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Comprehensive retrospective evaluation of existing *in vitro* chromosomal aberration test data by cytotoxicity index transformation

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ABSTRACT

New OECD test guidelines have been issued, in which the cytotoxicity index relative cell count (RCC) is replaced with a new index, RICC or RPD (relative increase in cell count/relative population doubling), with the goal of reducing the high proportion of false positive results in *in vitro* chromosomal aberration tests. Using a mathematical approach to estimate new indices from the RCC, we constructed an evaluation flow that quantitatively estimates how often the previous test conclusions change when applying the updated cytotoxicity criteria. The new evaluation flow was applied to a retrospective evaluation of 285 chemicals in two databases. The effects of the employment of new cytotoxicity indices are investigated at a large scale. Using the new evaluation flow, 90 chemicals were estimated as positive, 39 were designated as estimated negative (13 probably negative and 26 possibly negative), and 140 were designated as negative. Moreover, we also applied a prioritization index to indicate the likelihood of a chemical being re-evaluated as negative and assigned priorities for testing. Most of the chemicals that were designated as estimated negative and had negative results in the *in vivo* micronucleus tests were considered as false-positives that would be correctly judged under the new test guideline. Furthermore, statistical analysis of the frequency of estimated negatives revealed that the results for Ames-positive chemicals, especially those with a strong response, are unlikely to change. Therefore, we concluded that the new indices would likely reduce the proportion of false positive results and not increase the proportion of false negative results. This study is the first report of a comprehensive re-evaluation of test results in terms of new cytotoxicity indices. The evaluation flow we have developed facilitates efficient retrospective evaluation of genotoxicity.

1. Introduction

A high proportion of false positives in *in vitro* chromosomal aberration (CA) tests using mammalian cells have been reported [1]. Causes of false positives and methods to mitigate their effects have been discussed in international workshops [2,3]. Strong cytotoxicity due to overexposure to chemical substances is thought to be a cause of false-positive results [4]. Therefore, the Organization for Economic Co-operation and Development (OECD) published revised test guidelines for *in vitro* MN testing in 2010, in which relative cell count (RCC) was replaced with relative increase in cell count or relative population doubling (RICC/RPD), an index with which cytotoxicity can be more accurately calculated in view of cell proliferation [5]. Likewise, in 2011 ICH S2 (R1) reached the consensus that considering population doubling is preferred for measurement of cytotoxicity in *in vitro* mammalian genotoxicity tests [6]. Subsequently, revised editions of the OECD test guidelines for *in vitro* CA and MN testing describing three areas of modifica-

Abbreviations: OECD, Organization for Economic Co-operation and Development; RCC, relative cell count; RICC, relative increase in cell count; RPD, relative population doubling; PD, population doubling; CA, chromosomal aberration; MN, micronucleus; JEC, Japan Existing Chemical; DB, database; JOSHA, Japanese Occupational Safety and Health Act; LOGEL, the lowest observed genotoxicity effect level; NOGEL, no observed genotoxicity effect level.

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tion (changes in cytotoxicity indices, highest tested doses, and cell types) were published in 2014 [5,7–9].

While the proportion of false-positives is expected to be decreased significantly by the adoption of the new OECD test guidelines [10–14], caution is necessary when considering chemicals for which genotoxicity results may change upon re-testing. In *in silico* genotoxicity evaluations, the quality of the results of *in vitro* mammalian-genotoxicity tests significantly affect the usefulness of the *in silico* evaluations. First, knowledge of structure-activity relationships (SAR) related to genotoxicity based on past test results has been utilized for screening genotoxins [15,16]. Second, the read-across approach predicts the genotoxicity of test chemicals based on the past test results of similar chemicals. However, the inclusion of many false positive (non-DNA reactive) chromosome aberration inducers in the data sets has made it impossible to develop good SAR rules for most chemicals. Therefore, updating test results by re-experimentation is desirable because it will improve the accuracy and reliability of *in silico* evaluation; however, re-testing all existing chemicals would be extremely labor-intensive.

The impact of the change in the highest tested concentration in the new OECD test guidelines can be evaluated relatively easily by examining the test dose [12,17]. However, for the change in cytotoxicity index, evaluation has been difficult because of the lack of toxicity data at positive dose levels, although a large reduction in false positives is expected as a result of the adoption of the new index. In order to evaluate the effect of the change in cytotoxicity index on test results, we developed a method of estimating the results of tests conducted under the new OECD test guidelines based on past test data using cytotoxicity index transformation formulae [18]. After reviewing the results for 25 chemicals in the Japan Existing Chemical Database (JEC-DB) that were judged as false positive by Morita et al. *via* 3 strategies (scrutiny of the effects of extreme culture conditions, *in silico* analysis of genotoxicity, and review of *in vivo* genotoxicity and carcinogenicity literature for the chemical and related chemicals), we reported that 12 of the chemicals are likely to be negative [18].

