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# Chromosomal biomarkers of genomic instability relevant to cancer

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**It is generally acknowledged that a crucial event in the initiation and evolution of cancer is the acquisition of a genomic instability phenotype. This review focuses on mechanisms of chromosomal instability including aneuploidy, chromosome rearrangement and breakage-fusion-bridge cycles. The role of micronutrient deficiency, such as folate deficiency, in the causation of chromosomal instability is briefly reviewed and the concept of recommended dietary allowances for genomic stability is introduced. In addition, the techniques for measuring the various chromosomal instability events are discussed with a focus on the cytokinesis-block micronucleus assay as an almost complete system for measuring these various genetic mishaps.**

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▼ Cancer is a complex disease in which cells with altered gene expression grow abnormally, invade other tissues and disrupt their normal function. Several critical mutations in unique genes that accelerate cell division and inhibit cell death alter the balance towards the survival of the cancer phenotype and these events are now well documented for specific cancers [1–4]. However, the number of critical mutations accumulated within cancer cells cannot be explained solely by the normal point mutation rate and it has been proposed that mutations leading to hypermutability are central to the initiation of the cancer process [3–7]. Furthermore, although cancers are apparently clonal, there could be great genetic variability between cells within a cancer clone because genetic instability occurs at the early stage of cancer [3,8]. A consensus is emerging that a crucial early event in carcinogenesis is the induction of the genomic instability phenotype, which enables an initiated cell to evolve into a cancer cell by achieving a greater proliferative capacity and the genetic plasticity to overcome host immunological resistance, localized toxic environments and suboptimal micronutrient supply.

Several molecular mechanisms, such as point mutation, microsatellite instability, gene silencing and/or loss of heterozygosity of tumour suppressor genes, have been postulated as possible important initiating steps. However, evidence is emerging that altered gene dosage and gene expression via aneuploidy (abnormal number of chromosomes) and breakage-fusion-bridge (BFB) cycles leading to gene amplification and altered chromosome territories are crucial initiating events in cancer [4,9–12]. Examples of aneuploidy, chromosome rearrangement, chromosome deletions and gene amplification in cancer cells are given in Table 1.

Techniques for assessing aneuploidy and BFB cycles in mammalian and human cells are reviewed. The importance of the cytokinesis-block micronucleus (CBMN) assay as a tool for measuring genomic instability at the chromosome/molecular level is illustrated by examples showing its use to provide a comprehensive measure of chromosome breakage, chromosome rearrangement, chromosome loss, non-disjunction, gene amplification, necrosis and apoptosis. Our recent data suggest that folic acid deficiency induces BFB cycles in human cells and highlights the crucial role of micronutrients in the prevention of the genomic instability phenotype. The concept of optimal micronutrient status for genomic stability *in vitro* or *in vivo* is introduced and the validity of current biomarkers of genome damage for predicting cancer risk is discussed.

## Genomic instability in cancer – MIN and CIN tumours

Among the best studied mechanisms of genomic instability in cancer is that caused by mutation in DNA mismatch repair genes (e.g. *MSH2*, *MLH1*). This repair defect leads to the accumulation of point mutations in the DNA



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**Table 1. Examples of aneuploidy, chromosome rearrangement, chromosome deletions and gene amplification in specific cancers (Refs [56–66]).**

| Tumour type | Aneuploidy | Chromosome rearrangement[a] | Chromosome deletion | Gene amplification |
|---|---|---|---|---|
| Lung cancer | Ch 7 | 3q13.2 | 3p13–23 | 1p32–33, 1pter-33, 2p22–24 |
| Breast cancer | Ch 1 and 17 | 1q21–23 | 1p11–13 | HER2/neu protein receptor gene |
| Prostate cancer | Ch 7, 8 and 17 | 5q11 and 8p11 | 7q22 | 10q22 |
| Colorectal carcinoma | Ch 17 | 17p | 17p | 7p, 8q, 13q, 20q |
| Skin melanoma | Ch 7 and 10 | 1q11–q12 | 1p11–22 | 11q13, 22q11–13 |

[a]Site commonly involved in rearrangement.

sequence, which are readily observed in microsatellites – short DNA repeat sequences that are dispersed throughout the genome. Tumours exhibiting microsatellite instability are classified as MIN tumours [2,3,13]; these are distinguished from other tumours by having an apparently normal karyotype (i.e. normal complement and structure of chromosomes).

The vast majority of tumours, however, exhibit abnormal karyotypes involving either chromosomal rearrangement and/or aneuploidy. Those tumours that exhibit abnormal chromosome number are classified as CIN (chromosomal instability) tumours [2,3,13,14]. Abnormal chromosome number can initially occur by a variety of mechanisms, which include: (a) abnormal centriole number leading to multipolar mitoses, (b) chromosome loss at anaphase as a result of kinetochore defects, (c) malsegregation of chromosomes at anaphase as a result of defects in the separation of chromatids, (d) mitotic slippage caused by inhibition of mitosis leading to the formation of tetraploid cells, and (e) failure of cytokinesis following nuclear replication as a result of defects in microfilament assembly. It has recently been shown that different carcinogens can induce either MIN or CIN genomic instability *in vitro*, which supports the hypothesis that specific carcinogen exposure or DNA repair defects determines the type of genetic instability in the cancers they induce [13].

