Reliability of mutagen sensitivity assay: an inter-laboratory comparison

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Mutagen sensitivity is regarded as a genetic susceptibility phenotype for various cancers; it is cytogenetically based and probably involves a number of genes from different DNA repair pathways. This assay has been used in a number of laboratories in the field of epidemiology, where it has been investigated and appears to be a useful susceptibility biomarker for epidemiological studies assessing cancer risks at the population level. One concern about phenotypic assays, such as the mutagen sensitivity assay, has been that there could be wide variation in results depending on the timing of the assay (within individual variation), the individual performing the assay (within observer variation) and the laboratory where the assay has been performed (inter-laboratory variation). We conducted an inter-laboratory comparison study between the Memorial Sloan-Kettering Cancer Center and M. D. Anderson, in which we assessed all these concerns. We did not find any significant variation in any of the assays. The correlation was high for all tests. The good concordance rate between laboratories supports the continued use of the mutagen sensitivity assay by different laboratories, and demonstrates its potential to identify at-risk subgroups among normal individuals and cancer patients alike.

Introduction

The search for ways to measure cancer susceptibility has expanded geometrically as understanding of the human genome has evolved. Focus on genetic variation in the form of disease-associated mutations and polymorphisms has produced much data but few new understandings as yet. Genotype–phenotype correlations are currently being investigated in many laboratories in order to find potentially useful genotypes for association studies of disease among the general population. Although decrements in DNA repair capacity are probably involved in the carcinogenic process, to date there is no widely accepted consensus as to which measures of DNA repair, either phenotypic or genetic, might be useful to help predict individual susceptibility to carcinogenesis.

The bleomycin-induced mutagen sensitivity assay is an in vitro measure of DNA repair capacity developed by Hsu (1). This assay is an indirect measure of both DNA damage and DNA repair expressed as ‘breaks per cell’ (b/c) in short-term cultured lymphocytes. It is a relatively simple test in which a higher number of bleomycin-induced chromatid breaks indicates higher ‘mutagen sensitivity’ and lower DNA repair. For example, if the number of ‘breaks per cell’ is greater than the median of the controls, usually ~0.8, the examined subject is considered ‘mutagen sensitive’ (2). Mutagen sensitivity is regarded as a genetic susceptibility phenotype for various cancers; it is cytogenetically based and likely involves a number of genes from different DNA repair pathways. However, the specific genes accounting for the traits are still unknown (3).

Hsu (1) developed a functional or phenotypic assay, (the bleomycin-induced the mutagen sensitivity assay), for measuring ‘overall unrepaired DNA’, which has been found to discriminate between individuals with cancer and healthy controls (2). This assay has also been found to distinguish between individuals with cancer who will develop second malignancies and those who will not (4–6), as well as those with a family history of cancer and those without (7–12). It also became clear that mutagen sensitivity assay measured cellular responses based on environmental exposure and individual susceptibility (13). In the past 15 years, the mutagen sensitivity assay has been used in a number of laboratories in the field of epidemiology, where it has been investigated and appears to be a useful susceptibility biomarker for epidemiological studies assessing cancer risks at the population level (14). Large prospective studies have been conducted that demonstrate that chromosomal aberrations can predict cancer occurrence among a cancer-free cohort 15 years later (15,16); however, in these studies only baseline chromosome breaks were counted, no chemically induced mutagen treatments were applied as in this study.

One concern about phenotypic assays, such as the mutagen sensitivity assay, has been of the typical wide variation in results that may depend on the timing of the assay (within individual variation), the individual performing the assay (within observer variation) and the laboratory where the assay has been performed (inter-laboratory variation). As the mutagen sensitivity assay is somewhat time-consuming and expensive to conduct, we wanted to evaluate its reliability before continuing its use in epidemiological studies and going further to develop a higher throughput assay. Therefore, we measured the variability of the mutagen sensitivity assay in these contexts.

Materials and methods

Subjects

All subjects signed informed consent. All data were blinded as to subject status.
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Intra-individual variation
In the Memorial Sloan-Kettering Cancer Center (MSKCC) Molecular Epidemiology Laboratory we conducted the mutagen sensitivity assay at three separate times, ∼1 month apart on seven individuals, healthy women with family histories of breast cancer (21 × 50 metaphases = 1050 readings).

Intra-observer variation
In the MSKCC laboratory one observer read 50 metaphase spreads from 28 cancer patients in a study of soft tissue sarcoma assessed at two separate times, 1 week apart (1400 readings). We used the same slide from each individual for each set of readings in this part of the analysis as we were addressing the issue of reader variability.

Inter-laboratory variation
To measure inter-laboratory variability in assessing mutagen sensitivity, we digitally photographed 50 metaphases from nine healthy subjects (Zeiss Spotmatic and Kodak) and saved them on a CD. These images (450 readings) were read at MSKCC and also sent to The University of Texas M. D. Anderson Cancer Center Molecular Epidemiology Laboratory of Dr Qingyi Wei for evaluation. Both laboratories thus read the same, selected metaphases for identification of chromatid breaks.

