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Defining 'chromosomal instability'

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Most scientists agree that the majority of human solid malignant tumors are characterized by chromosomal instability (CIN) involving gain or loss of whole chromosomes or fractions of chromosomes. CIN is thought to be an early event during tumorigenesis and might therefore be involved in tumor initiation. Despite its frequent occurrence in tumors and its potential importance in tumor evolution, CIN is poorly defined and is used inconsistently and imprecisely. Here, we provide criteria to define CIN and argue that few experimental approaches are capable of assessing the presence of CIN. Accurate assessment of CIN is crucial to elucidate whether CIN is a driving force for tumorigenesis and whether a chromosomally unstable genome is necessary for tumor progression.

Genomic instability in malignant solid tumors

Molecular biology has unequivocally shown that tumors accumulate numerous mutated genes [1]. Underlying genomic instability might accelerate the accumulation of these mutations. Many theories regarding the timing and the impact of genomic instability (see Glossary) in cancer, such as the mutator phenotype [2], telomere dysfunction [3] or the aneuploidy hypothesis [4], have been proposed [5]. Among the most intriguing questions in this context are the following. (i) Does genomic instability represent an early or late event in tumor progression? (ii) Is genomic instability the driving force for tumorigenesis? (iii) Is an unstable genome necessary for tumor evolution? [6,7].

Among different forms of inherent genomic instability (Box 1), chromosomal instability (CIN) is the most prevalent form [8]. Despite the possible importance of CIN for tumor initiation and progression, it is surprising that CIN is poorly defined and that the use of CIN is frequently inconsistent and imprecise. For example, CIN is used to describe cancers that are shown, by cytogenetics or flow cytometry, to have an aneuploid or polyploid karyotype. It has also been used to describe cells that harbor multiple structural chromosomal rearrangements. Others describe CIN as frequent alterations in chromosome number [8–16].

Here we provide a definition for the widely used term CIN, define how it can be distinguished from genomic instability, aneuploidy and polyploidy and assess the suitability of current methods for the detection of CIN. The clarification of the type of chromosomal condition that is referred to by CIN and how CIN should be properly investigated and measured could contribute to the better assessment and treatment of CIN in pre-cancerous and early-stage lesions. For a discussion of the significance of unstable chromosomes in cancer and the mechanisms that might promote CIN, see Refs. [6,7,9,17–19].

Genomic versus chromosomal instability

The vast majority of malignant diseases have some underlying form of instability [9]. 'Genomic instability' refers to various instability phenotypes, including the CIN phenotype. Currently, instability phenotypes are best characterized for colorectal cancer (Box 1). Here, instability is subdivided into CIN and microsatellite instability (MIN or MSI) [8,9]; their occurrence is usually mutually exclusive in colorectal cancer cell lines [20].

The MIN phenotype occurs in ~15% of all tumors and is often not associated with chromosomal changes [21]. A third of these cases (~5% of all colorectal cancers) are associated with a family history of colorectal cancers and are often caused by mutations in mismatch repair genes [i.e. *human MutS homolog 2* (*hMSH2*), *human MutL homolog 1* (*hMLH1*), *human MutS homolog 6* (*hMSH6*), and *human postmeiotic segregation increased 2* (*hPMS2*)],

Glossary

Aneuploidy: Having an unbalanced number of chromosomes or large portions of chromosomes. An alteration in the number of intact chromosomes is termed whole-chromosome aneuploidy. Segmental aneuploidies refer to unbalanced regions of chromosomes, e.g. caused by deletions, amplifications or translocations.

Chromosomal instability (CIN): As described in the text, a poorly defined but often used expression. CIN should describe the rate (cell-to-cell variability) of gain or loss of whole chromosomes or fractions of chromosomes. This definition encompasses the rate of both whole-chromosome and segmental chromosomal aneuploidies.

Comparative genomic hybridization (CGH): A technology through which a test and reference DNA sample are differentially labeled to identify copy number changes in the respective test genome. In conventional CGH, both test and reference DNAs are hybridized to metaphase spreads. To improve resolution, the DNAs are now hybridized to immobilized DNA targets on an appropriate surface, referred to as array-CGH.

CpG island methylator phenotype (CIMP): The CIMP phenotype is characterized by extensive promotor methylation, methylation of *MLH1* and a strong association with the V600E mutation in *BRAF*; CIMP cells are chromosomally stable but exhibit MIN.