## Aneuploidy as an initiating event in cancer

Duesberg and colleagues [6,7] have suggested that the induction of aneuploidy, either by a chemical agent or by other means such as a genetic abnormality in the mechanism of chromosome segregation, might cause altered dosage of oncogenes and tumour suppressor genes. Their aneuploidy hypothesis of carcinogenesis also predicts that aneuploidy involving chromosomes that contain genes controlling the mitotic process leads to asymmetric segregation of chromosomes, initiating an autocatalytic karyotypic evolution generating preneoplastic and, ultimately, cancer cells (Fig. 1). This hypothesis would explain why cancer cells are often aneuploid; however, it would not necessarily explain the high frequency of chromosomal translocations and/or rearrangements and gene amplification often seen in cancer, which might be better explained by the BFB cycle mechanism of hypermutation (Figs 1–3). However, a potential unifying mechanism can be discerned from the recent observations that proteins involved in homologous recombination repair of DNA breaks, such as XRCC2 and XRCC3, are located at the centriole and that loss of function mutations in these genes leads to abnormal centrosome replication, multipolar mitoses and, therefore, aneuploidy [15,16]. In addition, unequal segregation of dicentric chromosomes caused by asymmetric exchanges following misrepair of double strand breaks could also lead to aneuploidy. The role of centrosome abnormalities in

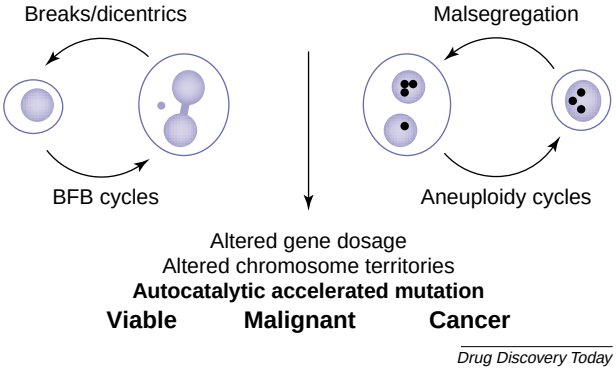


**Figure 1.** Hypermutability state induced by the generation of breakage-fusion-bridge (BFB) cycles and/or aneuploidy leading to altered gene expression and the evolution of the cancer phenotype. Duesberg and colleagues [6,7] have suggested that the induction of aneuploidy could cause altered gene dosage of oncogenes and tumour suppressor genes, as well as genes that control the mitotic process leading to further karyotypic instability. Alternatively, or simultaneously, chromosome breakage or telomere shortening could lead to gene amplification as a result of the BFB cycle mechanism [see Refs 20,38,39,66].

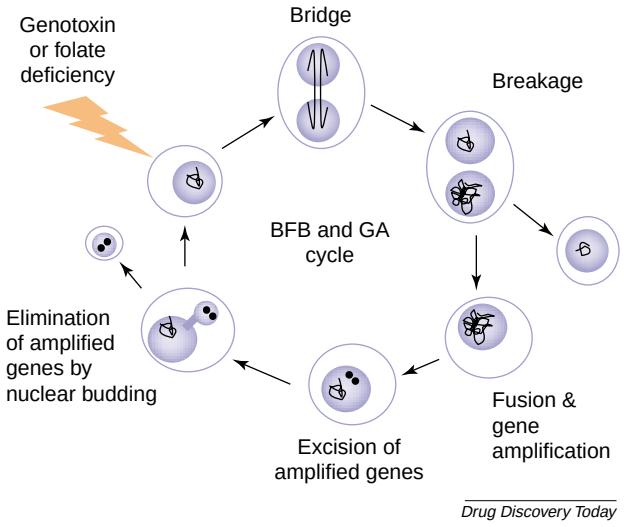


**Figure 2.** The breakage-fusion-bridge (BFB) and gene amplification (GA) cycle that leads to further nucleoplasmic bridges and the elimination of amplified genes by nuclear budding.

cancer, first proposed by Boveri [17], is now increasingly being confirmed for a variety of cancers, such as prostate cancer, in which the frequency of these abnormalities accumulates with progression to a more malignant state [18,19].

## Evidence for BFB cycles and folate-deficient normal cells

An initiating chromosome breakage event can lead to sustained genomic instability via the BFB cycle. This mechanism is particularly crucial for the evolution of the cancer phenotype because it provides the means for a cell to amplify genes that could, by chance, confer selective advantage against a stressful environment (e.g. micronutrient deficiency, cytotoxic drugs, exposure to carcinogens) and/or increase the capacity for cell proliferation or evade the immune response.

