Carcinogenicity Assessment for Risk Factors in Food: Current Issues and a Proposal

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In the current carcinogenicity assessment, the threshold is a major issue for genotoxic carcinogens. Carcinogenicity is usually assessed in combination with genotoxicity data. The current assessment methodology is based on the hypothesis that non-genotoxic carcinogens have thresholds but genotoxic carcinogens do not. However, it remains unclear in most cases as to how much the detected genotoxic potential is actually associated with the carcinogenicity at an organ level. To clarify this critical issue, in vivo genotoxicity has been investigated in transgenic rodents carrying reporter genes, which can simultaneously detect both genotoxicity and carcinogenicity on each organ basis. Studies of a number of genotoxic carcinogens have revealed good correlations between in vivo genotoxicity and carcinogenicity. However, some discordant results have been also found in some cases. Besides experimentally observed values of genotoxicity, MOA or statistics might be taken into account for biological or practical threshold. Then, statistical or mathematical evaluation can provide values of BMDL or MOE even for strictly defined genotoxic carcinogens. Another major issue is concerning extrapolation of animal data for human risk. For this purpose, WOE approaches based on MOA may be extremely useful. Experiments using transgenic rodents such as p53, nrf2 or CAR knockout mice might be helpful to elucidate the mechanisms of carcinogenicity. The other issues concern the development of screening or alternative methods. In the future, in silico and in vitro approaches will be powerful tools for screening genotoxic and carcinogenic potentials of a number of chemicals/agents.

Key words: food, carcinogenicity, assessment, risk factor

Introduction

Since carcinogenicity is recognized as one of the most serious toxicities, its refined assessment is necessary for preventing human health from cancer development. It has been reported that a number of chemicals and microorganisms in foods are known to be carcinogenic to humans and/or rodents\(^1\). Carcinogenic potentials are assessed by long-term bioas-
says using rodents as well as epidemiological surveys for humans. In long-term carcinogenicity bioassays, rats and mice have been used in general partly due to their small sizes and short life spans and also because they are mammals, like humans. The outcome of rodent long-term bioassay is basically assessed as either positive or negative for carcinogenicity. Previously, any chemical found to induce cancer in human or in animals after testing was in principle banned for use, and the law surround this has been called the Delaney clause\(^2\) in US. However, since then, scientific evidence has been accumulated on chemical carcinogens. The carcinogenic potential depends on the intensity of each chemical and also carcinogenic risk is determined due to the intensity and exposure level. Thus, the Delaney clause was repealed in 1996\(^2\).

Chemicals or microorganisms contained in food are principally categorized into two intended and unintended items. Food additives and pesticide residues are the former, and contaminants are the latter. In the current risk assessment, carcinogens are classified into genotoxic and non-genotoxic ones based on the genotoxicity data. Genotoxicity is believed to be linear and thus have no threshold. Basically, intended chemicals in food should not pose genotoxic potential and such non-genotoxic chemicals are usually assessed as the acceptable daily intake (ADI) on the basis of threshold named no-observed-adverse-effect-level (NOAEL). On the other hand, carcinogenic contaminants showing genotoxic potential are assessed by ways of other than threshold because such unintended chemicals are not completely excluded from environment in cases. Therefore, as low as reasonably achievable (ALARA) principle or virtually safety dose (VSD) based on linear extrapolation has been applied for unavoidable genotoxic carcinogens. Recently, non-threshold approaches such as threshold of toxicological concern (TTC)\(^3\-\(^{11}\) and margin of exposure (MOE)\(^11\-\(^{13}\) have also been introduced for both intended and unintended chemicals. Although genotoxicity is assumed in general to have no threshold, it remains unknown whether genotoxicity of some chemicals may have any no-observed-genotoxic-effect-level (NOGEL)\(^14\-\(^{17}\). Another current major issue on rodent carcinogenicity is how to extrapolate rodent data to human risk assessments, since such extrapolation is not fully supported scientifically in most cases. In this review, the current major issues on the carcinogenicity assessment for risk factors in food are discussed, and some approaches to solve the issues are proposed.

