *β-catenin* mutations are rare in non-MIN cancers[4]. These observations would be extremely difficult to explain if MIN occurred after APC inactivation, because, in that case, there would be no driving force for *β-catenin* mutation. Additionally, the spectrum of *APC* mutations that occur in most MIN cancers without *β-catenin* mutations is different from that in non-MIN cancers[32]. In particular, there is a higher frequency of alterations in simple repeat sequences in the MIN than in the non-MIN cancers. Again, this observation can not be explained unless MIN occurs before APC inactivation.

Interestingly, one gene that has been proposed as a potential initiator of CIN is *APC* itself[33,34]. Most *APC* mutations that are observed in patients lead to truncation of the encoded protein, with loss of the carboxy-terminal domain that normally interacts with microtubules. Mouse cell lines bearing similarly mutated *Apc* alleles harbour karyotypic abnormalities that are not seen in isogenic lines with wild-type *Apc* genes. However, these *Apc*-mutant cells actually tend to polyploidize in whole-genome increments rather than show the increased rates of losses and gains of one or a few individual chromosomes that is characteristic of cancers. It is therefore unlikely that the polyploidization observed in mouse cell lines with mutated *Apc* alleles represents the CIN that is found in human tumours. Indeed, some well-characterized human colon cancer cell lines with *APC* mutations have chromosome complements that have remained perfectly stable and invariant over thousands of cell divisions *in vitro*. One way of reconciling these disparate findings is to posit that, by promoting endoreduplication, *APC* mutations could provide a cancer cell with a 'genetic buffer' that allows it to tolerate and survive CIN, which itself might have a distinct genetic cause.

The perils of investigating CIN

Two recent studies by Haigis *et al.*[35] and Sieber *et al.*[36] have challenged the idea that aneuploidy (and, by inference, CIN) occurs at the early stages of colorectal tumorigenesis. Although both studies were well executed, they were not designed to detect the kinds of chromosome alterations that are likely to be present in early tumours.

All forms of instability result in a progressive phenotype as tumours mature. Even if the same level of instability were to exist in early benign tumours as in late advanced metastatic lesions, there would be marked differences in the number of genetic alterations that could be detected. Alterations accrue and become fixed in the cell with each wave of clonal expansion, so their quantification is further complicated by the variable number of generations that have occurred between the last bottleneck in tumour growth and the time of analysis, and the variability in microenvironments that the tumour cell encounters at various times during its life history.

There are two practical ramifications of this concept. First, a lower number of alterations per genome should be expected in early tumours than in late tumours, even when the instability is initiated early. Second, certain alterations in early tumours should be expected to only affect a subpopulation of the tumour cells before the clonal fixation. Both of these expectations have been experimentally verified. For example, it has been conclusively shown that pathogenic alterations can be more heterogeneous in adenomas than in carcinomas[37]. Furthermore, even though MMR deficiency occurs very early in the development of MIN tumours, as explained earlier, the resultant changes in simple repeat sequences are observed more commonly in late tumours[38,39].

Neither the study by Haigis *et al.* nor the study by Sieber *et al.* were designed to detect alterations that were not present in a high fraction of the tumour cells. The study by Sieber *et al.* did not use micro-dissected tissues, in which contaminating non-neoplastic cells had been removed. According to the methods described in their paper, LOH would not have been detected unless >75% of the neoplastic cells had lost an allele. Even so, they found ~9% of adenomas that had undergone LOH of one of the three chromosomes analysed. Haigis *et al.* analysed the variation in total numbers of chromosomes rather than LOH. The chromosome number in normal cells had such a large variance in these experiments that it would be difficult to identify a variant population if it did not affect the entire population of neoplastic tumour cells. Moreover, it has been shown