The recent work of Gisselson and colleagues [10,20], on primary cultures of solid tumours, confirmed that: (a) nucleoplasmic bridges, micronuclei and nuclear blebs were a common feature of a wide variety of cancer cells, and (b) the vast majority (71–86%) of these abnormal nuclear structures originated from unstable dicentric or ring chromosomes within the tumours. Therefore, the abnormal nuclear morphology that exhibits nucleoplasmic bridges, micronuclei and nuclear blebs is indicative of significant genomic instability within a cell.

Micronuclei (MN) originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [21]. The micronucleus index in human cells has become one of the standard cytogenetic tests for genetic toxicology testing. The CBMN assay is the preferred method for measuring MN in cultured human cells because scoring is specifically restricted to once-divided cells. These cells are recognized by their binucleated (BN) appearance after inhibition of cytokinesis by cytochalasin B [21]. Restricting scoring of MN in BN cells prevents confounding effects caused by suboptimal cell division kinetics and increases the reliability and sensitivity of this assay. Over the past 17 years, the CBMN assay has evolved into a comprehensive method for measuring chromosome breakage, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis (Fig. 4). This assay also has the potential of measuring frequency of multipolar mitoses caused by abnormal centrosome replication (Fig. 4); however, this approach has not been validated yet.

More recently, we have proposed that nucleoplasmic bridges (NPBs) between nuclei in BN cells should also be scored in the CBMN assay because they provide a measure of chromosome rearrangement that is otherwise not measured if only MN are scored [22]. The NPBs are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. It is rarely possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis and, ultimately, breakage of the NPB when the daughter cells separate. However, in the CBMN assay, BN cells with NPBs can accumulate because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes resulting in an NPB.

We have recently validated the use of NPBs as a biomarker of DNA damage in human WIL2-NS cells treated with hydrogen peroxide, superoxide or after co-incubation with activated human neutrophils [22]. NPB frequency in BN cells increased up to 20-fold relative to control in a dose-related manner with these DNA strand break inducing agents. NPBs were positively correlated with MN frequency in the same BN cells ($r > 0.82$, $P < 0.0001$). Over 60% of the BN cells with one or more NPB also contained one or more MN; this fits with the fact that acentric chromosome fragments (expressed as MN) and dicentric chromosomes (expressed as NPBs) are expressed simultaneously within a cell following exposure to agents such as ionizing radiation that induce double-stranded DNA breaks [23].

Over the past decade, another unique mechanism of MN formation, known as nuclear budding, has emerged. This process has been observed in cultures grown under strong selective conditions [24–26], which induce gene amplification. Shimizu *et al.* [27,28] showed that amplified DNA is selectively localized to specific sites at the periphery of the

nucleus and eliminated via nuclear budding to form MNi during S-phase of mitosis. Furthermore, DNA synthesis inhibitors, such as hydroxyurea, were shown to increase the rate of elimination of amplified DNA via this process. Amplified DNA might be eliminated through recombination between homologous regions within amplified sequences forming minicircles of acentric and atelomeric DNA (double minutes), which localize to distinct regions within the nucleus or through the excision of amplified sequences after segregation to distinct regions of the nucleus. This suggests that the nucleus could have a capacity to sense excess DNA that does not fit well within the nuclear matrix indicating a higher order DNA repair or nuclear housekeeping process. Shimizu *et al.* [27,28] have suggested that the nucleus eliminates excess amplified DNA by an active process that concentrates the amplified DNA to a peripheral point in the nucleus, following which this surplus DNA is budded out to form a micronucleus and eventually excluded from the cell altogether by extrusion of the micronucleus from the cytoplasm leading to the formation of a 'minicell'. The process of nuclear budding occurs during S-phase and the nuclear buds are characterized by having the same morphology as a micronucleus with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process.