**Known Carcinogens in Food**

Some of the known genotoxic carcinogens included in foods are listed in **Table 1**. According to the International Agency for Research on Cancer (IARC), environmental chemicals/agents are classified into 4 groups in terms of their carcinogenic potencies\(^1\). Agents of 1, 2A, 2B, 3 and 4 are carcinogenic, probably carcinogenic, possibly carcinogenic, not classifiable as to its carcinogenicity and probably not carcinogenic to humans, respectively\(^1\). Among 955 chemicals/agents so far evaluated by IARC, agents of 111 (12%), 65 (7%) and 274 (29%) have been judged as Groups 1, 2A and 2B\(^1\), respectively.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
<th>Rodent Carcinogenicity</th>
<th>IARC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B(_1)</td>
<td>Fungus</td>
<td>Liver</td>
<td>1</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Cooked food</td>
<td>Thyroid, Breast etc.</td>
<td>2A</td>
</tr>
<tr>
<td>Heterocyclic amines</td>
<td>Cooked food</td>
<td>Liver, Colon etc.</td>
<td>2A-2B</td>
</tr>
<tr>
<td>Nitrosamines</td>
<td>Sec Amines + Nitrite</td>
<td>Liver, Kidney etc.</td>
<td>2A-2B</td>
</tr>
<tr>
<td>Safrole</td>
<td>Cinnamon etc.</td>
<td>Liver</td>
<td>2B</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>Mushroom</td>
<td>Lung, Liver etc.</td>
<td>2B</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Fungus</td>
<td>Kidney</td>
<td>2B</td>
</tr>
<tr>
<td>Potassium bromate</td>
<td>Bread making process</td>
<td>Kidney etc.</td>
<td>2B</td>
</tr>
<tr>
<td>Maddor color</td>
<td>Maddor root</td>
<td>Kidney, Liver</td>
<td>3</td>
</tr>
</tbody>
</table>
Contaminants/Chemicals

Acrylamide, heterocyclic amines and nitrosamines are yielded as by-products during heat cooking process or in the stomach after ingested, and these are known to be genotoxic carcinogens categorized into IARC Group 2A or 2B. Aflatoxins, including an intensive mutagenic mycotoxin aflatoxin B₁, are well known hepatocarcinogens to rodents and classified into IARC Group 1\(^1\). Another mycotoxin ochratoxin A induces kidney tumors in rodents. Although the role of genotoxicity remained unclear, genotoxicity of ochratoxin A \textit{in vivo} has been recently detected in the dissected kidney of \textit{gpt} delta rats\(^{18,19}\).

Natural Plant Constituents

Natural plant constituents and the degradation products such as safrole and hydrazine are also known to induce tumors in rodents in addition to their genotoxicities\(^{20–22}\). In our recent investigation, safrole was confirmed to be genotoxic in the livers of \textit{gpt} delta rats\(^{23}\).

Food Additives

Madder color extracted from the roots of \textit{Rubia tinctorum} (madder root) has been used as a food coloring in Japan. In a carcinogenicity study, F344 rats fed a diet containing madder color extract for 104 weeks showed a significant increase in the incidences of renal and liver cell carcinomas in both sexes. Madder color extract was prohibited for use in 2004\(^{24}\) due to the carcinogenic data, taken together with its intensive genotoxicity, although its current IARC classification still remains Group 3. In extensive studies, rubiadin, a metabolite of madder color component is shown to play a major role in the carcinogenicity\(^{25}\). Potassium bromate is an oxidizing agent used as a food additive, mainly in the bread-making process. Potassium bromate induces renal cell tumors in rats\(^{26–29}\). Although potassium bromate possesses both initiating and promoting activities for rat renal tumorigenesis, the potentiality in rats seems to be weaker than in mice and hamsters. Potassium bromate showed relatively strong potential inducing chromosomal aberrations in contrast to its weak genotoxicity in microbial assays. Active oxygen radicals generated from potassium bromate were implicated in its toxic and carcinogenic effects form the results of 8-hydroxydeoxyguanosine production in the rat kidney\(^{30}\). Currently, its limited use is permitted under the condition of no residue.

Pesticide Residues

The major groups of pesticides, such as organophosphates, organochlorines, carbamates and pyrethroids have been reported to be carcinogenic in rodents in some cases\(^{31}\). Nevertheless, such pesticides are mostly permitted for their uses because of the data of absent genotoxicity.

