Determinants and Clinical implications of Chromosomal Instability in Cancer

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Abstract

One of the most common characteristics of cancer genomes is their aberrant architecture, ranging from small insertions or deletions to large chromosomal alterations. Chromosomal instability (CIN) underpins much of the intra-tumoural heterogeneity observed in cancers and drives phenotypic adaptation during tumour evolution. There is an urgent need to increase our efforts to target CIN as if it were a molecular entity. Indeed, CIN accelerates drug resistance acquisition, tumour relapse and treatment failure that plagues current therapies. Identifying novel strategies to modulate CIN and to exploit the fitness cost associated with aneuploidy in cancer is therefore of paramount importance for the success of cancer medicine. Modern sequencing and analytical methods greatly facilitate the cataloguing of somatic copy number alterations (SCNAs) and offer new possibilities to better exploit the dynamic process of CIN. Here we will review the principles governing CIN propagation in cancer, how CIN may impact on immune checkpoint blockade therapy and survey vulnerabilities associated with CIN that could offer therapeutic opportunities.

Introduction

Aneuploidy is one of the most striking and widespread features of human cancers, with the vast majority of tumours displaying various types of SCNAs including segmental aneuploidies, focal events, or whole-chromosome aneuploidies. Considering only the most frequent cancers, approximately 60% of lung tumors, 60-80% of breast tumors, 70% of colorectal tumors and 30 % of prostate tumors deviate from a diploid karyotype[1-6]. Tumours that do not feature gross aneuploidy often display hypermutation due to mismatch repair deficiency or POLE/POLD mutations, which may reflect the limits that cancer cells can handle in terms of genetic instability[7]. CIN refers to the ongoing acquisition of genomic alterations that can involve gain or loss of whole-chromosomes (w-CIN) or structural aberrations (s-CIN), which range from point mutations to small-scale genomic alterations and gross chromosomal rearrangements. However, aneuploidy (an aberrant genomic state) and CIN (the property of displaying a high rate of genomic changes) may differ in their prognostic value, a distinction that warrants careful investigation. In this review, we will discuss how CIN impacts upon tumour evolution, provide an overview of the causes of CIN in cancer with an emphasis on the mechanisms enabling CIN propagation, and strategies to target CIN in cancer.

CIN:opening Pandora's box

Mitotic causes of CIN

CIN cells acquire a high rate of SCNAs during cancer cell proliferation, creating genetic heterogeneity within the population. A myriad of defects can result in frequent missegregation of chromosomes during cell division. These mechanisms and their causative role in cancer have been reviewed in detail previously[8,9]. They include defects that directly impinge on the chromosome segregation machinery, such as altered microtubule spindle dynamics, mechanisms required to correct erroneous microtubule-kinetochore attachments, and defects affecting the mitotic checkpoint or sister-chromatid cohesion[8-14].

Supernumerary centrosomes are frequent in cancer and threaten genome stability by increasing the probability of creating merotelic attachments, a type of microtubule-kinetochore attachment defects that does not trigger the mitotic checkpoint[12,15]. Failure to cluster extra centrosomes into two poles leads to a multipolar division, most likely lethal due to an excessive loss of chromosomes[15-18] (Figure 1).

Genome-doubling or tetraploidization, which may arise from a cell division failure or endoreplication (re-replication without intervening mitosis) amongst several mechanisms[19], directly impair chromosome segregation fidelity during ensuing divisions due to the presence of extra centrosomes[15,20] (Figure 1). Tetraploidization is not only linked to cancer development but is also part of the normal development program of differentiated cell types such as hepatocytes, or megakaryocyte and placental trophoblasts which can become highly polyploid. In addition, tetraploidy is found in ageing tissues and in response to various stresses[21,22]. Genome doubling is a frequent feature of human cancers, reported in over 40% of lung, head and neck, breast, bladder, colorectal, oesophageal and ovarian cancers[2,23,24]. Of note, sequencing-based studies can identify tumors that have undergone WGD during their development, even if the ploidy is no longer tetraploid at diagnosis due to chromosome losses. This explains the possible discrepancy with cytometry-based studies where estimates are based on cells carrying an exact tetraploid DNA content. For example, computational approaches using genomics estimated that over 50% of breast cancer[2] had undergone WGD, while a large scale cytometry-based study detected tetraploid cells in 32% of tumors[25]. Genomics studies suggest that genome-doubling is a relatively early event in the evolution of several cancers and precedes the acquisition of additional SCNAs and subclonal expansion[23,24,26,27]**.** Tetraploid cells have also been detected in pre-malignant lesions in oesophageal, cervical, breast and head and neck cancers[25,28-30]. Genome doubling could therefore represent the CIN-initiating event in an important proportion of human cancers.

Structural defects trigger CIN

Aneuploid tumours almost invariably display both numerical and structural chromosomal aberrations. Pre-mitotic defects such as replication stress can generate chromosome fusions resulting in dicentric chromosomes (telomere fusion for example) and acentric chromosome fragments, both of which may be randomly distributed to daughter cells[31]. DNA bridges from dicentric chromosomes can also physically prevent cell division and generate tetraploid cells which are inherently prone to CIN[32-35]. Under-replicated regions may also prevent the physical separation of chromosomes during mitosis, leading to aneuploidy[36]. Numerical chromosomal aberrations can be symptomatic of DNA replication stress without underlying defects in the chromosome segregation machinery. Replication stress therefore provides an alternative route to generate complex karyotypes through the uneven distribution of damaged genetic material during division.

wCIN, sCIN and nuclear envelope defects

Recent studies indicate that missegregated chromosomes are prone to accumulate mutations and structural defects. For example, mitotic errors can result in lagging chromosomes during the partitioning of DNA into daughter cells, which may become trapped during cell division or isolated and form micronuclei. Both situations create a context whereby the DNA may sustain extensive DNA damage and chromosomal rearrangements including chromothripsis [37,38]. Interestingly, micronuclei and DNA bridges both display nuclear envelope (NE) disruption and therefore loose compartmentalisation with the cytoplasm, potentially exposing DNA to reactive oxygen species and cytoplasmic enzymes. In micronuclei, aberrant DNA replication correlates with NE collapse, the massive accumulation of DNA damage and chromothripsis[37-39]. Importantly these observations are not limited to *in vitro* analyses, as micronuclei displaying disrupted NE and DNA damage accumulation could readily be found on NSCLC paraffin samples[39].