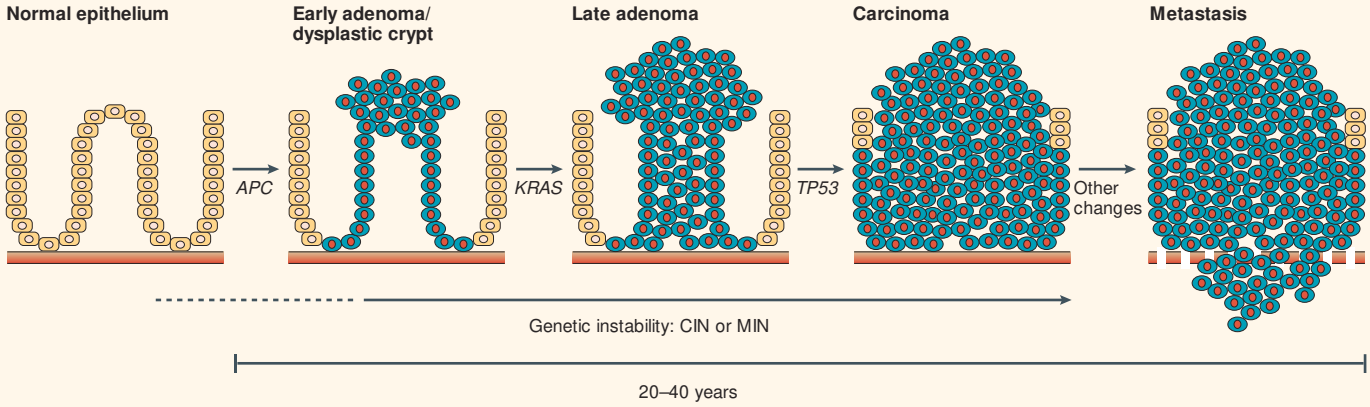

Figure 1 | **A step-wise model of colorectal tumorigenesis.** Colorectal cancers are believed to develop over the course of 20–40 years as a consequence of the episodic accrual of specific mutations in oncogenes such as *KRAS* and tumour suppressors such as *APC* and *TP53*. These mutations arise within the tumour in a characteristic sequence. A single cell within a heterogeneous population acquires a mutation in one such gene, and this mutation soon reaches fixation because of the growth advantage provided to the cell by the mutation. Genetic instability is thought to occur somewhere during the process of colorectal tumorigenesis to accelerate the rate of mutation in dividing cancer cells. Experimental evidence for the presence of chromosomal instability (CIN) in early tumours is limited because of the small size of samples and the possibility that chromosomal alterations can not yet be found in most cells in a young lesion (see text for details). Nonetheless, strong evidence does exist that CIN occurs early on in the tumorigenic process. Whether CIN is the first event in tumorigenesis, and therefore precedes mutation of *APC*, is still a matter of much debate.

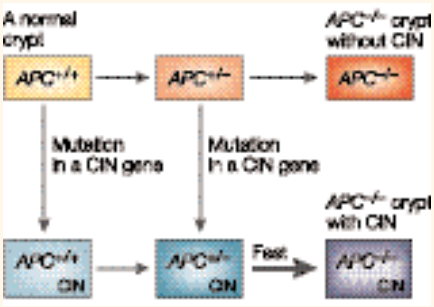

Figure 2 | **The timing of CIN.** Theoretically, the acquisition of a CIN mutation can occur before or after mutation of the tumour-suppressor gene *APC*. In either case, the process that leads to an $APC^{-/-}$ crypt without CIN will require two rate-limiting steps because mutations of both alleles of *APC* need to be inactivated (two hits). Initiation of CIN to lead to an $APC^{-/-}$ crypt can occur either before or after inactivation of the first *APC* allele. In either case, inactivation of the second *APC* allele would not be rate limiting because the rate of loss of heterozygosity (LOH) in a CIN cell is very high. It is possible that the two hits originally described by Knudson refer not to mutation of the two alleles of a tumour suppressor, but to the mutation of a CIN gene and the mutation of the first allele of a tumour suppressor.

that even when an LOH event is associated with loss of a whole chromosome in colorectal cancers, the number of that chromosome type per cell often does not change because the remaining chromosome is duplicated (see discussion above). In fact, the LOH events observed by Haigis *et al.* were not detectable by comparative genome hybridization (CGH), a technique that measures the number of each chromosome and that cannot detect LOH when one chromosome is reduplicated.

