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Clinical Experience With the Micronucleus Assay

Harinder S. Garewal, MD, PhD[1], Lois Ramsey, BS[1], George Kaugars, DDS[2], and Jay Boyle, MD[3]

[1] Arizona Cancer Center, VA Medical Center, Tucson, AZ 85723
[2] Medical College of Virginia, Richmond, VA 23298
[3] Johns Hopkins University, Baltimore, MD 21203

Abstract Because of the logistical and practical problems that make cancer prevention trials using cancer incidence as an endpoint virtually impossible to conduct for the majority of cancer types, there is a desperate need for valid intermediate markers of cancer risk to serve as surrogate endpoints in chemoprevention trials. A long and continually growing list of potential markers has been developed in the recent past. Unfortunately very few, if any, of them have been subjected to the usual quality control requirements for a laboratory test before being applied to clinical settings.

Modulation of micronuclei frequency has been reported in a number of chemoprevention trials involving the oral cavity, esophagus, lung, and lower GI tract; however, we have focused our efforts primarily on applying the assay to exfoliated buccal mucosal cells, since much of the published data deal with this site, and oral cancer prevention is the theme of one of our chemoprevention trials. After standardizing the definition of a micronucleus by literature review and direct exchange of slides and photographs with other investigators active in the field, we obtained smears from normal subjects, smokers with or without leukoplakia, and tobacco chewers with or without leukoplakia. Our summarized findings follow: (1) Micronuclei represent only one of numerous cytological abnormalities in exfoliated buccal cells that are manifest particularly in tobacco chewers. These include a high frequency of anucleate, binucleate, and multinucleated cells, abnormal shapes and sizes of nuclei, *etc.* (2) Intra-observer variability in the micronucleus count, assessed by counting the same group of slides on multiple occasions by the same observer, is in the range of ± 3 per 1000 nucleated cells, *i.e.*, 0.3%. Clearly, the impact of this can be extremely significant, particularly if the overall micronuclei frequency is low. (3) It is often difficult to find 1000 nucleated cells on a smear, especially if it is obtained from a tobacco chewer at the site of quid placement or at the site of a leukoplakic lesion. A large percent, often >50–70% of the exfoliated cells from such sites, will be anucleate. (4) Micronucleated cell frequency is very low in non-chewers. It is usually <0.5% (5 per 1000 nucleated cells), and frequently no micronucleated cells are present. (5) Smoking alone does not cause a significant increase in micronuclei frequency, irrespective of whether leukoplakia is present or not. In other words, non-chewers, with or without leukoplakia, who constitute a majority of the subjects of chemoprevention trials in the Western world, do not have the strikingly increased micronuclei frequencies reported by Stich *et al.* from studies conducted in Asia. In fact, the micronuclei frequency in this group is no different from lesion-free subjects. (6) Micronuclei frequency is increased in tobacco chewers, most significantly at the site of placement of the quid. These smears also have numerous other cytologic abnormalities, with micronuclei frequency being increased to about 0.8–1% (8–10 cells per 1000 nucleated cells).

Address correspondence to Dr. Harinder S. Garewal, Arizona Cancer Center, VA Medical Center, 3601 S. 6th Ave., Tucson, AZ 85723.

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In view of the above quality control and reliability data, clinical trials reporting reductions in micronuclei frequency of 2–3 per 1000 cells are virtually meaningless, especially if the phenomenon of "regression to the mean" is also taken into account. Data regarding tissues other than the oral cavity are scant, but essentially similar. These studies confirm the importance and need to subject putative intermediate markers to rigorous quality control assessment prior to generating literature on their modulation. © 1993 Wiley-Liss, Inc.

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The most convincing and "ultimate" proof of cancer preventive activity attributable to a specific intervention is the clear demonstration of cancer incidence reduction in a clinical trial setting. Unfortunately, such trials are not feasible for most types of cancer, since they would require an impossibly large number of subjects and an equally impossible long time frame. Although cancer is a common disease, specific types of cancer are still relatively infrequent events in an otherwise healthy population. Therefore, trials with cancer incidence as endpoints would necessarily involve several thousands of subjects followed for several decades. Such logistic difficulties have precluded cancer prevention trials with cancer incidence as an endpoint in all but a selected few malignancies. Nevertheless, decisions have to be made regarding the potential of interventions, such as chemopreventive agents, to prevent cancer. Presently, these decisions must be based on reviewing and analyzing a variety of indirect lines of evidence. Data from epidemiologic studies, laboratory and animal model experiments, and clinical intervention trials in humans, all using endpoints other than cancer incidence, are examples of such indirect evidence.