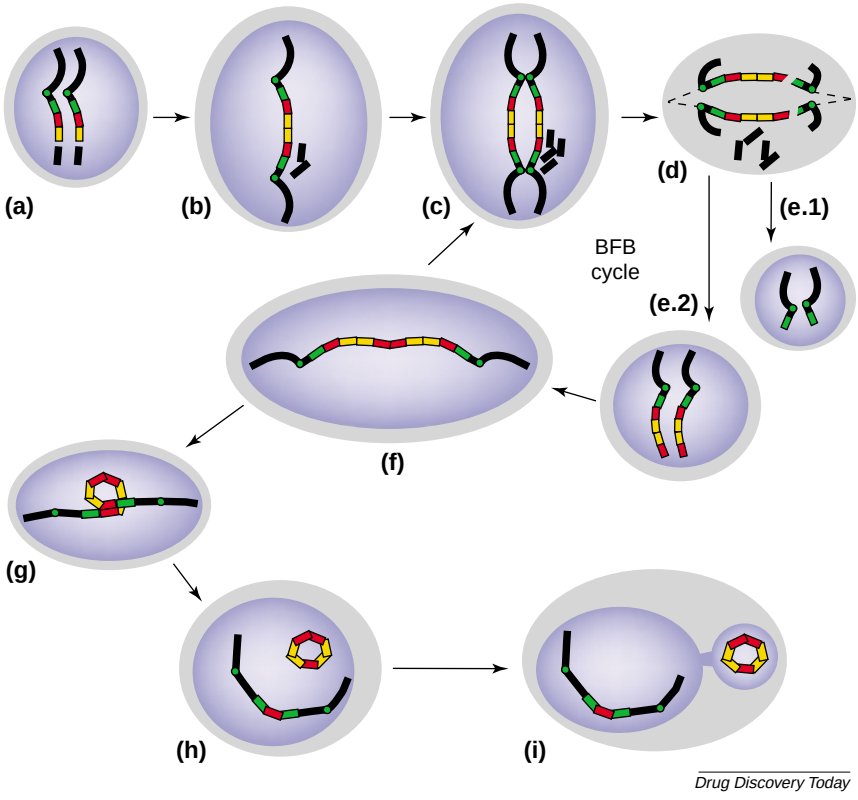


**Figure 3.** Gene amplification via breakage-fusion-bridge (BFB) cycles. Broken sister chromosomes **(a)** fuse at the 'sticky' ends to form a dicentric chromosome **(b)**. The dicentric chromosome is replicated in S-phase **(c)** and drawn to the cell's poles during anaphase (bridge) **(d)**. In this example, the dicentric chromosomes break in a non-central region and one daughter cell will receive chromosomes that contain two copies of the yellow and red genes **(e.1)**, while the other cell's chromosomes have a deletion in these genes **(e.2)**. The chromosomes with multiple copy number might fuse again **(f)** to propagate the amplification cycle (back to step **c**). Alternatively, recombination might occur between homologous sequences **(g)** and result in the 'looping-out' of a circular acentric DNA fragments or double minute **(h)**, which are subsequently extruded from the nucleus via budding **(i)**. Reproduced, with permission, from Ref [66].

We have observed nuclear buds in several previous experiments; however, these structures were never properly investigated or quantified. In a recent study on folic acid deficiency in long-term primary human lymphocyte cultures, we carefully quantified the inter-relationship between MN, NPBs and nuclear buds in an attempt to validate the use of these biomarkers and to determine more comprehensively the impact of folic acid deficiency on various aspects of genomic stability [29]. Briefly, lymphocytes ($5 \times 10^6$) from 20 (8 male, 12 female) asymptomatic volunteers (34–65 yrs) were cultured in duplicate in RPMI-1640 medium containing 5% dialysed foetal calf serum, 10 U $ml^{-1}$ interleukin-2 and either 12, 24, 60 or 120 nM folic acid for nine days. Medium was changed at days 3 and 6, cytokinesis was inhibited on day 8 and cells were transferred to microscope slides 24 h later (see [29,30]). We analyzed separately the pooled data from this study and verified that folic acid concentration correlated significantly ($P<0.0001$) and negatively ($r = -0.63$ to $-0.74$) with all these markers of chromosome damage, which were minimized at 60–120 nM folic acid, the latter being greater than the concentration of folate normally observed in plasma (10–30 nM), (Fig. 5). However, even more interestingly, we observed that the frequency of NPBs and nuclear buds correlated significantly and negatively with folic acid dose, suggesting that chromosome rearrangement and gene amplification are induced by folic acid deficiency. The strong cross-correlation between MN, NPB and nuclear bud frequency ($r = 0.75 - 0.77$, $P<0.001$) (Table 2) suggests a

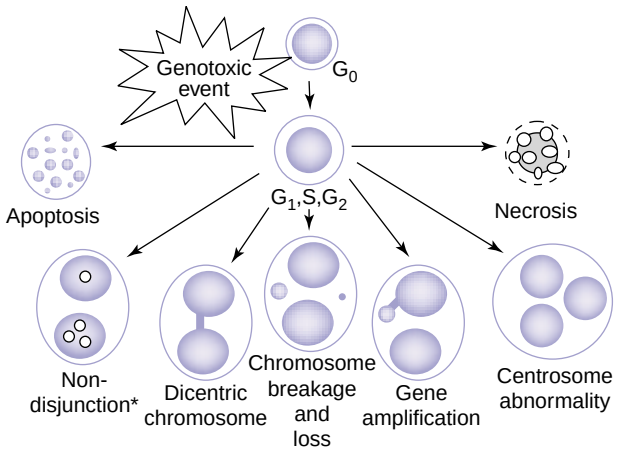


**Figure 4.** The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic and/or genotoxic agents. Using these biomarkers within the cytokinesis-block micronucleus (CBMN) assay it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, for example, dicentric chromosomes (NPB), gene amplification (nuclear buds), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells. Chromosome loss can be distinguished from chromosome breakage using pancentromeric probes or anti-kinetochore antibodies. *In addition, non-disjunction (malsegregation of chromosomes) can also be measured in BN cells using chromosome-specific centromeric probes. The CBMN assay has not been previously used to measure centrosome abnormalities but this is theoretically possible by scoring the frequency of tri- and/or quadrinucleated cells and confirming that their DNA content is 4N. Reproduced, with permission, from Ref [66].

