Cytokinesis-blocked micronucleus assay and cancer risk assessment

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Cancer risk assessment is a multidisciplinary process that goes beyond the scope of classical epidemiology to include the biological evaluation of individual differences to carcinogenic exposures. The inclusion of genetic biomarkers such as mutagen sensitivity or cytokinesis-blocked micronucleus (CBMN) assay end points into risk assessment models allows for a more comprehensive determination of cancer risk that includes known demographic (age and gender), lifestyle exposures (smoking and alcohol) and occupational or environmental exposures. The CBMN assay generates multiple correlated end points that, after applying data reduction methods, could be combined into a summary measure that incorporates information from each individual variable into a single (or possible multiple, uncorrelated) measure of risk. In this article, we highlight the use of the CBMN assay in radiosensitivity assessment. In addition, we demonstrate the potential use of the combined summary measures in cancer risk assessment as a result of chronic exposure to tobacco carcinogens. The simplicity, rapidity and sensitivity of the CBMN assay not only make it a valuable tool for screening but also the multiple end points simultaneously generated lead to a better understanding of the underlying mechanisms involved in the carcinogenic process that could in turn substantially improve risk predictions.

Introduction

Cancer is a multistage process that results from an accumulation of multiple genetic changes. The concept that genetic susceptibility to development of cancer is related to genomic instability was initially supported by rare disorders such as ataxia telangiectasia and xeroderma pigmentosum, which are associated with in vivo and in vitro chromosomal instability and defective DNA repair capacity (1,2). It is now established that maintaining the integrity of the genome is essential for normal cell function and any disruption in the process can lead to cell death or cancer development. Each genetic alteration or mutation, whether an initiating or a progression-associated event, can be mediated through a gross chromosomal change and therefore has the potential to be cytogenetically detectable (3). Cytogenetic assays are classical methods to detect chromosome aberrations (CAs), which have been used as end points of exposure to genotoxic agents. The conceptual basis has been the assumption that the extent of genetic damage reflects critical events for carcinogenesis, such as an impaired ability to remove damaged DNA or failure to correctly rejoin DNA breaks (4). Several investigators have reported a high incidence of chromosomal instability in lymphocytes of patients with different types of neoplasia (5–7). An increased frequency of CA in circulating lymphocytes is generally considered indicative of increased cancer risk for those exposed to DNA damaging agents. Hagmar et al. (8) and Bonassi and Abbondandolo (9) in two independent prospective studies reported a significant increase in the mortality ratio for all cancers in subjects who had previously shown increased levels of CA in their lymphocytes. Importantly, the data from both these studies, when pooled, indicate that the frequency of CA in peripheral blood lymphocytes (PBLs) is a relevant biomarker for cancer risk in humans, reflecting both early biological effects of exposure to genotoxic carcinogens and individual cancer susceptibility (10).