It is in this context that there is tremendous interest in developing so-called "intermediate endpoints" that can function as surrogate markers for cancer risk. These markers are measurable parameters: histological, genetic, biochemical, and others, which precede the formation of a malignant lesion and, when displayed, place an individual at a higher risk for cancer development. If one considers carcinogenesis a multistep process, then intermediate markers can be considered to be measurable events associated with specific stages of carcinogenesis. Chemoprevention studies would assess modulation of intermediate biomarkers by an intervention and link this assessment with an altered risk of cancer development.

Iwamoto, Obrams, and Schottenfelt [1], in a recent overview, stated that "to date, major research activities have been directed toward biomarker assay development without extending efforts to establish validity in human beings." This need for validation is well-recognized in that, at present, no intermediate marker has been proven to be "true" in terms of linking it to the final endpoint of invasive cancer [1,2]. Such validation clearly remains the primary goal of intermediate marker research. However, an equally important part of establishing validity in human beings is the need to subject all postulated markers and their assays to the usual quality control requirements of reliability and reproducibility, preferably prior to investigating their modulation in the context of a clinical trial. In the absence of such data, interpreting the significance of changes in a marker becomes quite problematic, leading to a great deal of confusion about the meaning of the results.

Numerous intermediate markers have been proposed based on findings derived from basic laboratory investigations [3]. As shown in Table I, these can be based on histology, quantitative enzymatic assays, immunohistochemical staining, indices of proliferation, and genetic assays. As indicated earlier, modulation of many of these markers, such as labeling index and ornithine decarboxylase activity, have been studied as parts of clinical intervention trials [4,5]. Nevertheless, because of the lack of quality control data, it is often extremely difficult to interpret the true significance of these "modulation" results. Wide variations, often of an order of magnitude or greater, exist between reports from different groups studying the same marker in the same organ [4,6,7]. The need for quality control is increasingly evident when these

TABLE I. Partial List of Proposed Intermediate Markers

Genetic Markers
Oncogenes and oncoproteins
Micronuclei frequency
DNA adducts
Cellular and Histologic Markers
Proliferation indices
Premalignant lesions (*e.g.*, leukoplakia, Barrett's esophagus, colon polyps)
Immunohistochemical proliferation indices (PCNA, Ki-67)
Differentiation markers
Growth factors and receptors
Cytokeratins
Involucrin
Lewis and extended Lewis antigens
Biochemical and Immunological Markers
Ornithine decarboxylase activity
Transglutaminases
Immunological parameters (*e.g.*, TNF production)
Micronutrient Status
Vitamins and trace elements
Levels of Chemopreventive Agents in Target Tissue

markers are taken from single institution trials to multi-institution studies, as has been the case, for example, for labeling index in colon cancer prevention studies.

In our laboratory, we have chosen to first pay attention to the quality control issues for each potential intermediate marker that we wish to study in a clinical trial, before attempting to analyze its modulation. Thus, we have studied and reported our findings on relevant quality control issues related to markers such as labeling indices, ornithine decarboxylase assays, and flow cytometry, these being some of the most studied and popular markers in intervention trials [4,8,9]. Although this approach involves more mundane and less glamorous data than reporting changes in the markers produced by specific interventions, we feel that in the long run, these quality control aspects will be critical for any marker to be validated in a large scale, multi-center trial, or in other words, for any marker to relate meaningfully and usefully to cancer risk and to its alteration by chemopreventive agents.

A popular marker, especially in oral cavity cancer prevention studies, is the frequency of micronucleated cells in exfoliated oral mucosal cells. Because of the ease with which these cells can be obtained, and the encouraging early results reported from studies conducted in Asia, this marker has become very popular in studies dealing with the prevention of aerodigestive

tract malignancies [10–12]. Its "modulation" has been reported in cells obtained from the oral cavity, lungs, and esophagus. Consequently, this is one of the markers we chose to monitor in our chemoprevention trials using beta-carotene in oral leukoplakia [13]. In the context of preparing for analysis of these trials, we conducted extensive quality control work with this assay to determine its potential applicability to oral cancer prevention studies conducted in the Western hemisphere.

