CINister thoughts

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Abstract

Chromosome instability (CIN) is the process that leads to aneuploidy, a known hallmark of human tumours for over a century. Nowadays, it is believed that CIN promotes tumorigenesis by shuffling the genome into a malignant order through translocations, amplifications, deletions (structural CIN), and gains and losses of whole chromosomes (numerical CIN or nCIN). The present review focuses on the causes and consequences of nCIN. Several roads can lead to nCIN, including a compromised spindle assembly checkpoint, cohesion defects, p53 deficiency and flawed microtubule–kinetochore attachments. Whereas the link between nCIN and tumorigenesis is becoming more evident, indications have emerged recently that nCIN can suppress tumour formation as well. To understand these paradoxical findings, novel reagents and more sophisticated mouse models are needed. This will provide us with a better understanding of nCIN and eventually with therapies that exploit this characteristic of human tumours.

Aneuploidy

Aneuploidy and CIN (chromosome instability) are different terms, but are often confused: aneuploidy is the state of a cell with an abnormal DNA content, and CIN is the process leading to aneuploidy. CIN can be classified into nCIN (numerical CIN), in which whole chromosomes are gained or lost, and structural CIN in which chromosome integrity is distorted, e.g. through translocations, deletions and amplifications. At least 70–80 % of cancers are aneuploid, and the majority display a combination of structural and numerical chromosomal abnormalities [1–3]. However, in the present review, I only discuss causes and consequences of nCIN.

Aneuploidy was first described in 1890 by David von Hansemann [4], who observed abnormal mitotic figures in several carcinoma samples. His findings were built upon by Theodor Boveri [5] who elegantly showed, by injecting two sperm into sea urchin embryos instead of one, that abnormal chromosome numbers can lead to abnormal development or to imbalances leading to death. As different chromosome distributions yielded different outcomes, he hypothesized that the individual chromosomes carry different information, thereby explaining how aneuploidy could drive tumorigenesis [5].

Assessing whole-chromosome aneuploidy and CIN

Aneuploidy can be measured by a range of different techniques depending on the material to be assessed. The classic assay to quantify whole-chromosome aneuploidy is karyotyping. Cultured or freshly isolated cells are treated with colcemid, which blocks the dividing cells in mitosis when the chromosomes are condensed, allowing for chromosome counting. In fixed tissue, the most common technique is hybridization of fluorescently labelled probes specific to a chromosome of choice [FISH (fluorescence *in situ* hybridization)]. This allows the number of copies of the chromosome in question to be counted in individual cells. A third technique to detect aneuploidy is quantification of micronuclei, which are formed from chromosomes that lagged behind during a previous mitosis. This technique is quite simple, but does not provide information as to which chromosomes are affected. A fourth and increasingly popular technique, mostly used on tumours, is CGH (comparative genomic hybridization). By hybridizing total genomic DNA from a tumour labelled in one colour, together with total genomic DNA from control tissue labelled in a second colour, to a microarray containing probes that cover the whole genome, the ratio of the DNA content from the tumour to the control tissue can be determined from probe to probe. The success of CGH critically depends on distinct differences in the chromosomal contents of the specimens assessed and is therefore only suitable to determine clonal aneuploidy as occurring in cancer. However, CGH is becoming increasingly sensitive and has been successfully used to compare DNA content from single cells, e.g. using DNA isolated from micro-dissected fixed tissue slices [6,7]. A fifth technique is the determination of DNA content by flow cytometry. This technique can only detect large differences in DNA content, such as tetraploidy, but allows for simple quantification of DNA content from large quantities of cells.

It is important to realize that all of these techniques involve end-point analyses. Thus they can only provide circumstantial evidence of CIN. To quantify ongoing nCIN, aneuploidy has to be measured at different time points in the

Key words: aneuploidy, chromosome instability (CIN), mouse model, numerical chromosome instability, spindle assembly checkpoint.

Abbreviations used: AD, Alzheimer's disease; APC/C, anaphase-promoting complex/cyclosome; CGH, comparative genomic hybridization; (n)CIN, (numerical) chromosome instability; DS, Down's syndrome; FISH, fluorescence *in situ* hybridization; MVA, mosaic variegated aneuploidy; RB, retinoblastoma; SAC, spindle assembly checkpoint; YFP, yellow fluorescent protein.