Genomic instability: Genomic instability includes CIN but also refers to other forms of presently known genomic instabilities, such as microsatellite instability (MIN) or CIMP. In addition, other, yet unknown, forms of instabilities might exist.

Loss of heterozygosity (LOH): LOH in a cell represents the loss of one parent's contribution to a portion of the cell's genome and is the result of a genomic change, such as mitotic deletion, gene conversion or chromosome mis-segregation.

Microsatellite instability (MIN or MSI): In diploid tumors, genetic instability that results from a high mutation rate, primarily in short nucleotide repeats. Cancers with the MIN (or MSI) phenotype are associated with defects in DNA-mismatch-repair genes.

Multiplex-FISH (M-FISH) or spectral karyotyping (SKY): Techniques for painting each chromosome of an organism in a different color, which is useful for deciphering numerical and structural chromosomal aberrations.

Polyploidy: A polyploid cell has more than two sets of chromosomes (two sets being the prevalent diploid state). A tetraploid cell has four sets of chromosomes, an octaploid has eight sets, and so on.

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Box 1. Genomic instability phenotypes in colorectal cancer

- Chromosomal instability (CIN): occurs in ~80–85% of all colorectal cancers [8,9].
- Microsatellite instability (MIN or MSI): associated with a germline mutation in a mismatch repair gene (i.e. *MLH1, MSH2, MSH6* and *PMS2*) causing hereditary nonpolyposis colorectal cancer (HNPCC); occurs in ~5% of all colorectal cancers [21].
- Sporadic MIN: in most cases, this phenotype overlaps with the CpG island methylator phenotype (CIMP) and is found in most tumors with mutations in the *BRAF* oncogene; occurs in ~10% of all colorectal cancers [22,23].
- Potentially other, presently not yet identified, forms of instabilities: some tumors display no signs of MIN, CIMP, or CIN [26]. Thus, a subset of tumors without any instability might exist, or these tumors exhibit a new form of instability that has not yet been identified. Large-scale sequencing efforts might have the potential to elucidate such a putative new form of instability.

resulting in hereditary nonpolyposis colorectal cancer (HNPCC) [21].

About two thirds of MIN-tumors occur sporadically, and these tumors usually have a V600E substitution in B-Raf [22]. Recent findings have shown that most cases of sporadic MIN overlap with the CpG island methylator phenotype (CIMP), which is characterized by extensive promotor methylation [23].

The available data indicate that the CIN phenotype is the most common instability phenotype, occurring in ~80–85% of all colorectal cancers [8]. The 'classical' pathway of colorectal tumorigenesis resulting in CIN(+), MIN(−) and CIMP(−) tumors involves the initiation of an adenomatous polyp through bi-allelic *adenomatous polyposis coli* (*APC*) mutations. The affected polyps become progressively larger and more dysplastic, often acquiring mutations in *KRAS*, a potent oncogene, and *SMAD4* (SMAD4 participates in cell signaling); loss of heterozygosity (LOH) and a mutation in the tumor suppressor gene *TP53*, by which stage the polyp has become an early carcinoma [24,25]. Genomic instability might occur at some point during the process of tumorigenesis. Intriguingly, some colorectal cancers display none of these instability phenotypes (i.e. CIN, MIN or CIMP) [26], and it is not known whether they have acquired a different form of genomic instability.

Aneuploidy and polyploidy versus chromosomal instability

In principle, there are two ways for cells to become aneuploid: they can develop alterations in the number of intact chromosomes, which is known as whole chromosome aneuploidy and originates from errors in cell division (mitosis). Alternatively, chromosomal rearrangements can occur, including deletions, amplifications or translocations, which arise from breaks in DNA and result in segmental aneuploidy.

Segmental aneuploidy is a well-established cause of tumor development. For example, telomere dysfunction and inactivated checkpoints can, through fusion-bridge-breakage cycles, result in unbalanced, nonreciprocal translocations, i.e. segmental aneuploids [3]. By contrast, the contribution of whole chromosome aneuploidy to cancer is controversial [17]. Some mouse models for spindle assembly checkpoint failure associated with chromosome number instability show early aging-associated phenotypes without an increased predisposition to spontaneous tumor development [18,27,28], whereas other mouse models support the idea that aneuploidy might promote oncogenesis [29,30]. The best evidence in favor of a causative role for a spindle checkpoint gene in tumorigenesis is the discovery that inherited mutations in *budding uninhibited by benzimidazoles 1* (*BUB1B*) result in chromosomal segregation defects and increased tumor incidence in humans [31].