THE MICRONUCLEUS ASSAY: HISTORICAL ASPECTS

The reader is referred to excellent reviews dealing with the historical aspects of the micronucleus test and its application in a variety of uses, such as screening chemicals for chromosome damage and mutagenic effects [14,15]. The test is based on the observation that mitotic cells with chromatid breaks or exchanges suffer disturbances in the anaphase distribution of chromatin, and this displaced chromatin may not be included in the nuclei of daughter cells, thereby becoming "micronuclei" in the cytoplasm. In the 1950s, the test was used to measure chromosome damage in plant cells, and was developed and extended in the early 1970s to animal cells. Considerable experience exists on the use of this assay in plants, animals, and in cultured cells [14,15]. In the early 1980s, primarily through the efforts of Stich and Rosin [16–20], the assay was applied to the study of carcinogen-exposed populations based on the underlying concept that the frequency of micronucleated cells might serve as an "endogenous dosimeter" of genotoxic damage occurring in the tissues. The frequency of micronucleated cells in exfoliated oral mucosal cells is dramatically increased by known, high-intensity genotoxic exposures, such as radiation treatment. Application of this assay to oral cavity cancer prevention studies is based on the theory that ongoing exposure to carcinogens will result in a significant increase in micronucleated cell frequency.

The first studies on the application of this assay to oral cancer prevention were reported by Stich, Rosin, and colleagues [18–20] with extremely encouraging results. Their studies involved very high-risk groups, primarily from India and the Philippines, where intense carcinogen exposure is common as a result of continuous betel nut chewing. Subjects with such habits, particularly if they had evidence of preneoplastic lesions, were found to have marked elevations in their micronuclei frequency, often in the range of 4–8%. Since the counting procedure is usually based on counting 1000 nucleated cells, such a frequency represents finding 40–80 micronucleated cells per smear. Thus, reducing this frequency to the range of 0.5 to 1% (*i.e.*, 5–10 micronucleated cells counted) appeared to be a reasonable and measurable goal. The latter, low frequency was described by the same investigators in "normal" individuals who neither smoked nor drank alcohol [16]. Stich and colleagues [17–20] reported a series of intervention trials in which a significant reduction in the frequency of micronucleated cells was achieved by intervening with vitamin A and/or beta-carotene. It is noteworthy that this reduction occurred in almost all subjects treated and did not correlate with the behavior of preneoplastic lesions that were targeted in these trials.

Based on the data of Stich and colleagues, the frequency of micronucleated cells quickly became one of the most frequently monitored immediate markers in clinical trials conducted in Western populations. In our own trials using beta-carotene, as mentioned above, this is one of the markers being monitored. However, we quickly became aware that, in contrast to the experience of Stich and colleagues, the frequency of micronucleated cells was rather low in our subjects, and the assay had several nuances necessitating quality control experiments to lay the groundwork for interpreting the clinical trial data. The low frequency of micronucleated cells was confirmed by other investigators; for example, Sarto *et al.* [21] reported frequencies in the range of 0.3 to 0.7% among a large group of smokers and nonsmokers. Results of these initial quality control experiments are summarized below.

DEFINITION OF MICRONUCLEUS

We accepted the standard definition that a micronucleus must resemble a nucleus in its texture, shape, and staining properties, but must be smaller and separate from the main nucleus. Initial examination of smears from subjects at risk, with or without preneoplastic

lesions, immediately confirmed the observation that micronuclei were only one of numerous cytologic abnormalities. A particularly vexing problem, not infrequently encountered, is the inability to find 1000 nucleated cells in a smear. Although this number is readily achieved in normal subjects, the percentage of anucleate cells in smears derived from tobacco chewers or from oral leukoplakic lesions, possibly representing those shed from the hyperkeratotic and parakeratotic layers, can be very high and often represent the majority of cells found on a slide. Furthermore, as recognized by other investigators studying the cytology of oral smears, fragmented nuclei derived from karyorrhexis, chromosome breakup, and nuclear pyknosis can be very common. Therefore, expertise in recognition and agreement with the definition of a micronucleus were first established by exchanging slides and photographs with other experienced laboratories and agreeing upon the definition of a micronucleus.

OBSERVER VARIABILITY

Since the frequency of micronucleated cells was considerably lower in our population than that reported by Stich *et al.* [18–20], in their high-risk groups, it was considered crucial that observer variability be addressed prior to assessing modulation by an intervention. Furthermore, since the best approach, and the one usually adopted in most clinical trials, is to have the same observer do the serial longitudinal counts in a particular study, intra-observer variability was considered to be the main issue to be addressed. Clearly, inter-observer variability will not be less than intra-observer variability.

To address the issue of intra-observer variability, a single slide set consisting of 11 slides was counted by the same individual on three different occasions. This generated 22 "serial pairs" of counts of the same slides. In these smears, most of which had counts less than 10 per 1000, or less than 1%, the average variability was ± 3 per 1000 (range 1–5). In serial counts, 9 of the 22 pairs (41%) were "decreased" and 5 (23%) were "unchanged." It is noteworthy that this "decrease" is virtually identical to the incidence of "decreased" micronucleated cell frequency reportedly caused by an intervention agent in at least one trial conducted in the US [11]. Obviously, the impact of observer variability will be significant, particularly in the low frequency smears from Western subjects. Taken together with the phenomenon of "regression to the mean" in a longitudinal study with serial assessment, it makes meaningful analysis of micronucleated cell frequency changes difficult.