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same specimen, for instance by karyotyping single cell clones from CIN cell lines, or by time-lapse microscopic imaging of chromosome segregation in cell lines, a laborious process.

Aneuploidy in disease

Systemic aneuploidy

CIN and aneuploidy have been linked to several diseases and syndromes. In some syndromes, aneuploidy is congenital and present in all somatic cells. The most well-known example of such a syndrome is DS (Down's syndrome) (trisomy of chromosome 21, occurring approx. 1 in 800 births). DS is associated with impairment of cognitive functions and physical growth, and early-onset AD (Alzheimer's disease) (starting at 30 years of age) [8]. Other examples of syndromes caused by systemic trisomies are Edwards' syndrome (trisomy of chromosome 18, occurrence 1 in 6000 births), Patau's syndrome, (trisomy of chromosome 13, 1 in 10000 births), Klinefelter's syndrome (XXY) and Turner's syndrome (monosomy of the X chromosome) [9–11], all affecting physical development and often cognitive functions. The mere existence of these syndromes indicates that systemic aneuploidy can be tolerated, albeit at cost.

Mosaic aneuploidy and disease

The most severe CIN-associated syndrome known is MVA (mosaic variegated aneuploidy). MVA is a rare recessive disorder in which patients encounter mosaic aneuploidy (mostly trisomies and monosomies of different chromosomes) in several tissues. MVA patients suffer from growth retardation, microcephaly and mental retardation, and often develop childhood cancers [12,13]. MVA is associated with mono- or bi-allelic mutations in the mitotic checkpoint kinase BubR1 [12–14]. These mutations indeed lead to a dysfunctional SAC (spindle assembly checkpoint) and therefore to gross aneuploidy, providing a molecular explanation for the observed mosaic aneuploidy [14].

AD is a progressive neurodegenerative disease of the brain and has also recently been associated with chromosome instability. As many DS patients develop brain pathology that closely resembles AD, there has been a distinct focus on trisomy of chromosome 21 in the brains of AD patients. Indeed, it has been found that trisomies for chromosome 21 (and chromosome 17) are more prevalent in AD-affected brains than in healthy age-matched brains [15,16]. The mechanism leading to AD-associated aneuploidy remains largely obscure, although a recent study has suggested that the expression of mutant APP (amyloid precursor protein), a common mutation in familial AD, might lead to microtubule dysfunction, hence facilitating CIN and ultimately neuronal cell death [17].

Cancer and aneuploidy

Cancer results from multiple genetic alterations that override safety checkpoints that normally protect against unconstrained proliferation. Whereas 70–80 % of human tumours are aneuploid, and aneuploidy has been associated with cancer for more than a century, the exact role that CIN plays in cancer is still ambiguous [1–3,18]. nCIN could contribute to tumorigenesis by facilitating deletion of chromosomes harbouring tumour suppressor genes, or by gain of chromosomes carrying oncogenes. Interestingly, the molecular pathways protecting CIN from occurring are frequently disrupted in human tumours. The rest of the present review focuses mostly on the relationship between nCIN and cancer.

Routes to aneuploidy

Cells are wired with several pathways that protect against nCIN, including the SAC, tight sister chromatid cohesion, centrosome number regulation, the p53 pathway and mechanisms preventing merotelic attachments, discussed in more detail below.