Current Issues

Test Methods

Currently, carcinogenicity bioassays on food additives and pesticides using both rats and mice are required to confirm species-specificity. However, for pharmaceuticals, the current guideline states that the long-term rat assay is essential. The long-term mouse bioassay is, however, no longer needed if alternative methods such as transgenic mouse assay\(^{32–40}\) or initiation-promotion assay are provided\(^{41–45}\). At the recent International Conference on Harmonisation (ICH) for pharmaceuticals, further revision is extensively discussed as to whether even the rat study can be waived in some cases. Although food factors differ from pharmaceuticals in terms of the latter being fully followed up in humans, these trends would more or less influence the carcinogenicity assessment of food factors in association with the movement of animal protection. Nevertheless, one of the most critical issues on the extrapolation of animal data for carcinogenicity to human risk still remains elucidated. Rodents have been reported to be susceptible to neoplastic lesions in the kidney, urinary bladder, stomach, thyroid, mammary gland, uterus, testis, adrenal gland, liver, ovary and pancreatic island\(^{46,47}\) and thus less relevant to human risk assessment in some cases. However, mode of action (MOA) approaches are needed to clarify the species specificity, except for α₂u-globulin-associated kidney tumors in male rats.
In contrast to carcinogenicity tests, although there are a number of test methods for evaluating genotoxicity, the Organisation for Economic Co-operation and Development (OECD) recently reorganized the test guidelines for genotoxicity. Genotoxicity is roughly classified into two distinct categories: direct DNA damage—mostly evaluated with the Ames test—and chromosomal aberration, including the mouse micronucleus test. Rat liver micronucleus assay and transgenic rodent assay carrying reporter genes have been developed very recently. Such genotoxicity assays could be promising test methods to evaluate genotoxicity in the target tissues and therefore have been extensively validated.

A threshold is suggested in vivo even in the genotoxicity, especially for chromosomal aberration including micronucleus tests. Because the existence of threshold would be experimentally determined with genotoxicity data, the overall understanding of genotoxicity and carcinogenicity could be crucial for the assessment of carcinogenicity.

### Assessment Methodology

Taken into account for carcinogenicity and genotoxicity, test chemicals are classified into four distinct categories, i.e., genotoxic carcinogens, non-genotoxic carcinogens, genotoxic non-carcinogens and non-genotoxic non-carcinogens. Based on this classification, genotoxic and non-genotoxic carcinogens are evaluated with and without thresholds in current risk assessment procedures, respectively. The discrimination into genotoxic and non-genotoxic carcinogens, however, is not simple; it is rather difficult because each assay is carried out separately. Namely, both assays are basically independent from one another, which raises a simple query as to how much the detected genotoxic potential can contribute to carcinogenicity. Methodologies that are currently used for carcinogenicity assessment are listed in Table 2. These methodologies possess both advantages and disadvantages in practical uses, and such disadvantages raise some issues to be resolved.

### Proposed Test Methods

#### Transgenic Rodents Carrying Reporter Genes

Recently, a new guideline on transgenic rodent mutation assays has been released from the OECD, in which MutaMouse, Big Blue mouse, lacZ plasmid mouse, gpt delta rodents and use of the λ cII transgene are stated as promising models. Among them, the gpt delta mouse was established by microinjection of λEG10 phage DNA (48 kb) into the fertilised eggs of C57BL/6J mice. Phage λEG10 carries about 80 copies of the transgene in a head-to-tail fashion at a single site of chromosome 17 and is maintained as a homozygote (i.e. the mouse carries about 160 copies of λEG10 DNA per diploid genome). Recently, gpt delta rats have been similarly developed in Sprague-Dawley rats and backcrossed to F344 and Wistar Hannover rats. The gpt delta rat has approximately 10 copies of the λEG10 vector integrated at position 4q24-q31 and is available as a hemizygote only. Mutation in the gpt delta mouse or rat can be assessed using 6-thioguanine and Spi selection, which respond primarily to point mutation and deletion, respectively.

To clarify the threshold issue, various chemicals, having both carcinogenic and non-carcinogenic properties, were investigated using gpt delta rodents about in vivo genotoxicity in the target tissues where tumors are induced.
because these rodent models would be powerful tools for the evaluation of both genotoxicity and carcinogenicity at the same organ level\textsuperscript{59,60}. Some genotoxic carcinogens showed significantly increased genotoxicity in the liver of \textit{gpt} delta rats, the target organ for carcinogenicity\textsuperscript{73}. In contrast, a genotoxic chlorinated water by-product, MX, failed to exert the \textit{in vivo} genotoxicity and carcinogenicity in \textit{gpt} delta mice\textsuperscript{59}. On the other hand, a known non-genotoxic carcinogen dicyclanil increased the \textit{in vivo} genotoxicity as well as oxidative DNA damage in female mice, in manners consistent with the sex specificity of its carcinogenicity, and thus albeit without clear evidence of direct DNA reactivity\textsuperscript{72}. It is well documented that ochratoxin A induces kidney tumors in rodents, but the involvement of genotoxicity on the kidney tumorigenesis remained investigated. However, significantly increased genotoxicity has recently been detected in the outer medulla but not in the cortex of the kidney in \textit{gpt} delta rats treated with ochratoxin A\textsuperscript{19}. It is also confirmed that such reporter gene-carrying rodents show similar carcinogenic susceptibility to the intact counterparts (data in preparation). Taken together, a combined subchronic toxicity/\textit{in vivo} genotoxicity study using such transgenic rodents is proposed as a short-term and advanced bioassay to detect genotoxic carcinogens. In terms of additional approaches to detect \textit{in vivo} genotoxic potential at organ levels, our proposed bioassay system may be more promising than a bioassay system simply extended from subchronic toxicity study\textsuperscript{77}.