NE integrity is also lost when dicentric chromosomes create ultrafine bridges, which can also lead to chromothripsis and hyper-mutation (kataegis) of localized chromatin regions[40]. NE loss exposes ultrafine bridges to a cytoplasmic nuclease creating single stranded DNA, the substrate for mutagenic APOBEC3 enzymes, which could explain the APOBEC mutational signature often found near rearrangement breakpoints[40,41]. The physical yet often transient isolation of DNA during CIN may contribute to the highly localized nature of APOBEC-driven mutations in cancer, as well as its appearance following the onset of CIN during tumour evolution[42-44].

Interestingly, cell migration through tight spaces and excessive cytoskeletal forces exert pressure onto the nucleus, leading to NE rupture, chromatin extrusion, DNA damage[45-47] and karyotypic abnormalities[47]. The process of epithelial-to-mesenchymal transition induced by TGF-β often associated with metastasis, was also linked to chromosomal instability and NE defects[48]. Physical constraints and paracrine effects associated with cancer cell dissemination therefore provide additional routes to genomic instability.

Cancer cell-extrinsic causes of CIN

Additional cell-extrinsic or non-genetic causes of CIN have also been proposed, besides mechanical forces upon the nucleus and paracrine induction of EMT described earlier. Glucose deprivation, hypoxia or acidification of the extracellular milieu, which mimic properties of the tumour microenvironment, induce genomic instability and aneuploidy[49,50]. Entosis, the process of cell engulfment by another cell, causes tetraploid and CIN by blocking division of the host cell, has been reported to be present at low frequency in human tumour specimens[51,52].

In summary, chromosome segregation errors can potentially trigger a chain of events resulting in extensive numerical and structural chromosomal aberration, and cause mutation acquisition. Indeed, there are numerous examples showing that aneuploidy itself can be a trigger for further chromosomal instability and rearrangements[53-55]. Aneuploid and tetraploid cells evolve to gradually accumulate further whole-chromosome and segmental aberrations with time[20,24,34,54-56]. Consequently, even infrequent missegregation events in cancer cells could induce a leap in cell fitness by causing profound copy-number changes and acquire point mutations.

Accelerated evolution through CIN and therapy resistance

CIN provides an efficient means to respond to various selective pressures, as exemplified by experimental data in various organisms[56-59]. Rare clones within karyotypically heterogeneous populations will often outcompete other cells only when facing selective pressure[57], and tetraploidization in particular facilitates the rapid acquisition of copy-number alterations and mutations in response to stressful conditions, leading to increased fitness[56,58]. CIN and tetraploidization also confer multidrug resistance, including for some of the most commonly used chemotherapeutic drugs[60,61]. Oncogene addiction, the basic principle for the efficacy of targeted therapies[62], can be circumvented by ongoing CIN. Elegant experiments using inducible mouse models showed that CIN (driven by MAD2 overexpression), when combined with $KRAS^{G12D}$ or HER2 oncogenes, consistently contribute to bypass oncogene addiction upon oncogene withdrawal and facilitates tumour relapse and persistance[63,64]. CIN thus offer an escape mechanism following targeted therapy, and suggests that the loss of driver oncogenic mutations from a copy-number event would not be as deleterious in cancer cells with ongoing CIN. This represents a conceivable scenario since ongoing CIN contributes to mutational heterogeneity by causing the loss of chromosomal regions previously harboring clonal mutations, which has been observed in lung[65] and breast[26] cancers.

CIN is also an important driver of parallel evolution. In NSCLC, focal amplification of driver genes takes place from different alleles in different tumour subclones, a process termed mirrored subclonal allelic imbalance, indicative of ongoing CIN[65]. Comparison of SCNAs in circulating tumour cells (CTCs) and metastatic tumours also revealed convergence towards common SCNA in patients from various cancer types[66]. Of note, neither studies observed convergence at the mutational level, suggesting CIN allows more rapid selection of driver events than other mutagenic processes in some cancers. Convergence at the copy-number level involving LOH or oncogene amplification have been reported in high-grade ovarian cancer[67]. The emergence of resistance during therapy can also proceed through parallel convergence. Resistance to ERK-inhibition can occur through parallel amplification of BRAF in divergent clones[52], while resistance to a high dose of a PI3Kα inhibitor arose through parallel convergence on PTEN loss[68,69]. CIN therefore allows cells to explore evolutionary trajectories during tumour evolution and adapt to therapy which underlies treatment failure. Radiation therapy[70], as well as many of the most commonly used chemotherapeutic drugs induce chromosomal instability *in vitro*[71,72]. CIN induction was observed for several classes of anticancer compounds targeting microtubules (Taxol), DNA damage response pathways (PARP and topoisomerase inhibitors) as well as DNA intercalating agents (cisplatin) or nucleoside analogues (Gemcitabine). Notably, in some cases CIN induction was exacerbated when using drug combinations below their respective IC50 values[71]. The efficacy of several drug used as standard of care could be linked to their common effect of driving excessive genomic instability in cancer cells. Based on this interpretation, what would make cancer cells exquisitely sensitive to several of these compounds is not their faster proliferation rate but rather the loss of various checkpoints, causing them to acquire additional SCNAs beyond a threshold compatible with cell survival (see *CIN Attenuation* section below).