In summary, only one molecular genetic study (Shih *et al.*, co-authored by our group) was specifically designed to detect chromosomal alterations of the type expected to occur in adenomas[31]. This study unequivocally identified gross chromosomal changes in adenomas significantly smaller than those examined in the two studies described above. Moreover, numerous cytogenetic studies support this conclusion, even in very small adenomas[40–47]. So, in our view, there can be little doubt that chromosomal abnormalities occur at a relatively early stage of colorectal neoplasia. Whether these abnormalities reflect an underlying CIN, and whether they occur before APC inactivation, are much more difficult questions to answer.

Quantifying CIN

Mathematical analyses have historically provided considerable insights into the nature of the genetic events underlying cancers and can provide an important framework for understanding the role of instability in tumour initiation and progression[48–51]. Consider the evolutionary dynamics of a single crypt. We are interested in comparing the rate with which cells in the colon can mutate the *APC* gene with and without CIN (FIG. 2). First, mutation of *APC* to achieve two hits should be considered. The first hit is likely to be a point mutation, and the second hit can either be a point mutation or a LOH event. The point-mutation rate of *APC* is a product of the error rate in DNA synthesis per nucleotide per cell division and the length in nucleotides of the *APC* gene. Denote this by u (see TABLE 2 for a definition of variables). The rate of LOH of APC in a normal (non-CIN) cell can be denoted by p_0. Denote by N the number of cells in a crypt that are at risk of receiving cancer mutations. These N cells are likely to be stem cells at the base of a crypt, as the high turnover rate of terminally differentiated colonic epithelium would probably prevent these latter cells from acquiring enough cancerous mutations within their short lifetime. In this way, colorectal cancers differ from cancers in other organs, such as in the breast and prostate, where there is no such rapid turnover. The probability that a single crypt, at time t, contains cells with both alleles of *APC* inactivated is a product of the rate of mutation of the first allele and the rate of mutation or LOH of the second, given by:

$$Y(t) \approx Nu(u + p_0)t^2 \qquad (1)$$

Time is measured in units of cell division. The probability increases as a quadratic function of time. The exponent, 2, is the same 2 as in Knudson's two-hit hypothesis: it takes two hits to inactivate a tumour-suppressor gene, in this case *APC*.

Let us now consider the possibility that a mutation responsible for CIN occurs either before the first or between the first and the second APC mutation. Denote by u_c the mutation rate toward CIN (mutation rate per nucleotide multiplied by the total number of nucleotides which could be mutated to give rise to CIN). In CIN cells, the rate of LOH is given by p, which can be approximated to 0.01, as indicated by experimental evidence on rates of centromeric loss[16]. Because of the increased error rate of chromosomal segregation, CIN cells will sometimes produce offspring cells that carry harmful (or lethal) alterations: we include a cost of CIN and denote by r the relative fitness of CIN cells. For example, if $r = 1$, then CIN has no cost; if $r = 0.9$, then CIN has a 10% cost, described by the net rate of proliferation of a cell. The probability that a crypt contains CIN cells with both alleles of *APC* inactivated reflects the probability of acquiring a CIN mutation, the potential cost of CIN and the accelerated rate of LOH given by CIN, and is approximately given by:

Table 2 | **Definition of variables**

Variable	Definition
u	Point-mutation rate of *APC* gene
u_c	Point-mutation rate to achieve CIN
p_0	Rate of loss of heterozygosity (LOH) of *APC* in a non-CIN cell
p	Rate of LOH of *APC* in a CIN cell
N	Number of stem cells in a crypt
t	Time (in units of cell division)
r	Relative fitness of a CIN cell [(1 – r) is the "cost" of CIN]

$$Y(t) \approx Nuu_c\left(2\rho + \frac{r\rho}{1-r}\right)t^2 \qquad (2)$$

Here, $\rho = (1 - 1/r)/(1 - 1/r^N)$ denotes the probability that a CIN cell with relative fitness, r, takes over a compartment of N stem cells. Equation (2) holds in certain limits; for a detailed discussion and how equation (2) was derived, we refer to REF. 46. Importantly, consider a simplifying assumption: if the cost of CIN is negligible, then $r \approx 1$, and the rate of LOH under CIN can be ignored because it occurs very quickly. Now, a good approximation for the probability that a crypt has cells with both alleles of *APC* inactivated is given by:

$$Y(t) \approx 2uu_c t^2 \qquad (3)$$

Note that both expressions for $Y(t)$ are quadratic functions of time. The inactivation of *APC* via CIN also follows a kinetic with two rate-limiting steps: one CIN mutation and one *APC* mutation. The second hit in *APC* is greatly accelerated by the presence of CIN and is therefore not rate limiting. It is possible, therefore, that the two hits originally described by Knudson refer not to mutation of the two alleles of a tumour suppressor, but the mutation of a CIN gene and the mutation of the first allele of a tumour suppressor (loss of the second allele would follow extremely rapidly and would not be rate limiting).

If a genetic pathway of tumorigenesis requires the inactivation of two or more tumour-suppressor genes, then it becomes increasingly likely that CIN precedes the inactivation of the first tumour-suppressor

gene in most cancers. Obviously, the elimination of each subsequent tumour-suppressor gene is greatly accelerated by the presence of CIN.

Whether or not CIN precedes *APC* inactivation can simply be decided by comparing $X(t)$ and $Y(t)$. If $Y(t) > X(t)$, then most neoplasias should be initiated by CIN cells. Consider all dysplastic crypts that have been generated up to time t: the fraction of crypts where CIN came before the first or second hit in APC is given by $Y(t)/[X(t)+Y(t)]$. We will next present some numerical examples after discussing the cost of CIN and the different types of CIN genes.

The wages of CIN

Let us now estimate the potential cost of CIN. The probability of losing a chromosome is given by p. Suppose losing a chromosome is lethal unless this chromosome is the second copy of chromosome 5 in a cell that has already sustained a mutation of *APC* in the opposite allele. This scenario poses a strong bias against CIN, but provides some upper limit for the cost of CIN in terms of relative fitness, r. As there are 46 chromosomes, $r \approx (1-p)^{45}$. For example, if $p = 0.01$ then $r = 0.63$. If $p = 0.002$ then $r = 0.91$.

The cost of CIN is much lower (that is, r is higher) in polyploid cells, because reducing the copy number of a particular chromosome from 4 to 3 would be expected to have only small effects on the relative fitness of a cell. So, tetraploidization could be an effective strategy by which cancer cells could reduce the cost of CIN. This discussion of the potential cost of CIN therefore mathematically formalizes the experimental hypothesis presented earlier, that the near-triploid state of cancer cells can be achieved by tetraploidization followed by individual chromosome loss.

In principle, CIN can have no cost at all or can even increase the relative somatic fitness of a cell for the reasons discussed above. The increased probability of deleterious mutations could be compensated by a faster cell-cycle time if a CIN-gene mutation causes a certain checkpoint to be less carefully monitored or the expression of a negative growth regulator to be suppressed. Therefore, it also makes sense to consider the effect of hypothetical CIN genes that do not reduce the somatic fitness of the cell.

Classes of CIN

Our poor understanding of the way that CIN is achieved forces us to consider numerous mechanisms by which CIN genes can become activated. We propose that three different classes of CIN gene should be considered according to the mutational events that are required for engaging CIN:

Class 1 — trigger CIN if one allele of the gene is lost (haploinsufficiency).
Class 2 — trigger CIN if one allele is mutated in a dominant (negative) fashion.
Class 3 — require mutations in both alleles to trigger CIN (recessive at the cellular level).