Mutagen sensitivity testing

In 1983, Hsu (11) proposed that environmental exposure induced chromosome damage varies along a continuum in the general population with higher levels observed in individuals with an inherent susceptibility to DNA damage. In 1989, Hsu et al. developed the mutagen sensitivity assay to detect potential variations in susceptibility to the effects of mutagenic agents. The assay measures the frequency of induced chromatid breaks in short-term cultured lymphocytes after exposure to different mutagens. This assay is considered an integrated biomarker that reflects the sensitivity to a mutagen, as well as DNA repair capacity in the study subjects (12). A number of investigators have reported that cancer patients express the mutagen-sensitive phenotype significantly more often than cancer-free control subjects (13–17). In addition, many laboratories use the mutagen sensitivity assay to compare mutagen induced DNA damage to circulating lymphocytes from subjects with cancer and subjects without cancer using cyogenetic end points. The original mutagen sensitivity testing by Hsu et al. (12) utilised bleomycin as the test mutagen, however, in the last three decades, a large number of mutagens have been applied to fresh or cryopreserved PBL cultures such as γ-rays, ultraviolet radiation, benzo[a]pyrene diol epoxide, hydrogen peroxide (H2O2) and heterocyclic amines [2 amino-1 methyl-6-phenylimidazo [4,5-b] pyridine] (18–21). Several studies investigated the genetic heritability of sensitivity to mutagens (22) and found that first-degree relatives of mutagen-sensitive individuals were also mutagen sensitive indicating an element of genetic susceptibility to such sensitivity (19). The cytokinesis-block micronucleus (CBMN) assay in human lymphocytes is one of the most commonly used
methods for measuring DNA damage. The success of this assay stems from the fact that it allows a large number of potentially genotoxic chemical agents to be analysed both in vitro and in exposed populations. Micronuclei (MN) originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. Compared to other cytogenetic assays, quantification of MN confer several advantages, including speed and ease of analysis, no requirement for metaphase cells and reliable identification of cells that have completed only one nuclear division, which prevents confounding effects caused by differences in cell division kinetics because expression of the genetic damage end points is dependent on completion of nuclear division (12). Because cells are blocked in the binucleated stage, it is also possible to measure nucleoplasmic bridges (NPBs) originating from asymmetrical chromosome rearrangements and/or telomere end fusions (23–26). Both MN and NPBs occur in cells exposed to DNA-breaking agents (27,28). In addition to MN and NPBs, the CBMN assay allows for the detection of nuclear buds (NBUDs), which represent a mechanism by which cells remove amplified DNA and are therefore considered a marker of possible gene amplification (29). The CBMN test is slowly replacing the analysis of CAs in lymphocytes because the damage end points are easy to recognise and score and the results can be obtained in a shorter time (30). In addition, information regarding other cellular events such as mitotic rate and cell death by apoptosis and necrosis can be simultaneously obtained from the same slides.

CBMN assay as a biomarker of radiosensitivity

Among the cytogenetic assays most frequently used for the assessment of in vitro chromosomal radiosensitivity and cancer risk, the CBMN assay for PBLs is characterised by high reliability and low cost. These and other characteristics (simplicity of execution, possibility of automation, discrimination between aneugenic and clastogenic exposure) have contributed to the worldwide success of this test for in vitro and in vivo studies of genome damage (31–33). Ionising irradiation (IR) induces DNA damage through direct interaction with DNA or indirectly, through damage induced by reactive species produced by ionizations elsewhere in the cell. IR-induced DNA damage can occur separately or in clusters and it includes base damage, single-strand breaks (SSBs), double-strand breaks (DSBs) and cross links (DNA interstrand or DNA–protein) (34). Base damage and SSB are usually repaired by the cell in an error-free manner. DNA DSB are considered to be among the most distinctive and detrimental forms of IR-induced DNA damage as the information content is lost on both DNA strands. Complex damaged sites and DSB are often non-repaired or misrepaired, resulting in CA and chromosomal instability (34–39).

Many inherited disorders predisposing to cancer show elevated levels of spontaneous CAs and an enhanced sensitivity to the induction of chromosomal aberrations by IR (40,41). Initially, chromosomal radiosensitivity was observed in cancer-prone chromosome fragile syndromes such as ataxia-telangiectasia, Fanconi’s anaemia and Nijmegen-breakage syndrome (1,2). In the last years, a large number of studies have shown that enhanced chromosomal radiosensitivity is also present in many other cancer-prone conditions and in significant proportions of cancer patients (42–44). The main causes of chromosomal radiosensitivity are defects in the pathways involved in DNA DSB repair and cell cycle checkpoint control (37–39,43). For the detection of in vitro chromosomal radiosensitivity with the CBMN assay, different in vitro irradiation procedures can be adopted. For a given dose, low-dose rate (LDR) irradiation will result in a lower MN yield compared to high-dose rate (HDR) irradiation, as more time is available for sublethal lesions to be repaired. Results obtained by different research groups confirmed that, compared to HDR, LDR allows a better discrimination between sensitive and non-sensitive individuals (40,45).