Mechanisms enabling CIN propagation

Aneuploidy tolerance

A fundamental difference between normal and transformed cells is in their ability to cope with genetic imbalances. The deleterious impact of aneuploidy on cellular proliferation has been documented for many cell types[73]. For example, in the haematopoietic compartment, aneuploid cells are outcompeted due to slower proliferation[74]. Aneuploidy also impairs organismal development and is the main cause of spontaneous abortions in humans, where most constitutive aneuploidies are embryonic lethal with trisomy 21 being a rare exception[75]. Aneuploidy has profound consequences of gene dosage by causing imbalances in the expression of hundreds to thousands of genes residing on the extra chromosome(s)[76]. This results in a number of aneuploidy-associated stresses that impair overall cellular fitness by causing metabolic changes and impacting on the protein turnover machinery[77-79]. Chromosome gains appear to be particularly detrimental to cell proliferation and tumours more frequently harbour chromosome losses than gains[54]. Therefore, it appears that the aneuploid state itself is not sufficient to transform normal cells and in fact aneuploid cells are largely under negative selection pressure[54,74]. Then how to reconcile this observation with the often-reported high proliferation rate of aneuploid cancer cells? As discussed below, aneuploidy tolerance mechanisms associated with cell transformation are thought to enable CIN propagation. There may also be an overestimation regarding the hyper-proliferative feature of cancer cells, symptomatic of *in vitro* analyses. The proliferation rate of primary human tumours based on radiographic measurements or derived from tumor marker levels, suggest their doubling times range from 30 days to several months (reviewed in [80]). These measurements reflect a combination of cellular proliferation and other factors acting upon cancer cells' fitness such as immuno-editing, which may mask their actual proliferative rate. Potential doubling time (Tpot) estimations derived from BrdU incorporation measurements, also suggest relatively slow doubling times from 1 to 2 days in head and neck cancer[81,82], 4.5 days in colorectal cancer[83] and 12.5 to 28 days in breast cancer[84,85]. Intravital imaging in immunocompromised mice also shows that cancer cell lines proliferate significantly more slowly *in vivo* than they do in cell culture[86]. The ability of cancer cells to proliferate *despite* aneuploidy, even at a slow rate, might be a crucial CIN determinant more physiologically relevant for tumour evolution.

What might then enable cancer cells to tolerate aneuploidy? Genetic alterations that improve protein turnover, hence alleviate proteotoxic stress, were reported to improve the fitness of aneuploid cells[87,88]. *TP53* disruption was proposed as an important mechanism enabling the propagation of chromosomal instability *in vitro* and in mouse models. In CIN+ colorectal cancers for example, TP53 and Adenomatous polyposis coli (APC) mutations, are the most significantly associated alterations[20,31,89-91]. Recent studies suggest that p53 does not invariably arrest cells following chromosome missegregation, and some aneuploidies can be propagated in a p53-proficient background[92,93]. One hypothesis is that p53 does not detect whole-chromosome aneuploidies *per se*, but some aneuploidies (involving specific chromosomes, or a combination thereof) generate a level of stress sufficient to induce p53 stabilisation[92]. On the other hand, the propagation of structural aberrations seems exquisitely dependent on p53 disruption and linked with the acquisition of complex karyotypes[92,93]. P53 stabilisation following chromosome missegregation has been linked to DNA damage resulting from the entrapment of the chromosome during cytokinesis or from the aberrant DNA replication and genomics rearrangements occurring within micronuclei[94]. The requirement for TP53 pathway disruption for CIN propagation may therefore be intimately linked to the co-occurrence of DNA damage at sites of chromosomal rearrangements that link numerical and structural aneuploidies.

However, classical DNA damage response signalling cannot completely explain CIN-induced p53 stabilisation, and in some experimental conditions stabilisation occurs without p53 phosphorylation at sites associated with DNA damage and cell cycle arrest cannot be reverted by ATM inhibitors[95]. We recently identified Caspase-2 (CASP2) as an upstream regulator of p53 following chromosome missegregation[96]. CASP2 was found to cleave MDM2 in response to chromosome missegregation, known to disrupt MDM2's ability to ubiquitinate p53 and targeting it for proteasomal degradation[97]. In colorectal cell lines, the CASP2 steady-state level was found to require BCL9L, which acts as a beta-catenin co-factor for CASP2 transcription. BCL9L mutational inactivation or CASP2 downregulation both conferred tolerance to chromosomal instability[96]. Importantly, reduced CASP2 levels also improved CIN tolerance in p53-deficient cells, by impairing generation of the pro-apoptotic product tBID. It remains unclear why CASP2 becomes active after chromosome missegregation. Several pathways therefore converge onto p53 and the apoptotic machinery to control CIN tolerance in cancer cells, determined by the ability of cells to cope with global transcriptional and metabolic changes and ongoing genomic rearrangements.