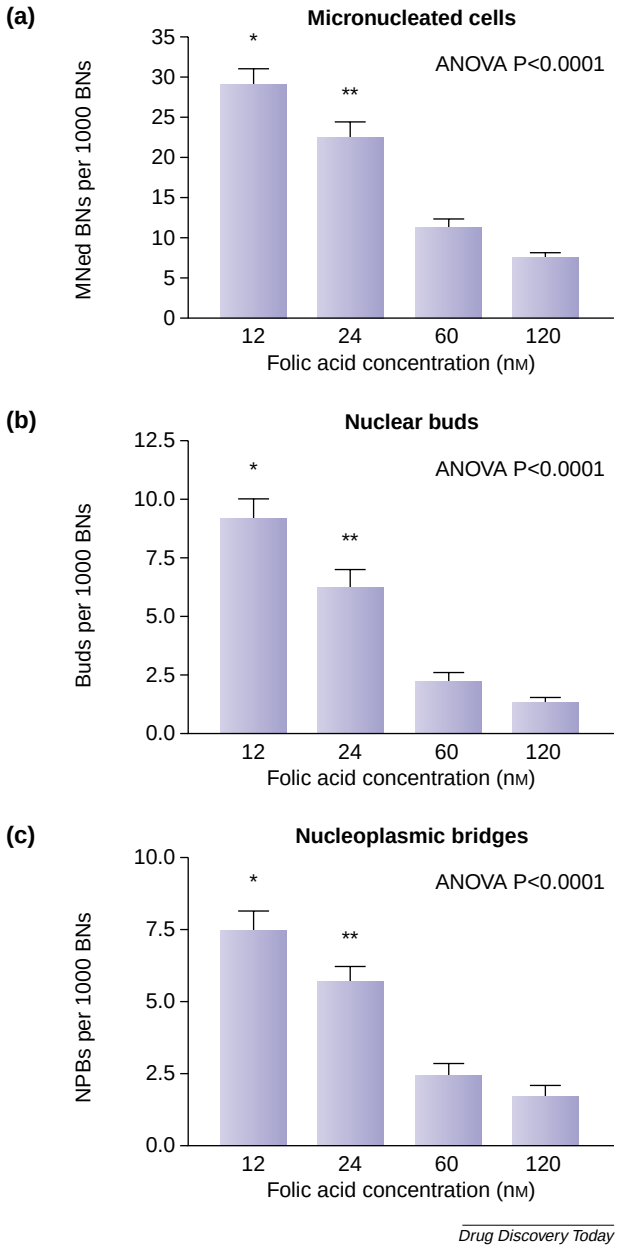


**Figure 5.** The effect of medium folic acid concentration on the induction of micronucleated (MNed) binucleated (BN) cells, nuclear buds (Buds) and nucleoplasmic bridges (NPBs) in primary human lymphocytes *in vitro*. N = 20. Data = mean ± SEM. * = P <0.01 versus 24, 60 and 120 nM. ** = P <0.01 versus 60 and 120 nM (Tukey's post test). Reproduced, with permission, from Ref [66].

common mechanism initiated by folic acid deficiency-induced DNA breaks, although coincidence of effects with other DNA damage events (e.g. folic acid deficiency-induced CpG hypomethylation) cannot be excluded.

## BFB cycles explain MN, NPB and nuclear buds induced by folic acid deficiency

Because folic acid deficiency is known to cause gene amplification and chromosome damage such as double stranded breaks [31–33], it is likely that these events contribute to the formation of MN, NPBs and nuclear buds in this system. Gene amplification is thought to be a key event in cellular resistance to drugs like methotrexate [34] and in tumour progression [35]. Several plausible models of gene amplification have been proposed (reviewed in [36,37]); however, the presence of NPBs in the cytokinesis-blocked cells provides support for the BFB cycle model described in the seminal work of McClintock in maize [38]. According to this theory, sister chromatids that have both undergone double-stranded breakage, fuse at a distal position (possibly telomeric) forming a dicentric chromosome with two copies of homologous genes positioned between the two centromeres. During anaphase, these dicentric chromosomes are drawn towards both poles and form (nucleoplasmic) bridges. During cytokinesis, these dicentric chromosomes,