However, aneuploidy is not synonymous with CIN; this point is best exemplified by humans with Down syndrome (DS). The majority of cases of DS are associated with a trisomy 21 in all cells [32]. However, because each cell harbors the same chromosomal composition without any variation, humans with DS do not have CIN, although each cell is aneuploid. Therefore, CIN should not be equated with aneuploidy. One might argue that the stable aneuploidy in DS cells facilitates the onset of cancer and CIN because epidemiologic data indicate that trisomy 21 predisposes patients to leukemia, especially acute megakaryoblastic leukemia (AMKL) [33]. However, gene dosage alterations caused by the trisomy 21 alone do not seem to be sufficient to cause AMKL, because current transformation models suggest that somatic mutations are also required in the X-chromosome gene, *GATA-binding protein 1* (*GATA1*), which encodes an essential transcriptional regulator of normal megakaryocytic differentiation [33]. Furthermore, although the risk for leukemia is increased in individuals with DS, solid tumors of childhood and adult nonhematologic cancers are significantly less frequent [34,35], suggesting that the trisomy 21 constitution does not, in general, predispose patients to malignant diseases or CIN. The same considerations are also true for polyploidy: the presence of triploid or tetraploid chromosome constitutions by itself does not make a cell population unstable.

The differences between aneuploidy, polyploidy and CIN can be further clarified by considering the cell-to-cell variability, the state and the rate of chromosomal changes.

Cell-to-cell variability, state and rate of chromosomal changes

The existence of genetic alterations in a tumor, even when frequent, does not necessarily indicate that the tumor is genetically unstable. For example, if a tumor is analyzed by comparative genomic hybridization (CGH) using DNA extracted from a large cell population, it is likely that several gains and losses will be identified [36]. Such gains and losses provide evidence for chromosomal rearrangements that might be extensive and complex. However, chromosomal imbalances observed by CGH do not enable conclusions to be drawn about the instability pattern, because the observed gains and losses could be the result of a stable, but aneuploid, clone that has obtained a growth advantage under certain selective pressures. Thus, an array-CGH profile describes a state of chromosomal alterations; therefore, the presence of multiple gains or losses within a tumor genome cannot be equated with CIN. Instead, the presence of CIN can be more reliably assessed by measurements of the cell-to-cell variability, which provides a good indicator for CIN but does not accurately

measure the rate of chromosome number alterations. Strictly, the determination of rate requires time-based measurements, which could establish how frequently the karyotypes of sister or daughter cells are not identical to each other or to their mother cells. Such rate experiments are feasible in cell culture experiments [8,9]; however, it is nearly impossible to perform such analyses on tissue samples obtained by surgery or fine needle biopsies. In summary, the rate of chromosomal changes indicates the degree of cell-to-cell variability, and this rate might differ significantly within a cell population with CIN.

CIN-high versus CIN-low

It is common practice to stratify MSI as either MSI-high or MSI-low depending on the number of markers that have microsatellite alterations [37–39]; similarly, there are CIN-high and CIN-low phenotypes [40,41]. It was suggested that severe deficiencies for proteins involved in DNA damage sensing and DNA repair pathways might accelerate aging, whereas less severe mutations in these same pathways might predispose individuals to cancer [19]. By analogy, one could argue that the aging phenotype in some mouse CIN models could be explained through a similar pattern. Therefore, CIN-low (i.e. a low rate of cell-to-cell variability), rather than CIN-high (i.e. a high rate of cell-to-cell variability), phenotypes might be important for cancer initiation. However, the identification of cells displaying the CIN-low phenotype probably requires particularly sensitive methods, such as the evaluation of very large cell numbers and assays providing full karyotypic information.

Methods for the analysis of CIN

The imprecise use of the term CIN is also a result of the inappropriate methods used to address the presence of CIN. Determination of CIN requires approaches capable of monitoring cell-to-cell variability or the rate of chromosomal changes. Analyses of the timing of CIN and the detection of the kinds of chromosome alterations likely to be present in early tumors is further hampered by the small size of samples and the possibility that chromosomal aberrations might not yet be present in most cells. For these challenging analyses, several methods exist, each with different strengths and weaknesses (Table 1).