Tolerance to Genome-Doubling

Genome-doubled cells appear inherently more tolerant to the gain or loss of whole chromosomes, possibly because the impact on overall gene expression is less than when it occurs in a diploid cell[24,98]. The greater ability of genome-doubled cells to buffer the negative impact of protein imbalances associated with aneuploidy, and their propensity to CIN due to the presence of extra chromosome(s), may explain why genome-doubling is such a common precursor of CIN in cancer appearing early in tumour development[23,24,27] (Figure 1). However the propagation of genome-doubled cells immediately following cell division failure is limited at least in part through a p53-mediated G1 arrest[99], which may explain why TP53 mutations are more frequent in genome-doubled tumours and occur prior to genome-doubling[100]. Activation of the Hippo pathway in response to an increase in centrosomes and microtubule nucleation was shown to contribute to p53 stabilisation[101]. However, TP53 disruption is not an obligatory step for the expansion of genome-doubled cells, and several mechanisms have been described allowing the bypass of TP53 activation. Growth factor signalling for example, promotes proliferation of tetraploid cells despite engagement of the p53-p21 axis[101]. This may be achieved in cancer through activating mutations in *PIK3CA* (encoding the p110α catalytic subunit PI3K), which were shown to confer tolerance to genome doubling (Martin-Berenjeno et al 2017, in press). In breast cancer, *PIK3CA*^{H1047R} mutations are predominantly clonal and occur prior to genome-doubling (Martin-Berenjeno et al. 2017, in press), with a similar association observed in colorectal adenocarcinoma (*PIK3CA* mutation)[27] and lung squamous-cell carcinoma (chromosome 3q amplification, which harbours the *PIK3CA* locus)[65]. Finally, overexpression of D-type cyclins, which link mitogenic signalling to cell cycle progression[102], allow the circumvention of a G1-arrest following tetraploidization by quenching p21 resulting from p53 transcriptional activation, preventing it from exerting its anti-proliferative function[103,104]. An important mechanism by which PI3K/AKT and ERK signalling contribute to bypass p53 stabilisation may be through the up-regulation of D-type cyclins[105].

CIN Attenuation

Cahill and colleagues proposed that genomic instability may contribute to tumour development only if it does not exceed a threshold beyond which it generates unviable

karyotypes. It follows the same principles observed in bacterial genetics and virology where an excessive mutator phenotype has catastrophic consequences[106]. This concept is supported by the finding that high SCNA burden and greater intratumour heterogeneity prior to therapy are associated with improved overall survival, while tumours with intermediate levels display a poor clinical outcome[107-109]. Accordingly, CIN can be either oncogenic or tumour suppressive in mouse models according to the level of instability, which is affected by the genetic context and the tissue[110,111]. Elevating chromosome missegregation rates increases cell death in various cancer cell lines and reduces their tumorigenic potential[112,113]. In addition, the efficacy of some cancer treatments that induce CIN such as taxol and radiation, is improved in cells where the basal rate of chromosomal instability is higher[70,112,114].

The requirement to reach an equilibrium of low CIN may explain the scarcity of mutations in genes whose disruption robustly induce CIN experimentally, since those would essentially be under negative selection. Analogous to Muller's ratchet principle that links mutation acquisition and species extinction, the accumulation of genomic alterations during tumour evolution may gradually increase CIN and lead to cancer cell death. It is thus possible that alterations that limit CIN might be selected for during cancer progression. Aneuploidy tolerance, although essential for CIN propagation, leaves cells vulnerable to extreme karyotypic changes, raising the question whether CIN levels can be modulated during tumour development to mitigate the impact associated with excessive instability.

We reported recently that deleterious mutations in various subunits of the Anaphase-Promoting Complex/Cyclosome (APC/C) are selected for in cancer, and showed that monoallelic inactivation of various subunits significantly reduced the rate of endogenous segregation errors in cancer cell lines[95]. APC/C dysfunction delayed mitotic progression only by 5 to 10 minutes, which was sufficient to significantly improve chromosome segregation fidelity, the fitness of tetraploid cells and reduce the frequency of merotelic attachment errors, considered a main cause of w-CIN[12] (Figure 1). Although cancer cells divide much less frequently *in vivo* than *in vitro*, intravital imaging studies suggest that the total duration of the mitotic phase itself is unchanged *in vivo* (~1h)[86], similar to that reported for various cell types in mouse embryos[115]. Pharmacological *in vitro* induction of extreme CIN rapidly selected for cells with APC/C mutations or reduced activity, translating into a 10-minute mitotic delay. The plasticity in mitotic duration, which merely affects the overall proliferation rate, offers an effective mechanism to attenuate many CIN-causing defects[95]. Delaying mitotic progression also improves tetraploid cell fitness by facilitating centrosome clustering which reduces the frequency of unviable multipolar divisions[16,95,116]. Mitotic biomarkers such as MPM-2 and phospho-Histone H3 may therefore not be optimal to determine the proliferation index on fixed samples. Secondary alterations that improve cell fitness by reducing CIN may therefore be acquired during tumour evolution. Mild alterations in mitotic duration due to genetic or epigenetic regulation of critical mitotic regulators may provide an effective mechanism to fine-tune the level of CIN to optimise cancer cell fitness.

A crucial determinant for CIN propagation therefore relies on the capacity of cancer cells to tolerate a given rate of instability, and disruption of this equilibrium is likely to impair cell fitness (Figure 2). For example many cancer cell lines that display a stable karyotype missegregate chromosomes at non-negligible frequencies, yet these events are not tolerated leading to cell death and clearance of aneuploid cells[96] (Figure 2). For example, APC-mutated organoids show a high rate of segregation errors (and would appear

aneuploid by FISH), but the aneuploid progeny is not propagated efficiently and the population does not become fully aneuploid unless TP53 is disrupted[91]. Reduced instability in evolved tetraploid cells can also be achieved by eliminating the extra centrosomes[15,104]. Buffering CIN rates is also a recurrent observation upon mathematical modelling the evolutionary dynamics of genetically unstable populations, and cell fitness is improved when CIN rates are reduced[117-119]. Identifying additional mechanisms driving CIN adaptation and tolerance may therefore reveal new strategies to target CIN therapeutically.

Interplay between immunosurveillance and CIN

A complex picture is emerging whereby CIN could impact on cancer cell recognition by the immune system in multiple and opposing ways.