In specific populations of breast cancer patients, enhanced chromosomal radiosensitivity is very evident (44,46–59); on the basis of this evidence, a link has been proposed between chromosomal radiosensitivity, deficient DSB repair and genetic predisposition to breast cancer. Chromosomal radiosensitivity has been observed in sporadic breast cancer patients as well as in patients with a family history of the disease. Since in some cases, MN frequencies of the less radiosensitive cancer patients may overlap with MN frequencies of the most radiosensitive healthy controls, an arbitrary cut-off value for chromosomal radiosensitivity has to be determined to allow the comparison of the rate of radiosensitive individuals between different populations. Most studies use the 90th percentile of the normal population as cut-off point for radiosensitivity. In the studies performed by Baeyens et al. (48,49), it was demonstrated that, using the 90th percentile of the control population as cut-off point for radiosensitivity, 61% of familial breast cancer patients were radiosensitive with the CBMN assay (45). For sporadic breast cancer patients, the percentage of radiosensitive patients was lower, 26%, but still significantly higher compared to the controls (49). No correlation was found between chromosomal radiosensitivity (using CBMN assay) and age of onset of the disease in breast cancer patients (49). The fact that enhanced in vitro chromosomal radiosensitivity is also observed among blood relatives of breast cancer patients with high MN scores points to the heritability of chromosomal radiosensitivity in breast cancer (19,60).

The involvement of mutations in the two most important breast cancer susceptibility genes—BRCA1 and BRCA2—in chromosomal radiosensitivity of breast cancer patients is frequently discussed in the literature. As several studies showed that ~40% of all breast cancer patients are characterised by an enhanced chromosomal radiosensitivity, a mutation in BRCA1 or BRCA2—detected in only 3–5% of all breast cancer patients—cannot account for such a high rate of radiosensitive cases. The fact that heterozygous BRCA1/BRCA2 mutation carriers would be more radiosensitive comes from mechanistic studies showing that the BRCA genes play a role in DSB repair (61,62). Literature data concerning the chromosomal radiosensitivity of heterozygous BRCA1/BRCA2 mutation carriers are limited and the conclusions also depend on the type of cytogenetic assay used to measure chromosomal radiosensitivity (63). With the CBMN assay, an enhanced radiosensitivity was found in PBLs of BRCA1/BRCA2 carriers (64–68). In the study performed by Baeyens et al. (68), breast cancer patients with a BRCA1 or BRCA2 mutation were on the average more radiosensitive than healthy women, but not different from breast cancer patients without a BRCA mutation, suggesting that mutations in BRCA1 or BRCA2 genes are not playing a major role in chromosomal radiosensitivity, as observed with the CBMN assay. MN studies performed on Epstein-Barr virus-transformed lymphoblastoid cell lines of
BRCA1 and BRCA2 heterozygotes also reported contradictory results (67,69). Whether individuals heterozygous for BRCA1/BRCA2 are in vitro more radiosensitive or not is very important for the medical management (mammography screening and cancer therapy) of these individuals.

Most of the above-reported findings support the view that enhanced chromosomal radiosensitivity of PBLs is a hallmark of breast cancer patients and may be a marker for low-penetrance genes conferring breast cancer susceptibility. As MN are the result of non-repaired or misrepaired ionising radiation-induced DSB, low-penetrant variations in genes involved in the processing and repair of these breaks may be involved in the genetic predisposition to breast cancer. To date, several genetic association studies have shown a correlation between single-nucleotide polymorphisms (SNPs) in DNA DSB repair genes, radiosensitivity and breast cancer risk (70–73). SNP studies performed by Willems et al. (72,73) in genes involved in non-homologous end joining, the main pathway for radiation-induced DSB repair (36,39), provided evidence that the variant alleles of the c.-1310C>G SNP and the c.2099-2408G>A in the XRCC6/Ku70 gene are risk alleles for breast cancer as well as for chromosomal radiosensitivity. Additionally, the combination of the variant ‘G’ allele of the c.-1310C>G SNP in Ku70 with a hormonal risk factor, reflecting susceptibility to oestrogen exposure, is associated with a more pronounced increase in breast cancer risk (73). Since breast tissue is receptive to oestrogen exposure and because oestrogen plays a dual role as a growth-stimulating hormone and as a DNA damaging agent, DSB repair is particularly important in breast tumorigenesis (74).