In the second phase of this inter-laboratory reliability study, the MSKCC laboratory reviewed 45 slides from 45 individuals—cancer patients and controls—and then sent these to be evaluated by the M. D. Anderson laboratory. Finally, the M. D. Anderson laboratory sent 45 slides from 45 individuals to be evaluated by the MSKCC laboratory. Each laboratory read 50 metaphases from each slide for each individual. Five slides were damaged in the exchange; these b/c values were considered negligible and data were not recorded for this analysis. At 70 h after culture initiation, bleomycin (0.03 U/ml; Sigma-Aldrich) was added to one culture; another culture remained as an untreated control. Due to extremely low values of spontaneous chromosome breaks in all control slides, these b/c values were considered negligible and data were not recorded for this analysis.

Statistical analysis
First we verified that our experimental values for breaks (b/c) satisfied the normality assumption based on Quantile–Quantile (Q–Q) plots and the Kolmogorov–Smirnov goodness-of-fit test. The overall distribution of the data is graphed in Figure 1 using scatter plots. Descriptive statistics such as mean, standard deviation (SD), correlation coefficient, coefficient of variation (CV) and 95% confidence intervals (CI) were examined for all types of variability and the intra-individual variability results are summarized in Table I.

Fig. 1. Scatter plots showing the reproducibility of the mutagen sensitivity assay (break/cell values) readings. Intra-individual variation (a) 1st versus 2nd sets, (b) 2nd versus 3rd sets and (c) 1st versus 3rd set of blood collection and mutagen sensitivity assays. Intra-observer variation (d) 1st versus 2nd set of readings of the same slide by the same researcher. Inter-laboratory variation: (e) selected metaphases and (f) unselected metaphases.
Pearson's correlation was applied as the distribution of the break/cell values was normal. Mean, SD and 95% CI values are of the difference between sets.

<table>
<thead>
<tr>
<th>Individuals/time points (~1 month apart)</th>
<th>Number of assay sets</th>
<th>Range of b/c values</th>
<th>Mean of b/c values (SD)</th>
<th>95% CI of the mean b/c values</th>
<th>CV of the mean b/c values</th>
</tr>
</thead>
<tbody>
<tr>
<td>First individual</td>
<td>3</td>
<td>0.40-0.68</td>
<td>0.56 (0.14)</td>
<td>(0.20-0.92)</td>
<td>8.33</td>
</tr>
<tr>
<td>Second individual</td>
<td>3</td>
<td>0.68-0.92</td>
<td>0.77 (0.13)</td>
<td>(0.45-1.09)</td>
<td>7.42</td>
</tr>
<tr>
<td>Third individual</td>
<td>3</td>
<td>0.28-0.36</td>
<td>0.33 (0.05)</td>
<td>(0.22-0.45)</td>
<td>2.67</td>
</tr>
<tr>
<td>Fourth individual</td>
<td>3</td>
<td>0.42-1.40</td>
<td>0.99 (0.51)</td>
<td>(0.28-2.26)</td>
<td>29.49</td>
</tr>
<tr>
<td>Fifth individual</td>
<td>3</td>
<td>0.33-0.65</td>
<td>0.49 (0.16)</td>
<td>(0.10-0.89)</td>
<td>9.24</td>
</tr>
<tr>
<td>Sixth individual</td>
<td>3</td>
<td>1.00-2.00</td>
<td>1.51 (0.50)</td>
<td>(0.26-2.75)</td>
<td>28.88</td>
</tr>
<tr>
<td>Seventh individual</td>
<td>3</td>
<td>0.76-0.82</td>
<td>0.79 (0.03)</td>
<td>(0.72-0.87)</td>
<td>1.76</td>
</tr>
<tr>
<td>First time point</td>
<td>7</td>
<td>0.36-1.00</td>
<td>0.65 (0.21)</td>
<td>(0.45-0.85)</td>
<td>32.84</td>
</tr>
<tr>
<td>Second time point</td>
<td>7</td>
<td>0.28-2.00</td>
<td>0.84 (0.59)</td>
<td>(0.29-1.39)</td>
<td>70.23</td>
</tr>
<tr>
<td>Third time point</td>
<td>7</td>
<td>0.36-1.52</td>
<td>0.85 (0.47)</td>
<td>(0.41-1.28)</td>
<td>55.51</td>
</tr>
</tbody>
</table>

Table II. Summary of statistical analyses of the reliability of mutagen sensitivity assay