Immune evasion may be particularly crucial for chromosomally-unstable tumours since the genomic alterations and stresses associated with aneuploidy may increase their immunogenicity. A recent analysis of 5,255 tumours and normal samples from TCGA revealed that high level segmental or whole-chromosome SCNAs in tumours correlate with reduced expression of gene signatures associated with adaptive immunity and cytotoxic $CD8^+$ T-cell/NK cells, suggestive of reduced immune infiltration[120]. Although these observations remain to be validated *in vivo*, it supports the notion that the tumour microenvironment of highly aneuploidy tumours is immunosuppressive, which is supported by a lower frequency of neoantigen editing in CRC[120].

General features shared by CIN cells may constitute an immunogenic trigger. This effect may in part be driven by endoplasmic reticulum-associated stress in polyploid cells resulting in extracellular exposure of calreticulin and recognition by cytotoxic T-cells and NK cells[121,122]. Pharmacological induction of CIN using an Mps1 inhibitor induced a pro-inflammatory gene signature, increased cytokine secretion, cell surface expression of NK-activating ligands and efficient clearance by NK92 cells in co-culture assays[93]. In mice, combining an Mps1 inhibitor with anti-PD1 therapy potentiated tumour regression, although it is unclear if immunogenicity was triggered by apoptotic cell death or by a feature of highly aneuploid cells caused by Mps1 inhibition[123]. Defects in nuclear envelope integrity from micronuclei DNA, chromatin bridges or during cell migration, were recently shown to allow DNA recognition by cytosolic cyclic GMP–AMP synthase (cGAS), a crucial sensor of double-stranded DNA that mediates type I interferon immune responses[124,125]. This led to a pro-inflammatory program downstream of STING (stimulator of interferon genes), known to promote anticancer T-cell responses[126]. ER-stress and transient cytosolic DNA exposure associated with CIN are two mechanisms that may trigger a cell-intrinsic innate immune reaction against chromosomally unstable cells.

Alternatively, CIN could generate tumour-specific neoantigens, which are targeted by activated T-cells in response to checkpoint blockade[127] or during adoptive T-cell therapy[128]. The efficacy of immune checkpoint blockade therapy has been associated with a high mutational burden from non-synonymous single-nucleotide variants (nsSNVs, causing single amino-acid substitutions), such as reported in melanoma, NSCLC, and cancers with DNA mismatch-repair deficiency[129]. As discussed earlier, CIN cells are prone to accumulate mutations, but this is unlikely to significantly increase nsSNV burden. Genomic rearrangements associated with CIN on the other hand, especially chromothripsis and chromoplexy[130], could potentially generate many frameshifts in a single catastrophic event. By analysing tumour mutational spectra in a pan-cancer study, we found that frameshifts may represent a strong trigger for antitumour T-cell reactivity[131]. Frameshifts result in the

expression of aberrant neopeptides of various lengths which, upon processing by antigen-presenting cells, can potentially generate a much larger number of neoantigens compared to point mutations. This may explain why renal clear cell carcinomas, which have a low nsSNV burden but a high frameshift burden, respond to checkpoint inhibitor therapy[131]. The contribution of complex rearrangements as a source of neopeptides, and their impact on checkpoint inhibitor efficacy warrants further investigation.

However, ongoing CIN during checkpoint blockade therapy may also lead to treatment failure. Indeed, checkpoint inhibitor resistance in NSCLC was recently linked to the loss of reactive cancer neoantigens through loss-of-heterozygosity[132]. This may be expected since CIN underpins the frequent loss of clonal mutations during NSCLC evolution[65].

Further studies are needed to understand the global impact of CIN on immunosurveillance, considering the metabolic stresses associated with aneuploidy, the immunogenicity associated with segregation errors as well as CIN's impact on neoantigen generation and elimination. While CIN induction *prior* to checkpoint blockade therapy may improve response, it may prove crucial to mitigate CIN during treatment to avoid resistance acquisition.

Leveraging CIN for cancer treatment

Challenges in identifying CIN biomarkers

A major limitation in our ability to specifically leverage CIN for prognostic and therapeutic purposes is the current lack of biomarkers to adequately capture the dynamics of the CIN phenotype, rather than the static nature of aneuploidy. DNA ploidy assessment using image-based cytometry or flow cytometry efficiently detect severe aneuploidies and tetraploidy, provide an indication of heterogeneity between tumour cells and are useful to determine absolute copy-number from sequencing data [133]. However, they lack resolution, fail to detect s-CIN or low w-CIN rates especially in near-diploid samples. Nuclear morphological defects on mitotic cells and micronuclei represent a surrogate for segregation errors. Cytogenetics methods relying on analysis of metaphase cells cannot be applied in a clinical setting, and FISH-based methods can only detect specific translocations or measure centromeric modal deviation for limited number of chromosomes at once[134]. Copy-number analysis using array-CGH or DNA sequencing from bulk samples essentially reveal clonally-selected alterations within any given tumour region, and fail to detect heterogeneity. This is illustrated by the illusion of diploidy observed when analysing highly aneuploid populations or after mixing defined aneuploid clones[64,135]. All those methods mostly report on the genomic complexity of cancer genomes, but not whether ongoing CIN is at play, not whether errors are tolerated and propagated.

Multi-region sequencing provides further insight into CIN dynamics, enabling to distinguish between clonal and subclonal SCNAs, and a high proportion of subclonal SCNAs is therefore indicative of ongoing CIN during tumour evolution. In NSCLC, tumours where the majority of SCNA events were subclonal displayed shorter disease-free survival, and observation independent of clinical factors in a multivariate analysis[65]. On the other hand, a high proportion of subclonal mutations, indicative of ongoing mutagenesis, had no prognostic value. CIN may therefore be a more important driver of cancer progression than an increased mutation rate, a provocative thought that warrants further investigation.