The SAC and aneuploidy

The SAC ensures correct chromosome segregation by delaying mitotic progression until all sister chromatids are correctly attached to opposing centrosomes, thus preventing nCIN. One single unattached kinetochore, the protein structure that sits on the centromeric DNA and recruits microtubules emanating from the centrosome, is sufficient to block a cell in metaphase [19]. In addition to attachment, the checkpoint appears to monitor tension between sister chromatids, ensuring bipolar attachment. The SAC comprises a set of roughly ten proteins (Mad1, Mad2, BubR1, Bub1, Bub3, Mps1, CENP-E, Rod, ZW10 and TaoK1). Several of these proteins are recruited to unattached kinetochores and send an inhibitory signal to CDC20, an activating subunit of the APC/C (anaphase-promoting complex/cyclosome). The APC/C is an E3 ligase that, once activated, degrades several mitotic proteins, for instance cyclin B1 and securin. Depletion of cyclin B1 inactivates CDK1 (cyclin-dependent kinase 1) and removal of securin activates separase, which together allow for sister chromatid cleavage and anaphase onset [1–3]. Several mutations in SAC proteins have been reported in human cancer, including BubR1, Mad2, Bub1 and ZW10 [1,2,20]. However, overexpression of SAC components in cancer is more frequent. One clear example is overexpression of Mad2, which can be a consequence of loss of the tumour-suppressor gene RB (retinoblastoma) [21]. Indeed, Mad2 overexpression increases aneuploidy in RB-proficient mice and tissue culture cells [22]. Conversely, restoration of normal Mad2 levels in RB-deficient cells rescued the CIN phenotype of these cells, suggesting that RB loss indirectly contributes to nCIN-driven tumorigenesis. RNA expression profiling from tumours suggests that many other mitotic proteins are overexpressed in cancer, e.g. the Aurora kinases A and B, BubR1, Bub3 and Mad1 [1]. Although overexpression of SAC proteins has been shown to stimulate nCIN and predispose mice to tumorigenesis, the question remains whether such up-regulation directly provokes tumour development in humans as well, or that it is a secondary effect of a hyperactivated cell cycle machinery.

Aneuploidy due to lack of sister-chromatid cohesion

Before chromosome segregation, sister chromatids are held together by a ring-like structure called the cohesion complex. Defects in the cohesion complex are found in cancers [23] and seem to play a causal role in congenital disorders such as Cornelia de Lange syndrome and Roberts' syndrome, both of which are associated with mosaic aneuploidy [24]. Defects in the cohesion complex result in premature separation of the sister chromatids and are revealed as railroad tracks in metaphase spreads: instead of X-shaped chromosomes with a clear connection between the sister chromatids at the centromere, sister chromatids are physically separated. Three recent studies have linked the loss of tumour suppressor RB to diminished sister chromatid cohesion and mitotic chromosome condensation, offering an additional explanation for the RB-deficiency phenotype [25–27]. Mitogen deprivation normally blocks cells in a robust G_1-phase arrest. Cells devoid of the RB gene family are still sensitive to mitogen deprivation, but arrest in G_2-phase instead [28]. Mitogen stimulation reverses this arrest, but cells that re-enter the cell cycle are frequently aneuploid and show evidence of reduced sister chromatid cohesion [25]. Although the cohesion complex is intact in these cells, condensin II, a direct RB interactor, fails to localize to centromeres in RB-depleted cells. This leads to reduced sister chromatid cohesion and thus to subtle aneuploidy [26]. These findings were confirmed by a mouse study showing substantial acceleration of p53 loss-driven lymphomagenesis by concomitant RB inactivation. The resulting tumours were highly aneuploid [27], indicating that decreased sister chromatid cohesion indeed leads to a CIN phenotype and predisposes to cancer.

p53 and aneuploidy

nCIN has severe consequences in tissue culture studies, either killing cells within a few cell divisions [29] or blocking cells in the G_1-phase following a missegregated chromosome [30]. If aneuploidy is highly lethal, why then is such a large fraction of human tumours highly aneuploid? One protein that could explain this apparent paradox is p53. Over 50 % of all human tumours show mutations in the coding sequence of p53 and an even larger fraction show defects in the p53 pathway. Its role is most well known as a transcription factor that is activated upon DNA damage, thereby inducing expression of genes involved in cell-cycle arrest (such as $p21^{CIP1}$) and apoptosis [31]. However, p53 counteracts the effects of nCIN as well. For instance, it was found that p53 deficiency can delay early embryonic lethality of Mad2-deficient embryos [32]. Another simple, but elegant, experiment showed that whole-chromosome aneuploidy was tolerated better in p53-deficient HCT116 cells than in their p53-proficient isogenic counterparts [33]. Finally, it was shown that tetraploid cells were only capable of tumour formation when p53 was absent [30]. Together, these findings strongly suggest that part of p53's tumour-suppressive effect comes from suppressing nCIN either by killing or arresting aneuploid cells.