FREQUENCY OF MICRONUCLEATED CELLS

In order to estimate the range of micronucleated cell frequencies likely to be encountered in studies conducted in the US, 69 oral mucosal cell smears were obtained from tobacco chewers and 31 from non-chewers. Of the 69 chewer smears, 46 were from "light" users who were young, male rugby players chewing less than a can per day for at least 5 days per week. The remaining 23 were from miners in Virginia with a more continuous and heavy daily habit (1–3 cans/day) extending over several years. In preliminary experiments, we noted that the frequency of micronucleated cells was no different in cigarette smokers than non-smokers. Consequently, although most of the 31 non-chewer smears were from cigarette smokers, they were analyzed as a group.

As shown in Table II, the micronucleated cell frequency was significantly greater in chewers versus non-chewers ($p < 0.01$). It should be noted that, although higher than non-chewers, the highest frequencies in our study population ($0.92 \pm 1.5\%$) were still many-fold lower than those reported by Stich and colleagues from Asia (4–8%). There was also a difference be-

TABLE II. Micronucleated Cell Frequency in Tobacco Chewers and Non-Chewers

Subjects*	MNCF (%)
Chewers (69)	$0.51 \pm 1.0^{\dagger}$
"Heavy" (23)	0.92 ± 1.5
"Light" (46)	0.31 ± 0.51
Non-Chewers (31)	$0.09 \pm 0.12^{\dagger}$

* Number of smears examined is in parentheses.
† $p = 0.006$
MNCF = micronucleated cell frequency

TABLE III. Micronucleated Cell Frequency: Lesion Versus Non-Lesion

Smears*	MNCF (%)
All lesions (28)	0.36 ± 0.56
All non-lesions (72)	0.39 ± 0.95
Chewers	
Lesion (19)	0.46 ± 1.1
Non-lesion (50)	0.53 ± 1.1
Non-Chewers	
Lesion (9)	0.14 ± 0.15
Non-lesion (22)	0.07 ± 0.11

* Number of smears examined is in parentheses. No statistically significant differences between lesion versus no lesion were present in any paired comparison.

tween the "heavy" and "light" chewers. Comparisons between subjects with preneoplastic lesions (leukoplakia) and those without are shown in Table III. There was no statistically significant difference between any of the groups based on the presence or absence of lesions. Similarly, we were unable to demonstrate a statistically significant difference between smears derived from lesion versus non-lesion sites. It should be emphasized that, although the mean frequency among non-chewers with lesions (0.14%) appears to be double that in non-chewers without lesions (0.07%), it was not statistically different. Practically, these numbers represent 1.4 cells per 1000 versus 0.7 cells per 1000, a difference that is not only statistically meaningless, but clinically meaningless as well.

CONCLUDING REMARKS

The search for, and validation of, intermediate markers is of unquestionable importance in cancer prevention research. Our experience with the micronucleus assay, as with labeling index and ornithine decarboxylase activity, serves to emphasize the importance of quality control experiments with each assay as a part of the validation process. Availability of this information will allow us to more meaningfully evaluate the utility of the micronucleus assay in oral cancer prevention research conducted in Western populations. The data on intra-observer variability at the low frequencies encountered in non-chewing subjects already suggest that risk assessment and modulation results will most likely be of little value when applied to these cases. Whether substantial changes can be produced in tobacco chewers by chemopreventive interventions remains to be demonstrated. Clearly, these changes must be greater than the intra-observer variability of the assay for the results to be valid and useful.

These findings are similar to those encountered when an attempt was made to apply this assay to colonic mucosa. In the face of reader variability, the frequency of micronucleated cells in colonic crypts was found to be too low in the various risk categories, *e.g.*, patients with polyps or cancers versus normal, to apply the assay as an intermediate marker (Einspahr and Alberts, personal communications). Attempts to correlate micronuclei frequency with risk categories have also been unsuccessful at other sites, including the esophagus [22]. Because the impact of intra-observer variability is greatest when the total counts are low, small-magnitude increases or decreases in frequency must be interpreted with great caution.

In summary, our approach to intermediate markers has evolved into requiring quality control data to be generated prior to the application of the marker to measure modulation in a clinical setting. We hold the view that the most fundamental quality control requirement of reproducibility must be explored for any putative marker in order to be able to ascertain the significance of modulation data. Hopefully, this approach will be adopted for other markers as they are proposed for use in clinical trials.

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