Analysis of circulating tumour cells (CTCs) or tumour-derived cell-free DNA (cfDNA) from liquid biopsies offers an amenable way to track SCNA evolution during cancer progression and treatment[66,136-138]. In particular low-coverage sequencing on CTCs provides a non-invasive way to assess tumour heterogeneity at the single cell level to infer CIN[66]. Obviously, single cell genomics provides the ultimate level of resolution to fully appreciate the extent of heterogeneity, and represents the most promising avenue to develop clinically applicable biomarkers for CIN[139]. Combining DNA image cytometry and multi-region sequencing could be exploited to drive he identification of robust biomarkers capable of capturing CIN dynamics particularly in samples with low cancer cell fraction, which is crucial if we are to leverage CIN for stratification purposes or to exploit it for direct therapeutic intervention.

CIN in Clinical trials

Considering the pervasiveness of CIN in cancer and the consequences of tumor heterogeneity for cancer treatment, there is currently a very limited number of clinical trials (reported on clinicaltrials.gov) that either directly investigate the impact of CIN, explore ways to leverage CIN therapeutically or monitor CIN during disease progression or therapy. One trial (NCT03096418) is directly investigating whether paclitaxel increases CIN levels in breast tumors, as suggested from initial studies[114], and whether breast cancers with CIN may be more sensitive to further instability resulting from neoadjuvant therapy. In this study, the level of aneuploidy and CIN will be measured by parallel methods including whole-genome sequencing and FISH on independent core samples per biopsy. In addition, clinical response will also be correlated with tumor levels of paclitaxel (measured by HPLC) as well as proliferative (Ki-67) and mitotic (phospho-Histone H3) biomarkers. A recently completed trial (NCT00512642) involving Lung Imaging Fluorescence Endoscopy (LIFE) to detect early lung lesions in high risk patients involved the collection of analysis of p53 status and genomic instability (aneuploidy) when lesions were found. Studying CIN in pre-malignant and early disease could be further explored for specific cancer types, such as in a current study investigating the correlation between ploidy and recurrence in early rectal cancer (NCT03039595). Another interesting line of investigation worth exploring is to examine the occurrence of CIN in resection margin as predictor of relapse, similar to what has been done in a study of oral squamous cell cancer[140].

Perspectives for Targeting CIN cancers

Given the far-reaching consequences of CIN for treatment success and outcome, several approaches have been explored to target CIN, taking advantage of features associated with the aneuploid state or their capacity to sustain further instability.

Reducing fitness of aneuploid cells may be achieved using compounds that exacerbate the proteotoxic stress (the Hsp90 inhibitor 17-AAG) and metabolic stress (the AMPK agonist AICAR) associated with aneuploidy, which have shown some selectivity against aneuploid and CIN cells[87,141]. Aneuploid and CIN cell lines were recently found to contain higher levels of ceramides, a class of pro-apoptotic sphingolipids synthesised on the ER[142], and consequently were more sensitive to pharmacological increase in ceramide levels[143]. This may explain the reported synergy between conditions that increase ceramide levels and paclitaxel, which induces chromosome missegregation at clinically relevant doses[114,143-145].

Increasing chromosome missegregation rates to generate unviable karyotypes is another avenue actively explored. Several groups have developed Mps1 inhibitors aimed at causing massive aneuploidy by ablating the mitotic checkpoint, which again seems to synergise with paclitaxel[112,123,146]. Identifying cancer types exquisitely sensitive to Mps1 inhibitors may prove challenging and relies on the premise that unwanted aneuploidy in normal tissues would not be propagated. Mps1 inhibitor efficacy may therefore be restricted to cancers where paclitaxel has proven effective. The success of Mps1 inhibitor monotherapy may also be limited by the rapid acquisition of resistance as observed *in vitro* through Mps1 mutations, APC/C dysfunction and aneuploidy tolerance acquisition[95,96,147]. Forcing cells with extra-centrosomes (such as genome-doubled cells) into a catastrophic multipolar division, by preventing centrosome clustering, is also being explored for example by targeting of the non-essential kinesin HSET[15,16,148]. By accelerating mitosis, Mps1 inhibitors also impair efficient centrosome clustering and promote multipolarity[16,95,116]. Phase I studies are currently ongoing for Mps1 inhibitors (NCT02366949, NCT02138812, NCT02792465).

Targeting tolerance mechanisms in combination with approaches aimed at increasing CIN rates may represent an efficient way to limit resistance acquisition and possibly improve response to DNA damaging agents that also drive excessive CIN. Targeting pathways that converge onto p53 are particularly relevant, either by reactivating p53 in CIN tumours, disrupting cyclin D-p21 interaction or by blocking signalling pathways that induce tolerance. For example, low doses of PI3Kα inhibitors which dampen the low-level pathway activation upon oncogenic activation of *PIK3CA* may reduce CIN tolerance and tumour heterogeneity, and limit the generation of drug-resistant clones.

Reducing tumour heterogeneity by directly suppressing chromosome missegregation may be confounded by the complexity of the CIN phenotype in established tumours, and CIN may only be temporarily reduced as was reported upon targeting a CIN-driving process using an MCAK inhibitor[149].

Further studies are needed to understand the evolutionary trajectories of heterogeneous CIN populations in response to various treatments, which may uncover new targetable dependencies. A deeper understanding of the biological processes affecting the fitness of CIN cells combined with the ongoing cataloguing of cancer mutations associated with subclonal expansion may also identify additional druggable targets. In addition, whether acute induction of extreme CIN will potentiate antitumour immune responses or drive resistance to checkpoint blockade warrants further investigation.