Centrosomal abnormalities and aneuploidy

In the majority of dividing cells, centrosomes form the opposing poles to which the sister chromatids migrate during anaphase. Centrosome number is tightly controlled, with centrosomes duplicating during S-phase and segregating to different daughter cells in mitosis. Many aneuploid tumours show abnormal centrosome numbers as well [34]. Although intuitively, one would expect that an abnormal number of centrosomes would result in multipolar spindles that give rise to aneuploid cells, this does not seem to be the main route [35]. Whereas multipolar spindles do occur, they tend to have a lethal outcome for the resulting daughter cells. Instead, in the majority of these cells, supernumerary centrosomes cluster together during mitosis allowing a 'normal' bipolar spindle to form [36]. However, the increased number of centrosomes does result in an increased frequency of lagging chromosomes, independently from the number of chromosomes present in these cells [35]. Therefore it seems that an abnormal centrosome complement can contribute to an nCIN phenotype as well.

Merotelic attachments and aneuploidy

Merotelic attachments occur when a single kinetochore is bound by microtubules from opposing centrosomes. These faulty attachments arise frequently in early mitosis, and if not resolved before anaphase, they can result in chromosomes that lag behind during cytokinesis. As the sister chromatid is subjected to pulling forces from both poles, it will mislocalize to the incorrect daughter cell in 50 % of the cases. Furthermore, these delayed chromosomes frequently end up in micronuclei, which might make them more susceptible to missegregation in the next mitosis as well. Merotelic attachments do occur in 'normal' cells (in 0.5 % of dividing primary human fibroblasts), but occur more frequently in cancer cells [37]. For instance, Caco2 or MCF-7 cells, both highly aneuploid cancer cell lines, show a dramatic increase in the number of merotelic attachments, when compared with non-CIN cancer cell lines (e.g. HCT116) [38]. Structural defects to the chromatin or spindle machinery, defects in the machinery that corrects these flawed kinetochore attachments, such as deregulated Aurora B, INCENP (inner centromere protein), survivin or shugosin expression, but also extra centrosomes can explain the elevated incidence of merotelic attachments in such cells [35,37]. Merotelic attachments fulfil both requirements of the SAC (kinetochore attachment and intracentromere tension) and are therefore not sensed by the checkpoint. As they can occur in any cell, they are probably contributing significantly to nCIN in early and late lesions.

Models for aneuploidy and nCIN

What are the consequences of nCIN and the resulting aneuploidy? Both 'stable' aneuploidy (as a model for congenital aneuploidy disorders, such as DS) and nCIN have been modelled.

Models for systemic, congenital aneuploidy

'Stable' aneuploidy has been modelled in mouse and yeast. These studies revealed that extra copies of chromosomes (diploids in haploid yeast, or several defined trisomies in otherwise diploid mouse cells) affected cell proliferation significantly. In yeast, this was attributed to a delayed G_1-phase. The main affected pathways in these cells involved ribosome biogenesis and sugar metabolism. Similar results were found for aneuploid mouse embryonic fibroblasts. Supposedly, the extra chromosomes are actively transcribed, explaining the need for extra rRNA and energy. The further imbalance to the cell physiology resulting from aberrant expression of other proteins from the extra chromosome might lead to a general stress response in the aneuploid cells, thus delaying the cell cycle [39,40].