<table>
<thead>
<tr>
<th></th>
<th>Individuals (n)</th>
<th>Readings (n)</th>
<th>Correlation*</th>
<th>P-value</th>
<th>Mean (SD)</th>
<th>95% CI</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-individual variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second time of the assay (b/c)–first time of the assay (b/c)</td>
<td>7</td>
<td>350</td>
<td>0.63</td>
<td>0.13</td>
<td>0.19 (0.48)</td>
<td>(−0.64, 0.25)</td>
<td>0.34</td>
</tr>
<tr>
<td>Third time of the assay (b/c)–second time of the assay (b/c)</td>
<td>7</td>
<td>350</td>
<td>0.91</td>
<td>&lt;0.01</td>
<td>&lt;0.01 (0.26)</td>
<td>(−0.24, 0.24)</td>
<td>0.97</td>
</tr>
<tr>
<td>Third time of the assay (b/c)–first time of the assay (b/c)</td>
<td>7</td>
<td>350</td>
<td>0.39</td>
<td>0.39</td>
<td>0.20 (0.43)</td>
<td>(−0.60, 0.20)</td>
<td>0.28</td>
</tr>
<tr>
<td>Intra-observer variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b/c from first reading–b/c from second reading</td>
<td>28</td>
<td>1400</td>
<td>0.90</td>
<td>&lt;0.01</td>
<td>−0.04 (0.30)</td>
<td>(−0.18, 0.10)</td>
<td>0.59</td>
</tr>
<tr>
<td>Inter-laboratory variation</td>
<td>Selected metaphases</td>
<td>9</td>
<td>450</td>
<td>0.84</td>
<td>&lt;0.01</td>
<td>0.07 (0.24)</td>
<td>(−0.11, 0.25)</td>
</tr>
<tr>
<td></td>
<td>Unselected metaphases</td>
<td>85</td>
<td>4250</td>
<td>0.80</td>
<td>&lt;0.01</td>
<td>−0.01 (0.18)</td>
<td>(−0.05, 0.03)</td>
</tr>
</tbody>
</table>

Mean, SD and 95% CI values are of the difference between sets. P-values are of the correlation.

*Pearson’s correlation was applied as the distribution of the break/cell values was normal.

**t-test compares the mean to 0, thus tests the significance of the departure of the difference from zero.

Figure 1 also shows the high positive correlation between different readings. To test the hypothesis that there were no intra-individual, intra-observer and inter-laboratory difference in b/c values, the standard paired t-test was employed. Test statistics related to correlation and paired t-test for the difference in the means of the b/c values were demonstrated in Table II.

Results and discussion

The distributions of b/c reading differences were centred at zero and we did not find any noteworthy patterns in variation (Figure 1). The correlations were very high and every 95% confidence interval for the reading differences included zero.

Intra-individual variation

We were interested in whether the timing of the blood collection had any influence on the number of breaks/cell of the study subjects. Intra-individual variation did not vary significantly over time. P-values of 0.34, 0.97 and 0.28 for each comparison demonstrate that there were no significant differences between readings due to intra-individual variation and that no trend over time was detected (Table I). This result is in accordance with some previous findings that have examined the specificity of the mutagen sensitivity assay and found that was not influenced by confounders, such as gender, and diet (19–21). Others emphasize that mutagen sensitivity might be considered as a complex expression of toxicological responses, therefore exposure to alcohol and smoking can be important contributors to initiate these processes (22). The low frequency of b/c values ≥1 (23%) in our study population showed that subjects possibly have not been exposed to these mutagenic factors or to a lesser extent than other population reported.

Intra-observer variation

The repeated evaluation of two sets of metaphases from 28 individuals with the same technician reading the slides demonstrated no significant difference in b/c values. Table II shows the similarity of the empirical cumulative distributions from the two metaphase readings. McIntyre et al. (23) also examined reader variability of the mutagen sensitivity assay and found that k value (Kappa statistic) was fair. However, our intra-observer variability was excellent, so training may play a difference.

Inter-laboratory variation

Two laboratories have reviewed metaphases. In one set of analyses, where researchers from each laboratory read the exact same, selected metaphase spreads, there was no significant difference in the readings (P-value = 0.39; Table II).

In a second set of analyses, one slide from each individual was independently scored by a technician from each laboratory without preselection of specific metaphases from the slide and found no statistically significant differences between readings (P-value = 0.51; Table II). Our evaluation shows that two separate laboratories have surprisingly good reproducibility of results for the mutagen sensitivity assay. As laboratory personnel of both laboratories were trained by using same technique, our future plan will be to explore inter-laboratory concordance between different settings and countries.

The application of bleomycin, a radiomimetic drug capable of inducing both single- and double-strand chromosome breaks (independently of cell cycle phases) in standard lymphocyte cultures allows investigators seeking cancer risk assessments to quantify individual overall DNA repair capacity
within a relatively short time period. The mutagen sensitivity assay can also be adapted to accommodate different mutagens that offer additional information about the involvement of specific DNA damage and repair pathways. For example, using benzo[a]pyrene diol epoxide (24), gamma radiation (25) and ultraviolet light (26) that are well-known carcinogens as the test mutagens can help address the susceptibility to cancer with a specific aetiology. These make the assay potentially useful for larger scale molecular epidemiological studies. Furthermore, mutagen sensitivity may be an important susceptibility biomarker showing consistent association with cancer risk, variability among controls and good reproducibility. The good concordance rate between laboratories supports the continued use of the mutagen sensitivity assay by different laboratories, and demonstrates its potential to identify at-risk subgroups among normal individuals and cancer patients alike.

It would also be valuable to extend inter-laboratory studies to other laboratories in other countries with potentially different environmental exposures. This type of study could also add important insights into the applicability of the mutagen sensitivity assay.

References