Single cell approaches

The key strength of single cell approaches is their ability to perform analyses on a cell per cell basis, whereas the molecular or multiple cell methods are performed on a merged population of cells in which chromosomal instability is often masked. Interphase fluorescence *in situ* hybridization (FISH) enables the rapid screening of hundreds of cells and provides cellular chromosomal copy numbers for the regions included in the respective probe set [36]. Especially popular are chromosome-specific centromere probes, because their use usually results in signals with high fluorescence intensities, thus facilitating evaluation. Additional or missing chromosome-specific centromere signals are usually interpreted as evidence for whole chromosome aneuploidy [8,10–15]. By contrast, region-specific probes are used to assess the presence of selected regions on chromosome arms. A missing signal might indicate either segmental aneuploidy or whole chromosome aneuploidy; probe sets can be tailored to distinguish between them [36]. For example, if probes for both the long and short chromosome arms (p- and q-arms) are applied and both probes simultaneously have a reduced or an increased signal, loss or gain of the entire respective chromosome is likely. The disadvantage of interphase FISH is that only a limited number of probes can be used simultaneously. Thus, chromosomal copy number changes could be missed.

Karyotyping, either by traditional banding analysis or by 24-color FISH [i.e. multiplex FISH or spectral karyotyping (SKY)], yields information about chromosomal copy numbers, chromosomal structural changes and cell-to-cell variability [36]. However, the preparation of metaphase spreads from solid tumors is labor intensive and requires skilled multidisciplinary teams including surgeons, pathologists and cytogeneticists. Furthermore, metaphase spreads from even short-term cultures might acquire culturing artifacts, which can mask characteristics of the tumor.

Lagging chromosomes or chromosome fragments can be excluded from the main daughter nuclei during cell division and form small nuclei within the cytoplasm; these are termed micronuclei. Thus, the presence of micronuclei is a surrogate marker for CIN; unlike other approaches, this detection method is based on cell morphology without direct visualization of chromosomes or genomic regions. Micronuclei counting is a very popular method of CIN analysis because it enables the rapid evaluation of hundreds of cells [15,16]. However, it cannot distinguish whole chromosome aneuploidy from segmental aneuploidy.

By contrast, array-CGH easily identifies both whole chromosome and segmental aneuploidies [36]. Recently developed protocols for the isolation of individual cells and unbiased whole genome amplification enable these analyses to be performed using DNA derived from as little as a single cell [42,43]. Therefore, single cell array-CGH currently offers the best resolution for the assessment of CIN and the presence of whole chromosome or segmental aneuploidies. However, single cell array-CGH is not amenable to automation and therefore does not represent a high-throughput approach.

Multiple cell approaches

Several cytogenetic and molecular techniques are available that use a multiple cell approach (Table 1). Although these technologies are extremely valuable for the detection of aneuploidies, they allow, at best, only indirect conclusions to be drawn regarding the presence of chromosomal instability.

Conventional array-CGH usually uses DNA from multiple cells and therefore describes the state, rather than cell-to-cell variability, or the rate, of chromosomal changes in a tumor. However, array-CGH offers the opportunity to detect and evaluate low-level mosaicisms within populations containing as few as 10–20% abnormal cells [44]. Because of this ability, it should be possible to estimate whether chromosomal imbalances are present in all cells or in just a subset.

Table 1. Current methods for the assessment of chromosomal changes in solid tumors[a,b]

Method	Chromosomal change detected	Cell-to-cell variability (rate)[c]	State[c]
Interphase-FISH with centromere probes	WCA	++	++
Interphase-FISH with chromosome arm/region-specific probes	WCA and SA	++	++
Karyotyping (banding analysis/24-color karyotyping)	WCA and SA	++	++
Micronuclei counting	WCA and SA cannot be distinguished	+++	∅
Single cell-array CGH	WCA and SA	+++	+++
Conventional array-CGH (DNA extracted from multiple cells)	WCA and SA	+	+++
Digital PCR	Allelic imbalance as a surrogate for SA	++	∅
Flow cytometry	WCA and SA cannot be distinguished	+	+++
Loss of heterozygosity	SA	∅	+++
SNP-array	WCA, SA, LOH and LOH without copy number change	∅	+++

[a]FISH, fluorescence *in situ* hybridization; WCA, whole chromosome aneuploidy; SA, segmental aneuploidy; CGH, comparative genomic hybridization; LOH, loss of heterozygosity.
[b]The first five rows indicate single-cell approaches; the last five rows indicate multiple cell approaches.
[c]The usefulness for assessing the cell-to-cell variability or the rate of chromosomal changes (which best indicates chromosomal instability) and the state of chromosomal alterations (which does not provide accurate data about a possible instability) is outlined in the following rating scale: +++, best currently available approach; ++, less suitable, however, still provides valuable information; +, reveals, at best, some indirect information. ∅, unsuitable.