Using our method, it is possible to perform a retrospective evaluation of any cytotoxic index data. However, at present, our method has only been applied to a subset of chemicals in a single database; therefore, its general applicability has not been verified. Furthermore, while results have been obtained suggesting that changes in cytotoxicity indices are useful for reducing the proportion of false positive-results, the risk of increasing the proportion of false-negative results has not been determined. In addition, it is possible that the number of chemicals that were false positives under the old guidelines and determined to be possibly negative under the new guidelines will increase, in which case re-testing all chemicals unequivocally under the new test guidelines would be inefficient. Therefore, we developed a method to quantify the possibility of changes in test results and determine evaluation priorities. Here, we have thus elaborated on the retrospective evaluation method previously reported. Moreover, by estimating the degree of cytotoxicity under conditions when test results for chromosomal aberrations are positive, we have developed new indices quantifying the likelihood that test results will change using the new OECD test guidelines. Additionally, using our newly developed evaluation flow, we conducted a comprehensive retrospective evaluation of 37 chemicals in the existing chemicals mutagenicity database based on the Japanese Occupational Safety and Health Act (the JOSHA-DB), in addition to 248 chemicals in the JEC-DB, and confirmed the effect of the new test guidelines. We believe that this report will serve as a basis for comprehensive retrospective evaluation of *in vitro* genotoxicity tests, resulting in more accurate databases and more appropriate chemical use.

2. Methods

2.1. Databases used for retrospective re-evaluation

We retrospectively evaluated the Japanese Chemical Substances Control Law database (JEC-DB) and Japan Occupational Safety and Health Act database (JOSHA-DB) because (1) test results were obtained under the OECD-GLP or compliant Japanese test guideline under GLP and (2) measured RCC data were available. All chemicals in the DBs were assessed using Chinese hamster lung (CHL/IU) cells under short-term treatment (with and without metabolic activation) and/or continuous treatment (24 h, without metabolic activation). When evaluating chromosomal aberration test results, an aberration rate of 5–10% was determined to be equivocal, whereas a rate of 10% was determined to be a positive result [20]. If a chemical was categorized as equivocal in the original call and the result showed reproducibility, the chemical was considered positive. The historical control range has seldom exceeded 4% in *in vitro* CA testing [21]

2.1.1. JEC-DB

The Japanese Chemical Substances Control Law is a law concerning regulation of the investigation and manufacture of chemical substances that aims to prevent pollution of the environment with chemical substances that may affect human health or ecosystems. We evaluated all 248 chemicals listed in the JEC-DB [19] by Morita et al. [17].

2.1.2. JOSHA-DB

The hazard investigation system of the Occupational Safety and Health Act was created to prevent the occurrence of occupational diseases such as occupational cancer among Japanese workers by evaluating the mutagenicity and carcinogenicity of novel chemicals before they are produced or imported. For the chemicals evaluated by the hazard investigation system, the Ames test is normally performed first, after which chromosomal aberration tests may be required if a strong positive result is obtained. In this study, we retrospectively evaluated all 37 chemicals in the JOSHA-DB, some of which were also included in the JEC-DB (see below for details), with strong positive results in the Ames test and chromosomal aberration test results published after revision of the OECD test guidelines in 1997 [22,23].

4-Aminophenol (CAS No. 123-30-8) and 2-vinylpyridine (100-69-6) were included in the JEC-DB and JOSHA-DB. Because the lowest tested doses of 4-aminophenol and 2-vinylpyridine were the lowest observed genotoxicity effect levels (LOGEL) in the JOSHA-DB, we used the data from the JEC-DB for both chemicals. Therefore, 37 of the 39 chemicals included in the JOSHA-DB were evaluated. Finally, by evaluating the JEC-DB and JOSHA-DB, 285 chemicals were selected for retrospective evaluation.

2.2. Transformation formulae for cytotoxicity indices

The formulae used to transform RCC [5,7] into RICC/RPD are shown below [18].

$$\mathrm{RICC} = \frac{2^{E/D}}{2^{E/D}-1} \times \mathrm{RCC} - \frac{1}{2^{E/D}-1} \quad (1)$$

$$\mathrm{RPD} = \frac{1}{\log\left(2^{E/D}\right)} \times \log \mathrm{RCC} + 1.0 \quad (2)$$

In the formulae above, D is the cell doubling time and E is the experimental time. Thus, when using CHL/IU cells (D = 15 h) with