MAD2 is an example of a class-1 gene[20], *BUB1* is a class-2 CIN gene[18], and *BRCA1* and *BRCA2* are class-3 CIN genes[52]. If there are n_1 class-1 genes and n_2 class-2 genes, then the mutation rate towards CIN is given by $u_c = 2n_1(u+p_0)+2n_2u$. The ease with which CIN can be triggered (reflected by the magnitude of u_c) would intuitively be expected to have an impact on the likelihood of CIN mutation occurring before *APC* mutation. Our analysis shows that class-3 genes cannot contribute to a CIN that precedes inactivation of APC; this mutational pathway would be too slow. However, class-3 genes could become important when considering genetic pathways of tumour progression that require inactivation of several tumour-suppressor genes.

Numerical examples

Our calculations show that the presence of a fairly small number of CIN genes in the human genome could ensure that CIN mutations come before the inactivation of the first tumour-suppressor gene on the way to cancer. In this section, we calculate the minimum number of CIN genes that are required for CIN to initiate colon cancer (or any other cancer that starts with the inactivation of a tumour-suppressor gene).

First, we discuss the situation in which the CIN phenotype has a negligible cost. In this case, CIN will precede APC inactivation provided that $u_c > N(u+p_0)/2$. This result is obtained by comparing equations (1) and (3). For this inequality to hold, the number of potential class-1 genes per genome must exceed $N/4$ and the number of potential class-2 genes in the genome must exceed $(N/4)(1+p_0/u)$. If there are $N = 4$ stem cells per crypt and p_0 is of the same order as u, then only one class-1 or two class-2 genes per genome would be needed to ensure that CIN precedes APC inactivation. If p_0 were 10 times greater than u, then one class-1 gene would still be needed but three class-2 genes would be needed to get a CIN phenotype that preceded APC inactivation.

Let us now suppose that CIN has a significant cost, given by $r \approx (1-p)^{45}$. If there are $N = 4$ stem cells per crypt and the rate of LOH in a CIN cell is given by $p = 0.01$, then the existence of at least three class-1 genes in the human genome would ensure that a mutation in any one of them would precede APC inactivation in most dysplastic crypts. For an LOH rate of $p = 0.001$ in CIN cells, only two class-1 genes would be sufficient for CIN to precede APC inactivation. If p_0 were of order u, then five class-2 target genes per genome would be needed if the resultant instability determined by any of them were $p = 0.01$, but only three such genes would be required if the resultant instability was $p = 0.001$. For $p_0 \approx 10u$ and $p = 0.001$, we need 12 class-2 genes to get a CIN phenotype that preceded APC inactivation. Many other specific cases can be calculated. Our equations provide the general answer.

Conclusions

The results from these mathematical models corroborate experimental evidence for the presence of anenploidy in early colorectal cancers. Given our presumptive approximations for the parameters constraining the number of cells in a colon and their mutational rates, there might only be a small pool of potential CIN genes necessary for chromosomal instability to be an early event in tumorigenesis. However, important questions remain unanswered. What exactly is the nature of this instability? Further elucidation of the genetic basis for CIN will hopefully provide us with a mechanistic understanding of this process. How many genes like *BUB1* or *MAD2* are mutated or lost in cancers and provide a genetic basis for CIN? So far, only a handful of genes have been found, and these seem to be inactivated only rarely. Crucial to our understanding of CIN is the differentiation between processes required for the maintenance of genomic integrity in normal cells and those that are abrogated and a cause of CIN in cancers. Does the early onset of CIN in cancers imply that instability is more important for the initiation than the maintenance of cancers? The answer to this question will be important in our attempts to gain insight into the evolutionary process of cancer and will also help to determine whether the inhibition of chromosomal instability should be a new avenue to pursue when therapeutically targeting neoplasias.

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