Although the prognostic value of aneuploidy has been demonstrated for several indications, deriving robust approaches to assess whether ongoing CIN is taking place within a given near-diploid or aneuploid sample may be crucial to efficiently exploit it in a clinical setting. Indeed, aneuploid cancer cells are not invariably chromosomally unstable and can maintain a stable yet abnormal karyotype. Discriminating between CIN+ and CIN- regardless of the ploidy status will potentially inform on the response to therapy and chances of relapse.

Conclusion

Development of robust biomarkers capable of capturing CIN dynamics is crucial if we are to leverage its potential for stratification purposes and to exploit it for direct therapeutic intervention. Tackling CIN is essential for the success of personalised medicine, a problem

that is only just beginning to be understood from a therapeutic perspective. Great attention has been given to the extremely diverse causes of chromosomal instability, but tolerance mechanisms, ripe for exploitation, are starting to emerge as being crucial determinants for its propagation.

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FIGURE LEGENDS

FIGURE 1 : Merotely, tetraploidy and CIN attenuation.

A) Several types of mitotic defects can lead to chromosome missegregation. Illustrated are merotelic attachments, whereby one of the sister chromatids is attached to opposite poles (magenta). These errors are not detected by the mitotic checkpoint, hence mitosis proceed without delay, resulting in lagging chromosome that can be missegregated to daughter cells. Severe defects (excessive CIN) generates a high frequency of unviable aneuploid karyotype that deviates greatly from a 2n diploid content (2n +/- x), due to the loss or gain of too many chromosomes (red daughter cells).

B) Infrequent segregation errors involving fewer chromosomes likely generate viable progeny (orange daughter cells), whose proliferation will then depend on various tolerance mechanisms. The frequency of segregation errors can be attenuated by acquiring secondary alteration that will improve mitotic fidelity. APC/C dysfunction is one mechanism allowing cells to delay mitotic progression, giving more time for endogenous mechanisms to correct attachment errors.

C) Supernumerary centrosomes in tetraploid cells (4n) frequently generate multipolar spindles and merotelic attachment. Failure to cluster extra centrosomes into two poles will lead to a multipolar division (4, or 3 daughter cells as shown here) with severe and random chromosome losses (4n - x). The presence of extra centrosomes also greatly increases merotely.

D) Tetraploid cells avoid multipolar divisions by achieving centrosome clustering, which requires the kinesin HSET. Tetraploids are believed to be more tolerant to segregation errors because it has a milder impact on overall protein stoichiometry, compared to a diploid cell. Delaying mitotic progression provides more time to achieve centrosome clustering, and reduces the frequency of segregation errors, improving tetraploid cell fitness and the propagation of a sustainable rate of CIN (yellow daughter cells).

FIGURE 2 : Impact of CIN tolerance and attenuation on the propagation of cells with complex karyotypes.

A) Cells without CIN and functional stress response pathways will maintain a stable karyotype. Rare stochastic segregation errors will be outcompeted but may persist.

B) CIN in the presence on functional stress response pathways including p53 will prevent the propagation of cells with complex karyotypes. Only aneuploidies involving specific chromosomes may be tolerated and will proliferate at a much slower rate.

C) CIN tolerance allows rare stochastic error from an otherwise karyotypically stable population, to be efficiently propagated. Additional numerical and structural aberration could be acquired and propagated.

D) Aneuploidy tolerance combined with high chromosomal instability will generate an increasing number of cells with unviable karyotypes and is therefore tumour suppressive.

E) Alterations causing a transient or less penetrant CIN phenotype will reduce the frequency of unviable karyotypes. CIN cells may also acquire secondary mutations to reduce the rate and severity of chromosome segregation errors, improving fitness.