**Table 2. Correlation matrix for DNA damage biomarkers of the CBMN assay induced by varying folic acid concentration [66].**

| | | [Folic acid] | MNed BNs | NPBs |
|---|---|---|---|---|
| **NuclearBuds** | **P** | <0.001 | <0.001 | <0.001 |
| | **r** | –0.68 | 0.76 | 0.75 |
| **NPBs** | **P** | <0.001 | <0.001 | |
| | **r** | –0.63 | 0.77 | |
| **MNed BNs** | **P** | <0.001 | | |
| | **r** | –0.74 | | |

Abbreviations: CMBN, cytokinesis-block micronucleus; NPB, nucleoplasmic bridges; Mned BNs, micronucleated binucleated cells.

which span both daughter nuclei, are thought to break unevenly and might form a chromosome with two copies of one or more genes and a chromosome (fragment) with no copies of these genes. The chromatids with multiple copy number of these genes might fuse again during interphase forming a dicentric chromosome (doubling again the gene copy number within the chromosome), which is then replicated during the next nuclear division leading to the next BFB cycle and further gene amplification (see Figs 2 and 3).

The BFB cycle model has been validated as a mechanism for gene amplification in various systems, including: (i) coformycin-induced amplification of the adenylate deaminase gene in Chinese hamster cells [24], (ii) *N*-(phosphonyacetyl)-L-aspartate-induced amplification of the CAD gene in human fibrosarcoma cells, which is enhanced by the expression of *Vpr*, a HIV accessory gene [25], (iii) methotrexate-induced amplification of the dihydrofolate reductase gene in Chinese hamster cells [26], and iv) actinomycin D-induced amplification of the multi-drug resistance 1 gene in Chinese hamster cells through the induction of fragile sites which determined the initiation and size of amplicons [39]. Folic acid deficiency-induced fragile site expression and DNA hypomethylation could have also contributed to the promotion of gene amplification and resulted in elimination of this DNA by nuclear budding in our system. For example, the induction of hypomethylation by 5-aza-2′deoxycytidine has been reported to enhance *N*-(phosphonylacetyl)-L-aspartate-induced amplification of the CAD gene in Syrian hamster kidney cells [40].

Therefore, the genomic instability phenotype can be readily recognized by examining cells for abnormal nuclear morphology indicative of BFB cycles, for example, MN, NPBs and nuclear buds. In addition, genomic instability can also be manifested by a high rate of aneuploidy and multipolar mitoses which are detectable by fluorescence *in situ* hybridization (FISH) or cytologically, respectively. In our experience, one of the better methods for measuring and/or observing BFB cycles and non-disjunction/chromosome loss is the CBMN assay. The carcinogenic potential of a chemical or physical agent should be based on the capacity to induce BFB cycles and aneuploidy, which is best assessed in this assay because observation and/or quantitation of MN formation, nuclear budding, non-disjunction and NPB formation is efficiently and accurately achieved in binucleated cytokinesis-blocked cells. The generation of BFB cycles and nuclear budding is best observed in a 9-day culture because this will enable a sufficient number of nuclear divisions to occur for gene amplification to be observed using the nuclear budding cytogenetic end-point.

The results with folic acid show quite clearly that micronutrient deficiencies can on their own cause the type of genomic instability observed in cancer. The fact that we could observe significant increments in MN, NPBs and nuclear buds over the physiological concentration range of folic acid (12–120 nM) supports the hypothesis that folate is important in the prevention of cancer. These observations have (a) provided further impetus for the concept that the recommended dietary allowances should be based on the prevention of genomic instability (as explained below) and (b) highlighted the potential importance of micronutrient concentration in culture medium as an important modifier, not only of spontaneous chromosome abnormality but also of chemically induced genome damage. These points have important implications in the relative risk assessment of chemical/radiation exposure depending on micronutrient status *in vitro* or *in vivo*.

## DNA damage biomarkers as predictors of cancer risk

Because cancer can take from several years to decades to develop it is not always practical to perform prospective epidemiological studies over such long periods. Therefore, there is justifiable interest in determining whether biomarkers of DNA damage might be predictive of cancer risk. Table 3 lists some of the DNA damage biomarkers that are currently used to study human cells *in vitro* or *in vivo*. However, the significance of these biomarkers in terms of predicting cancer is largely unknown. Taking smoking as an example, one can measure environmental exposure (i.e. number of cigarettes smoked), internal dose (e.g. cotinine concentration in the blood), molecular dose as a biomarker of exposure [e.g. polycyclic aromatic hydrocarbon (PAH) adducts in DNA of sampled tissue], preclinical biological effects, biomarkers of effect (e.g. chromosome aberrations, tumour suppressor gene inactivation), and, ultimately, clinical disease (i.e. lung cancer). The biomarkers of exposure and effect and clinical disease can all be largely influenced by susceptibility factors, which include polymorphisms

**Table 3. DNA damage biomarkers**

- DNA strand breaks (e.g. SCGE assay)
- Chromosome aberrations
- Cytokinesis-block micronucleus assay
- Aneuploidy
- Telomere shortening
- Apurinic sites
- DNA adducts
- DNA oxidation
- DNA methylation
- Nuclear p53
- Point mutation assay (HPRT)
- Mitochondrial DNA mutation
- DNA damage in germ cells

Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyl transferase; SCGE, single cell gel electrophoresis.

that alter the activity of relevant DNA repair, carcinogen metabolism and apoptotic pathway genes, as well as dietary factors that alter the activity of these genes.