Models for nCIN

As discussed above, nCIN induced by abrogation of the SAC results in cell death within a few cell divisions in tissue culture cells and has a deleterious effect on cell proliferation [29]. However, the vast majority of human tumours show massive numerical aneuploidy, indicative of nCIN. Therefore tumour cells must somehow have undergone selection to circumvent the growth inhibitory signals. To gain more insight into the consequences of nCIN *in vivo*, several mouse models have been developed. The majority of these mouse models, in which nCIN has been induced by abrogating components that play an essential role in the SAC, have reinforced the correlation between nCIN and tumorigenesis. However, the results also raise at least five unaddressed issues that I briefly discuss next. (i) Inactivation of the SAC through the germline (knockouts for Mad1, Mad2, BubR1, Bub3, Bub1, Rae1, Cdc20 and CENP-E) unequivocally results in early embryonic lethality and massive nCIN, confirming that the SAC is essential for the developing embryo [41–47]. This is consistent with the lethality in cell lines, but these findings do not fortify further the link between aneuploidy and cancer. (ii) A reduction in SAC proteins (i.e. heterozygousity) does predispose to spontaneous cancers (Mad1, Mad2, CENP-E) or to carcinogen-induced cancer (BubR1, Rae1, Bub1 and Bub3). However, cancer penetrance is quite low (average of 30%) and tumour latencies are very long (18 months or longer), despite massive aneuploidy in tissue and mouse embryonic fibroblasts isolated from those models. If nCIN was the driving force behind these tumours, one would expect much stronger phenotypes from an evidently compromised checkpoint, given the observed aneuploidy. (iii) Further reduction in the efficacy of the checkpoint does not seem to aggravate the cancer phenotype, but instead provokes a progeria-like phenotype (BubR1 hypomorph) [48]. Furthermore, in some models, nCIN can even suppress tumour formation: CENP-E heterozygousity reduces the incidence of spontaneous liver tumours and delays tumorigenesis in CENP-$E^{+/-}$/$p19^{Arf-/-}$ mice [46]. Likewise, whereas the number of colon tumours is increased in $Bub1^{+/-}$/$Apc^{min/+}$ mice, the number of small intestinal tumours is reduced in mice with the same genotype, when compared with $Bub1^{+/+}$/$Apc^{min/+}$ mice [49]. Finally, a 90% reduction in Bub1 protein levels (Bub1 hypomorphic allele) in $PTEN^{+/-}$ (phosphatase and tensin homologue deleted on chromosome 10) mice reduce the incidence of prostate neoplasia [50]. (iv) Overexpression of Mad2 or Aurora A in the mouse provokes similar cancer predispositions as partial reduction of SAC components, suggesting that up-regulation affects the checkpoint as well. However, it remains obscure how such overexpression deregulates the checkpoint [22,51]. (v) Despite the similarities between the current mouse models (nCIN occurring, modest cancer phenotypes), several differences have been reported as well. This is of particular interest, as all models are aimed at generically taking out the SAC. But whereas these checkpoint mutations were present in all tissues in these models, tumours arose in different tissues from model to model. This suggests that either the targeted SAC components have specific roles in addition to checkpoint regulation or that the SAC is differentially regulated from tissue to tissue. Both options open intriguing research avenues, but require novel reagents and mouse models targeting the SAC in a tissue-restricted fashion.

Outlook

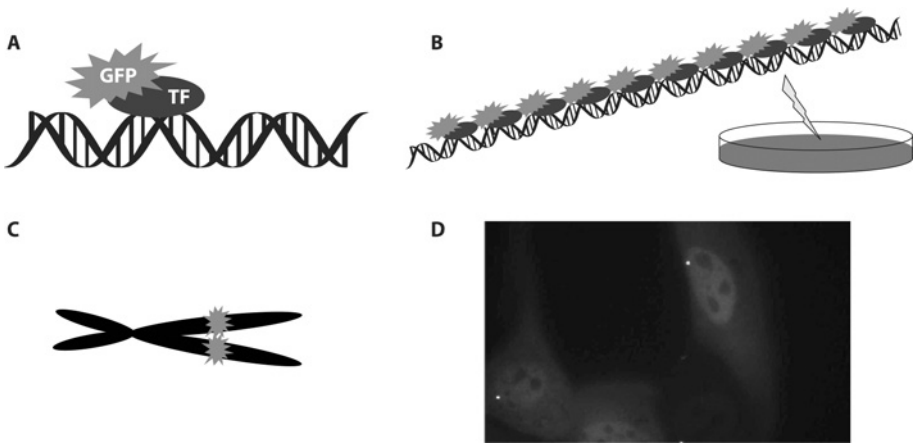
Since the first observation of aneuploidy a century ago, the link between chromosome instability and disease has become increasingly clear. The molecular mechanisms involved in nCIN, such as the SAC and chromosome segregation are being unravelled. The comparison of non-transformed cell lines and cancer cell lines is showing that nCIN is dramatically increased in the latter, presumably mostly due to merotelic attachments. Mouse models designed to assess whether nCIN can be causative for cancer suggest this to be the case. However, some results remain ambiguous and require additional investigation. In order to develop therapies that prevent or cure CIN-instigated disease, we need more insight into the direct consequences of CIN *in vivo*. So which should be the next steps forward?