Digital polymerase chain reaction (PCR) or digital single nucleotide polymorphism (SNP) analysis is a PCR-based approach in which the alleles within a tumor sample are individually counted [45]. Digital PCR identifies allelic imbalances, which reflect gains and losses of particular chromosomal regions. The presence of allelic imbalances has been used as a marker for CIN in early-stage tumors [46].

Flow cytometry efficiently allows the identification of the relative nuclear DNA content and ploidy level of many cells. A flow cytometer measures cells in suspension that flow in single file through an illuminated volume where they scatter light and emit fluorescence; the intensity of the fluorescence correlates with the DNA content. All cells that have equal quantities of the cellular content being measured (e.g. DNA) form a defined peak. Similarly, cells with increased or decreased DNA content because of aneuploidy are visible as additional peaks. However, flow cytometry does not provide reliable information about cell-to-cell variability; moreover, the presence of cells with different ploidy levels does not necessarily indicate CIN.

In cancer, LOH is identified by the presence of heterozygosity at a genetic locus in an organism's germline DNA and the absence of heterozygosity at the locus in the cancer cells. LOH can arise by several pathways, including deletion, gene conversion, mitotic recombination and chromosome loss. Because LOH analysis is performed using DNA from multiple cells, it describes a state but not a rate of variability. In recent years, analyses that use SNP arrays have become popular because they offer the unique opportunity to detect, simultaneously, DNA copy number changes and LOH [47]. The hybridization of single cell amplification products to SNP arrays has not been reported, however, implying that this array-platform is not yet suitable for the estimation of CIN.

Important criteria for a CIN definition

Many published results reporting CIN describe the state, rather than the cell-to-cell variability or the rate, of chromosomal changes. However, CIN is not synonymous with the state of aneuploidy that is observed in a static image of the chromosomal content of a cancer cell. Therefore, several parameters should be included in a description of chromosomal changes to determine the presence of CIN.

- Because CIN refers to the rate with which whole chromosomes or large portions of chromosomes are gained or lost in cancers, the rate should be described as cell-to-cell variability or variability between cell populations.
- The rate of chromosomal changes in a test cell population should be compared with a reference cell population. This comparison will be especially important in the detection of the CIN-low phenotype.
- To achieve accurate quantification, the number of cell divisions that a population has undergone should be related to the rate of chromosomal change.
- An optimal CIN assay should reliably measure not only the rate of whole chromosome changes but also the rate of other chromosomal changes, such as rearrangements, deletions, insertions, inversions and amplifications. Thus, the assay should be capable of determining the presence of both whole chromosome and segmental aneuploidies.
- Polyploid cells should be identified, and results should be recorded separately from those obtained for diploid cells. The fitness cost of CIN is much lower 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 [6]. Therefore, tetraploidization could be an effective strategy by which cancer cells could reduce the cost of CIN. Tetra- and triploid cells might therefore be more prone to chromosomal changes, which should be considered in determining the presence and degree of CIN.
- Appropriate statistical means should be used to establish if the rate of chromosomal changes in a test population differs significantly from a reference population.

Concluding remarks

Many published studies have assessed aneuploidy and polyploidy and equated these observations with

chromosomal instability (CIN). However, conclusive evidence for an increased rate of instability can only be achieved by specialized technologies capable of measuring cell-to-cell variability, and the description of such results should contain several parameters as outlined above. Because of frequently used yet insufficient technologies, the existence of CIN in early tumor stages remains elusive [6,7].

Further experimental developments are needed to accurately assess CIN in cell populations. CIN has been formally shown only 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 (e.g. deletions, insertions, inversions and amplifications). However, available cytogenetic data indicate that these latter changes are at least as common as losses or gains of whole chromosomes (see the Mitelman Database of Chromosome Aberrations in Cancer; http://cgap.nci.nih.gov/Chromosomes/Mitelman). It is essential for future research to discern if such changes reflect underlying CIN, which is probably the result of mechanisms other than disturbance of the chromosome segregation machinery, as is the case for whole chromosome instability [10,11]. Further refinements in single cell array-CGH [42,43] might enable researches to address this question.

Future research and a more careful assessment of CIN should help refine our knowledge about its timing in tumorigenesis and to establish the exact role of CIN in tumor initiation and progression.

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