One would expect, in theory, that biomarkers that measure events on the direct causal path to cancer would be the ones most likely to be predictive. However, because cancer involves multiple mutations it is not clear whether a generic biomarker that is predictive of genomic instability leading to hypermutation would be as important as a common event in cancer cells, such as inactivation of p53 or apoptosis. An important limitation of DNA damage biomarkers in human studies is the relevance of the accessible tissue in which DNA damage is measured (e.g. erythrocytes, lymphocytes, exfoliated epithelial cells) to the cancer studied (e.g. breast, prostate, colon). Ideally, measurements are conducted in the same tissue.

The validation of a single biomarker of DNA damage for cancer prediction requires a long period of study often involving the effort of numerous laboratories. The process of validation has been reviewed recently [41]; however, this would involve three key stages: (a) the development of standardized protocols that take into account sample acquisition, sample storage, expression time, (b) determining the most important methodological, demographic, life-style and genetic variables that influence the index measured, and finally (c) the test in case-control and prospective studies of the sensitivity and specificity of the biomarker to predicting risk of cancer generally and of a specific cancer.

The only cytogenetic biomarker that has been through most of the stages outlined previously is the technique of classical metaphase analysis for measurement of chromosome aberrations (i.e. chromosome breaks and rearrangements) in human lymphocytes. The results of the Italian and Nordic cohort prospective studies have shown a significant 2.3–2.6-fold increased risk for cancer in those individuals with a high level of chromosome aberrations (CAs) compared with those with low levels of CAs [42]. Furthermore, the risk for high versus low levels of CAs was apparent regardless of exposure to known carcinogens suggesting that increased chromosome damage is itself a causal factor in cancer.

## The CBMN assay in lymphocytes – a case study of a maturing biomarker of DNA damage

The micronucleus assay in human lymphocytes is one of the most commonly used methods for measuring DNA damage rates in human populations because it is relatively easier to score MN than chromosome aberrations. Micronuclei originate mainly from chromosome breaks or whole chromosomes that fail to engage with the mitotic spindle when the cell divides. The current methodology is based on the CBMN assay (Fig. 4) in which lymphocytes from a blood sample are cultured *ex vivo* and the once divided cells are recognized by their binucleate appearance after cytokinesis-block with cytochalasin B [43].

In combination with chromosome specific centromeric probes, the cells can also be analyzed for non-disjunction and aneuploidy of specific chromosomes that might be important in cancer (e.g. chromosomes 7 and 17) or in Alzheimer's disease (e.g. chromosome 21). For example, this approach has been used recently to demonstrate that individuals who develop Alzheimer disease have a significantly higher propensity in their lymphocytes to malsegregate chromosome 21, leading to elevated frequencies of chromosome 21 triploidy [40]. This supports the hypothesis that perhaps mosaicism of chromosome 21 aneuploidy could lead to accelerated ageing of selected portions of specific tissues within the body, such as the brain.

The CBMN assay has also been successfully used to show that individuals who develop breast cancer, and their relatives, exhibit elevated sensitivity to the DNA-damaging effects of ionizing radiation [45]. This sensitivity was indicative of a defect in double-strand break repair and was observed in 10 of 11 cases of BRCA1 mutation carriers [46]. These data suggested that the assay was useful not only as a biomarker of spontaneous damage to DNA but also as a measure of DNA repair phenotype. Given the large number of different BRCA1 mutations and the 120+ DNA repair genes [47] it might be more practical to study the phenotype than to try to unravel or predict phenotype from complete genotypic information and micronutrient status.

The wide-spread use of the CBMN assay has made it feasible to launch an international collaborative project, the HUMN project [48] (http://humn.org), with the following

objectives: (1) determining the important methodological, demographic and life-style variables that influence this index of DNA damage, (2) establishing standardized protocols for the assay, and (3) ultimately to determine the capacity of this biomarker to predict cancer risk, as well as the risk of other degenerative diseases, such as Alzheimer's disease. The scope of this project is such that it has the participation of at least 40 laboratories in more than 16 countries with a total of base-line MN frequency data on more than 6000 subjects and in excess of 60,000 person years in terms of relevance for prospective epidemiological studies. Having established the network successfully it was possible to collate and analyze the various databases, which led to three important observations: (1) base-line frequencies of MN frequency varied greatly between populations, (2) age and gender were the two most important demographic factors influencing MN frequency, and (3) the scoring criteria used were probably the most important method variable influencing the score obtained [49]. Exposure to genotoxic agents, host factors, methods and scoring criteria explained 75% of the total variance. A more recent analysis of the data showed that current smoking status produced an increase in MN frequency only in those smoking 30+ cigarettes per day [50]. This initial study also provided a reference range of 'normal' MN frequency values. In the second stage of the project, standardized protocols are being developed and the extent of variation in slide scoring between scorers and laboratories is currently being assessed. During 2002, a prospective cohort study is being performed in Europe in the EU CancerBiomarkers programme, in which the predictivity with respect to cancer risk of MN frequency in lymphocytes measured using the CBMN assay will be determined.