FIGURE 1

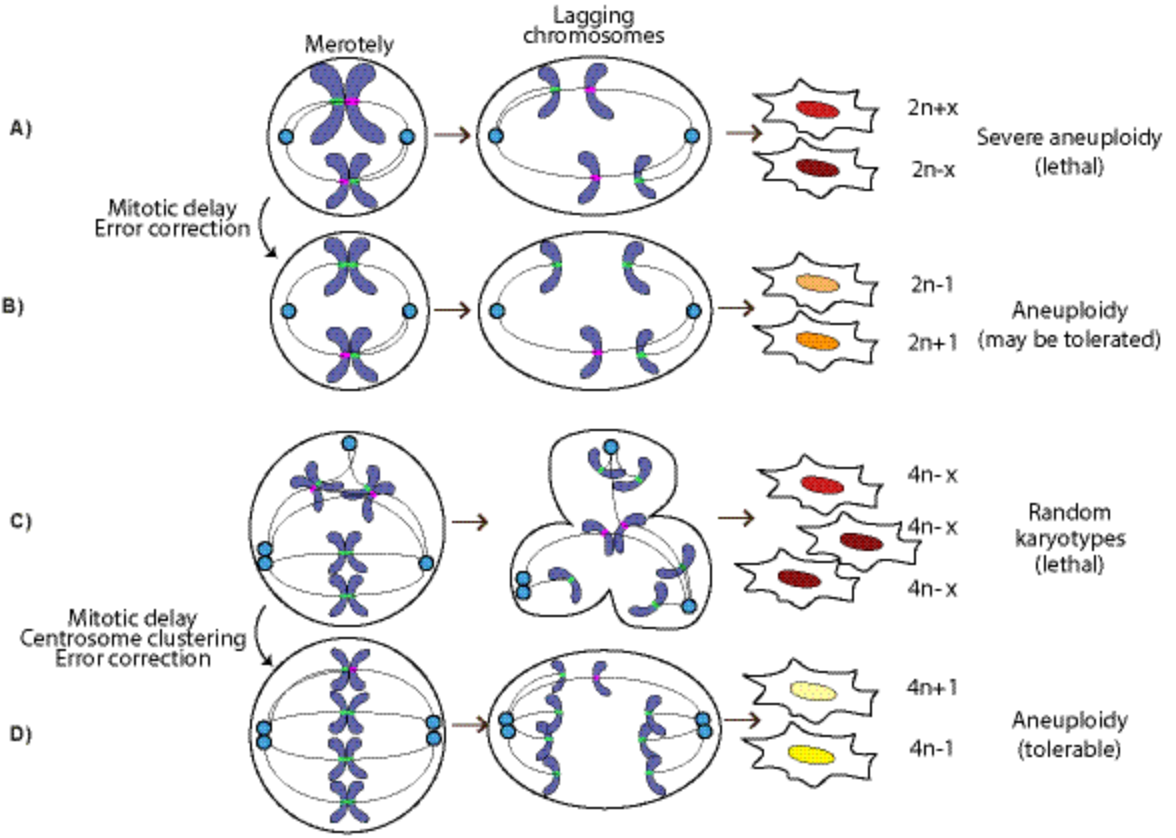
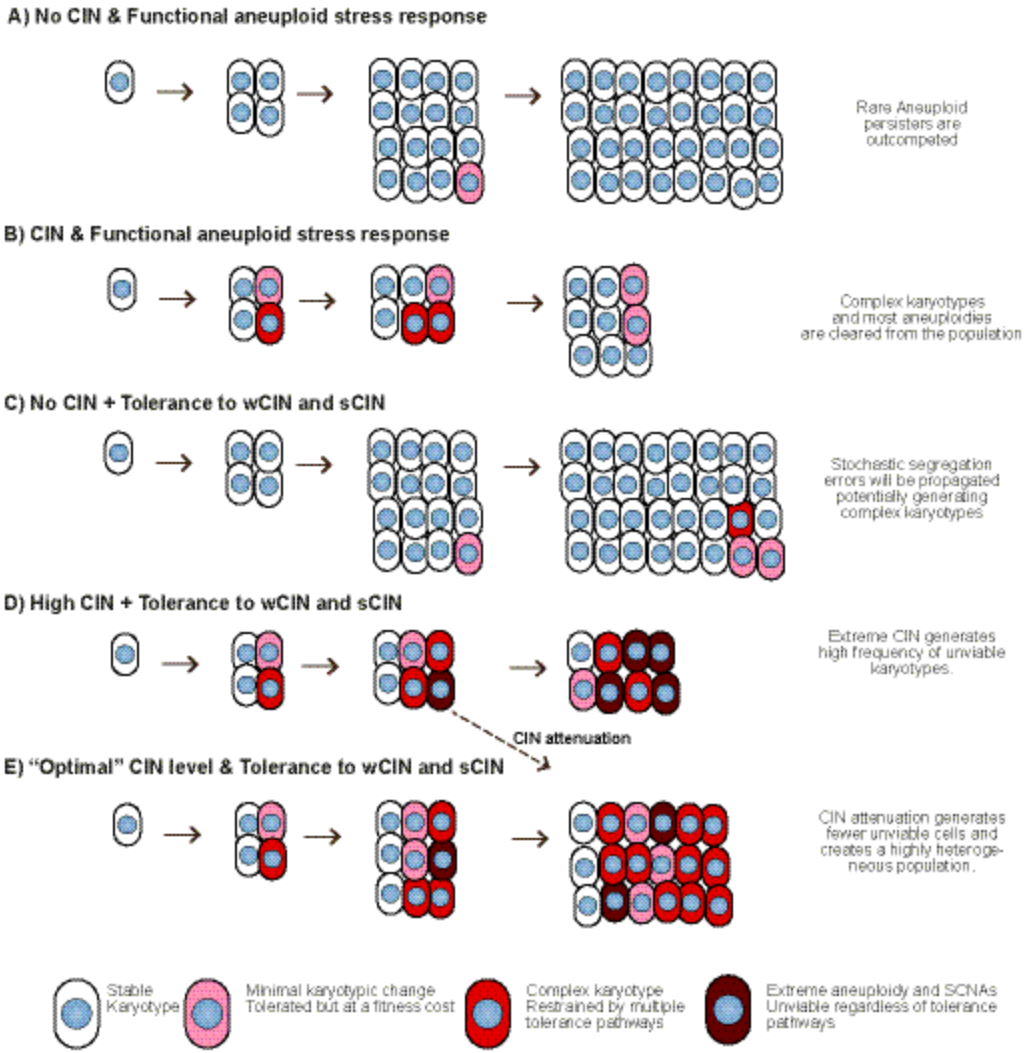

Merotely
Lagging chromosomes
A)
2n+x
2n-x
Severe aneuploidy (lethal)
Mitotic delay
Error correction
B)
2n-1
2n+1
Aneuploidy (may be tolerated)
C)
4n-x
4n-x
4n-x
Random karyotypes (lethal)
Mitotic delay
Centrosome clustering
Error correction
D)
4n+1
4n-1
Aneuploidy (tolerable)

FIGURE 2


A) No CIN & Functional aneuploid stress response
Rare Aneuploid persisters are outcompeted
B) CIN & Functional aneuploid stress response
Complex karyotypes and most aneuploidies are cleared from the population
C) No CIN + Tolerance to wCIN and sCIN
Stochastic segregation errors will be propagated potentially generating complex karyotypes
D) High CIN + Tolerance to wCIN and sCIN
Extreme CIN generates high frequency of unviable karyotypes.
CIN attenuation
E) "Optimal" CIN level & Tolerance to wCIN and sCIN
CIN attenuation generates fewer unviable cells and creates a highly heterogeneous population.
Stable Karyotype
Minimal karyotypic change Tolerated but at a fitness cost
Complex karyotype Restrained by multiple tolerance pathways
Extreme aneuploidy and SCNAs Unviable regardless of tolerance pathways