CBMNN assay as predictive biomarker of susceptibility to tobacco carcinogens

One of the classic examples of genetic host susceptibility as a modulator of individual’s risk for development of cancer is exposure to tobacco smoke carcinogens. This notion is supported by the fact that only a fraction of long-term smokers (~15%) will develop lung cancer in their lifetimes (75) even though smokers are exposed to a cocktail of >60 different carcinogens in cigarettes smoke (76). Several biomarkers have been used to evaluate susceptibility to the carcinogenic effects of benzo[a]pyrene; however, no assays specifically evaluated susceptibility to the nicotine-derived nitrosamine 4-(methylnitosamino)-1-(3-pyridyl)-1-butaneone (NNK), which is a potent inducer of lung adenocarcinoma (77). It has been reported that the total level of NNK in mainstream smoke is 3 to 15 times that of benzo[a]pyrene (78). NNK is an aromatic compound that induces lung cancer independent of route of administration, in both susceptible and resistant strains of mice (79). NNK induces DNA cross links, DNA adducts; and increases the frequency of chromosome aberrations (80,81). DNA adducts are generated by NNK through the methylation or the pyridyloxobutylations pathway. Bulky adducts are repaired by nucleotide excision repair, un repaired DNA damage can block DNA replication and thereby result in the generation of double-strand DNA breaks. Removal of methylated adducts leads to the creation of apurinic/apyrimidinic sites that subsequently lead to strand breaks. If not repaired or illegitimately recombined, DSBs will be expressed as chromatid-type aberrations in M1. Likewise, a similar process may give rise to aberrations in M2 with O6MeG/T-derived secondary lesions being the trigger (82,83). El-Zein et al. (84) used the CBMN assay to evaluate susceptibility to NNK among smokers who developed lung cancer cases and smokers who remained disease free. They reported that lung cancer cases and controls exhibited significant differential sensitivity to the genotoxic effects of NNK where the lymphocytes from patients were significantly more sensitive to NNK clastogenic effects than were those from the smoker controls. When analysed on a continuous scale NNK sensitivity phenotype based on induced MN, NPBs and NBUDs was associated with a 2.3, 45.5 and 10.0-fold increased risk for developing lung cancer, respectively (84). When other events of toxicity [such as MN in mono-nucleated (MN-Mono) cells, cellular apoptosis, necrosis and nuclear division index] were included in the analysis in an effort to fully evaluate the CBMN cytome (CBMN cyt) assay as a comprehensive method for determination of genotoxicity; the lung cancer cases showed significantly higher levels of MN in mono-nucleated cells, apoptosis and necrosis than controls (P < 0.001). These observations support the hypothesis of breakage-fusion-bridge cycle mechanism of hypermutation during carcinogenesis (reviewed in refs 27,28). Gisselsson et al. (85) reported that abnormal nuclear morphology (associated with MN, NPBs and NBUDs) is indicative of significant genomic instability within cells and is a common feature of a wide variety of cancers. An increase in the frequency of these chromosomal damage end points in a surrogate tissue, such as PBLs, would imply constitutional sensitivity to genetic damage. In addition, to evaluating DNA damage end points, the CBMN cyt assay allows for evaluation of cell death events. The tobacco carcinogen NNK has been shown to induce apoptosis, either through induction of ROS (86) or through release of arachidonic acid (87). In the study by El-Zein et al. (88), the lung cancer cases had a lower induction of apoptosis by NNK as compared to the controls indicating the possibility of defective apoptotic machinery in the cases, thus allowing for the survival of damaged cells. NNK induces phosphorylation of Bcl2 facilitating the interaction between Bcl2 and c-Myc which in turn stabilises c-Myc protein and enhances its global inhibition of apoptosis contributing to lung cancer development and proliferation of tumour cells (89). Crott et al. (90) reported that the frequency of MN, NPBs and NBUDs correlated significantly and negatively with folic acid concentrations, suggesting that these chromosomal end points may be induced by folic acid deficiency. In addition, Kimura et al. (91) showed a significant impact of methyltetrahydrofolate reductase (MTHFR) C677T polymorphism and folic acid concentration on MN, NPBs and NBUDs in human lymphocytes. Similarly, our group has recently reported that the level of genetic damage end points are modulated by SNPs in the folate pathway genes especially when interactions between the SNPs are considered (92).