## Diet is an important variable in DNA damage biomarkers

We have also been using the CBMN assay to try to understand how diet influences base-line DNA damage rate in lymphocytes. This type of study is essential not only in terms of setting dietary recommendations but also in interpreting the biomarker data when they are used in biological dosimetry of exposure to genotoxins or to predict cancer risk.

Our earlier studies in vegetarian and non-vegetarian populations, who had wide differences in plasma folate, vitamin B12, vitamin C and vitamin E concentrations, indicated quite clearly that folate and vitamin B12 were more important determinants of micronucleus (MN) frequency than vitamin C or vitamin E [51]. Subsequent studies in men aged (50–70 yrs) and young adults (aged 18–20 yrs) confirmed these early observations. However, in addition,

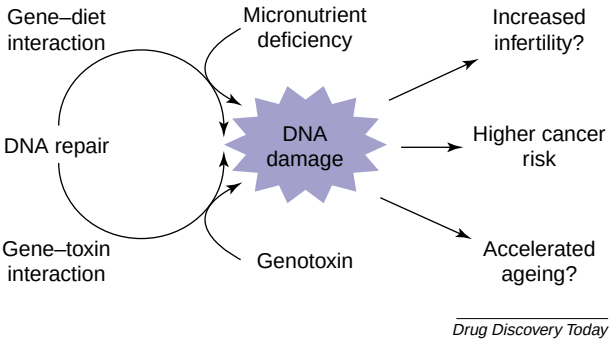


**Figure 6.** The various possible interactions between genotoxin exposure, micronutrient status and genetic background that determine DNA damage rate and diseases caused by accelerated genomic instability.

they also showed that MN frequency was not only significantly and negatively correlated with serum vitamin B12 but also significantly and positively correlated with plasma homocysteine, a risk factor for cardiovascular disease [52,53]. These cross-sectional data showed that MN frequency was minimized when plasma homocysteine was <7.5 μmol $l^{-1}$ and serum B12 was >300 pmol $l^{-1}$, the latter being double the concentration associated with anaemia. Using a placebo-controlled intervention trial, we also demonstrated that supplementation over three months with 3.5-times the Australian recommended dietary allowance (RDA) for folic acid (700 μg) and vitamin B12 (7 μg) reduced MN frequency in lymphocytes by 25% in young adults who were in the top 50% of MN frequency but had no influence on MN frequency in those in the low 50% of MN frequency before the intervention [53]. These results illustrate that dietary deficiency of folate and vitamin B12 is an important determinant of DNA damage rate and that above RDA intakes of these vitamins might be required in large subsets of a population to minimize DNA damage rate.

These results have an added important implication in that it is now feasible and plausible to consider the concept of recommended dietary allowances (RDAs) using genomic stability as the main parameter on which to set these intake levels [54]. The importance of this approach is supported by the fact that: (a) several micronutrients, such as zinc, magnesium, folic acid and vitamin B12, are required as co-factors in DNA metabolism, (b) deficiency in these co-factors could induce important chromosomal mutations that increase cancer risk, and (c) genomic stability is crucial for normal function of cells. We can envisage a future when the ability to use DNA damage biomarkers for predicting cancer risk will be considerably improved because of a better understanding of how dietary factors modulate genomic stability.

## The future

The future for the use of DNA damage biomarkers will consist of a better integration of classical cytogenetic and non-cytogenetic methods with molecular probes, leading to automated simultaneous and multiple measurements, not only of events that lead to a hypermutable state but also of specific mutations on the causal pathway of cancer. The use of cDNA arrays and proteomics will enable us to better understand which genes are upregulated and downregulated following specific genotoxic insults and which of these correlate best with DNA damage events on the causal pathway of cancer. The main challenge is to make the full loop in predicting cell phenotype (normal or cancer) from the large amount of information generated from array strategies. Equally important will probably be the shift from considering not only gene–toxin interaction in predicting DNA damage and cancer outcomes but to integrate this approach with gene–diet interaction studies (Fig. 6). This comprehensive approach, combined with more robust techniques, will hopefully be fruitful in identifying better not only the cancer risk of large genetic sub-groups but also individuals, which is the ultimate goal.

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