Development of novel mouse models

Given the early lethality caused by the systemic induction of nCIN, conditional mouse models will be essential for inducing nCIN in a tissue of choice, either early or later in development. By combining these conditional alleles with the wide variety of Cre-recombinase lines available, virtually every tissue can be targeted for checkpoint abrogation in a defined setting, allowing the consequences of nCIN in adult tissue to be determined. Tissue-specific abrogation of SAC proteins might also provide more insight into the remaining issue of putative non-checkpoint roles of SAC components, as in these models the effects of loss of different SAC components can be compared within the same tissue. When such models provoke early-onset cancer with high penetrance, the tumours can be used to identify additional genetic alterations allowing aneuploid cells to survive and circumvent the proliferation defects normally imposed by aneuploidy.

Figure 1 | **Design of an *in vivo* chromosome tracker**

(**A**) An artificial transcription factor (TF), labelled with a fluorescent probe (GFP, green fluorescent protein), binds to a unique DNA sequence. (**B**) A tandem repeat of this unique DNA sequence is introduced into the genome of (mouse) embryonic stem cells. (**C**) Expression of the fluorescent transcription factor will label the chromosome harbouring the tandem repeat. (**D**) 3T3 cells expressing YFP-tagged TetR (Tet-repressor) in cells harbouring a Tet-binding repeat



Better insight into the *in vivo* causes of nCIN

Ultimately, new mouse models should aim to mimic the cancer situation as closely as possible. Even though nCIN has successfully been modelled by partial abrogation of the checkpoint, SAC inactivation is rarely found in human cancers [1,2]. We therefore need to identify the molecular instigators of nCIN in cancer. This knowledge might soon emerge from high-throughput characterization (e.g. sequence, epigenetic state, proteome) of aneuploid tumours, along with the progression of the SAC field. Another way of identifying candidates is by genetic screens [e.g. RNAi (RNA interference) screens, mutagenesis screens] in which aneuploid cells can be specifically enriched for. Subsequently, this knowledge can be used to develop disease-relevant mouse models.

Development of novel reagents

Still, these new mouse models will mainly provide circumstantial evidence for the consequences of nCIN, as the actual ongoing rate of instability cannot be assessed *in vivo*. As discussed above, most techniques to assess CIN frequencies are laborious, retrospective and currently only available for cell lines. Therefore we also need novel reagents that allow for the quantification of nCIN *in vivo*. For instance, a sophisticated mouse model that allows for intravital imaging of chromosome segregation (e.g. employing fluorescently labelled H2B–tubulin) to visualize missegregation events *in vivo* might provide instant assessment of the consequences of nCIN for the daughter cells. It will be important to restrict the expression of those markers to a proliferating cell compartment as mitosis in the adult mouse is not as frequent as in cell lines. Another useful tool to develop would be a chromosome tracker, aiming at tracking a single chromosome. This could be achieved by the introduction of a long tandem repeat into the genome combined with an artificial fluorescent transcription factor [e.g. the Tet-repressor bound to YFP (yellow fluorescent protein)] that binds this repeat (Figures 1A and 1B). When both are present, the transcription factor will bind to the repeat, allowing for *in vivo* FISH (Figures 1C and 1D). Both models could be combined as well, providing information about missegregation events and aneuploidy.

Integrate

Ultimately new mouse models for nCIN can be combined with mouse models to visualize chromosome instability. Only then can the direct consequences of chromosome instability be assessed *in vivo* in a tissue of choice, where microscopy is possible. However, given the rapid advances in the microscopy field and fluorescent proteins, imaging mitosis, missegregation and its consequences might be closer than we think!

Acknowledgements

I apologize to all whose work on nCIN and aneuploidy was not cited because of space restrictions. I am very grateful to Sophia Bruggeman and Steve Pettitt for critically reading this paper and to Peter Sorger and members of the Allan Bradley laboratory for fruitful discussion.

Funding

This work is supported by an EMBO long-term postdoctoral fellowship and previously by a Dutch Cancer Society fellowship.

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Received 9 August 2010
doi:10.1042/BST038xxxx