CBMN assay and cancer risk assessment

Cancer risk assessment is a multidisciplinary process that goes beyond the scope of classical epidemiology to include the biological evaluation of individual differences to carcinogenic exposures. The inclusion of genetic biomarkers of cancer risk, such as mutagen sensitivity or CBMN biomarkers, allow the researcher to complete a comprehensive risk assessment for cancer for known demographic (age and gender), lifestyle exposures (smoking and alcohol), occupational and environmental exposures (asbestos and wood dust) and medical comorbidities (chronic obstructive pulmonary disease).
It is hypothesised that the inclusion or addition of genetic biomarkers into existing cancer risk assessment models can improve the discriminatory power of the original model. For example, Spitz et al. (93) published a risk model for lung cancer using a White population of 1851 lung cancer cases and 2001 controls. In 2008, the same group extended their original model with the inclusion of two known biomarkers of lung cancer risk (host-cell reactivation and mutagen sensitivity) using 725 White lung cancer cases and 615 controls (94). The expanded model increased the discriminatory power (as measured by the area under the receiver operating characteristic curve, AUC) from 67 to 70% ($P = 0.006$) among former-smokers and from 68 to 73% ($P = 0.048$) among current smokers. Although the increases in discriminatory power were modest, they were comparable to increases in power reported for the Gail model for breast cancer with the inclusion of SNP data (95). The CBMN assay has also been shown to be an important biomarker for lung cancer risk assessment. El-Zein et al. (84) reported that the positive predicted valued (PPV = predictive probability of being a lung cancer case) were 96, 98 and 100% when using the 95th percentiles of spontaneous and NNK-induced MN, NPBs and NBUDs, respectively.

Assays such as the CBMN method produce multiple and correlated outcomes, which are only increased when the assay is performed with and without exposure to different mutagens. Therefore, when such biomarkers are used to develop a comprehensive cancer risk assessment model, the existence of multiple correlated markers must be taken into account and data reduction methods could be used to derive a summary measure that incorporates information from each individual variable into a single (or possible multiple, uncorrelated) measure of risk. One such method is Principle Components Analysis (PCA), which involves a mathematical procedure to transform a number ($p$) of (possibly) correlated variables into a set of uncorrelated variables called ‘principal components’. Although the entire amount of variability of the original set of variables is explained in the total set of $p$ principal components, the majority of this variability is explained in only $k$ components (where $k \leq p$). The first principal component is the linear combination with maximum variance, and each succeeding component is the linear combination with remaining maximum variance. El-Zein et al. (88) used PCA to derive a summary measure defined by the first principal component for NNK-induced chromosome damage end points (binucleated cells with MN, NPBs and NBUDs) for lung cancer risk. They observed an AUC = 97.9% (95% confidence interval, 95.9–99.0), PPV = 94.8% and a negative predictive value (NPV = predicted probability of being a non-case) of 92.6%. They further reported an increased in the discriminatory power when MN in mono-nucleated cells were included in the PCA:AUC = 99.1% (95% confidence interval, 97.9–100.0), PPV = 98.7% and NPV = 95.6%.

The CBMN assay shows great promise as a method for cancer risk assessment. The ability to identify high-risk subgroups (such as high-risk former-smokers and current smokers) is clinically imperative, where such individuals might benefit from increased screening surveillance that is not appropriate for low-risk individuals. Further unravelling of the underlying repair mechanisms contributing to cancer predisposition could in the future contribute to the development of a predictive multivariate biomarker. Although the current cancer risk assessment models show relatively modest levels of discriminatory power (60–80%), the potential public health benefits of such models is tremendous. Several studies investigated the use of MN assay buccal exfoliated cells as a minimally invasive method for monitoring genetic damage in human populations with promising results (96–98). However, future studies should also investigate the use of this assay as a tool of early detection of certain cancers. Lung cancer is an ideal cancer for such studies where genetic damage could be detected along the respiratory tract of apparently healthy smokers as a result of both field carcinization and field injury that precede the development of cancer by many years. Examining accessible tissue along the respiratory airway (such as buccal, nasal and bronchial tissues) could potentially lead to development of clinically suitable non-invasive tools for early detection of the lung cancer. Therefore, inclusion of a biomarker such as the CBMN cyt assay in cancer risk assessment has the potential to substantially improve risk predictions. In addition, the simplicity, rapidity and sensitivity of the CBMN cyt assay make it a valuable and useful screening tool in clinical and epidemiological studies.

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References


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