\textbf{In Silico Tools}

Several \textit{in silico} models based on quantitative structure activity relationship (QSAR) have been developed in the assessment of genotoxicity for pharmaceuticals as well as industrial chemicals\textsuperscript{78–85}. To overview how such \textit{in silico} models are used internationally in the regulatory assessment of pharmaceutical impurities, both statistics- and knowledge-based (expert system) tools recommended in the guidelines or used practically by various regulatory agencies, as well as other existing programs, were analyzed\textsuperscript{78}. As a result, the available \textit{in silico} tools for genotoxicity and carcinogenicity prediction showed promising results, and thus the regulatory application of QSAR methods has been constantly growing. For regulatory purposes, it is recommended that predictions of genotoxicity/carcinogenicity should be based on a battery of models, combining high-sensitivity models (low rate of false negatives) with high-specificity ones (low rate of false positives). Recently, some \textit{in silico} tools for screening non-genotoxic carcinogenicity have also been designed, besides genotoxicity\textsuperscript{86}.

\textbf{In Vitro Cell Transformation Assays}

A short-term cell transformation assay has been developed to detect initiating and promoting activities of chemical carcinogens, using BALB/c 3T3 cells or Bhas 42 cells established from BALB/c 3T3 cells transfected with \textit{v-Ha-ras} gene\textsuperscript{87–92}. In comparison of the sensitivity between both assays, Bhas 42 cell assay was as sensitive as BALB/c 3T3 cell assay for the detection of initiating activities of arsenic compounds\textsuperscript{89}. For the detection of promoting activities, its sensitivity was equivalent to that of the two-stage BALB/c 3T3 cell transformation assay where the target cells were initiated with sub-threshold dose of 3-methylcholanthrene, confirming that Bhas 42 cells behave as initiated cells in the transformation assay\textsuperscript{89}. Taken together with the fact that Bhas 42 cell transformation assay is shown to be superior to classical BALB/c 3T3 cell transformation assay in cost and labor performance, Bhas 42 cell transformation assay has been validated for OECD test guideline\textsuperscript{87}.

\textbf{Toxicogenomics-based Systems}

Gene-based prediction models for early assessment of potential carcinogenicity of chemicals have been developed in rats by using toxicogenomics database\textsuperscript{93–97}. It has been reported that prediction accuracies for carcinogenicity were high enough, and false positive predictions were almost completely eliminated\textsuperscript{95}. Interestingly, similar positive predictions were obtained in several genotoxic as well as non-genotoxic carcinogens, indicating that the expression profiles of selected candidate biomarker genes might be common characteristics in the early stage of carcinogenesis for both genotoxic and non-genotoxic carcinogens\textsuperscript{95}. Such toxicogenomic models might be useful for the prospective screening of carcinogenicity of compounds and prioritization of compounds for carcinogenicity testing\textsuperscript{95}.
Proposed Assessment Strategy

There are two major issues, namely threshold and extrapolation to human risk in the current carcinogenicity assessment. Several possibilities are suggested on the critical issue that genotoxic carcinogens may also have the threshold. First, if a set of genotoxicity data for a compound carcinogenic to rodents proved to be falsely positive, the compound is no longer called a genotoxic carcinogen, indicating the existence of a true threshold like a non-genotoxic carcinogen. It may be difficult to confirm whether the genotoxic potential found in an assay is false positive, however, false reactions in a number of genotoxicity data could obviously exist, judging from some discrepancy between in vitro and in vivo assays, as well as single dose and repeated dose in vivo studies. Second, it is unclear as to how much the detected genotoxicity contributes to the carcinogenicity found in long-term rodent assays. This point could be important to understand organ-, species- and sex-differences of carcinogenicity. Third, it is well known that the carcinogenesis process per se involves multi-stages such as DNA damage/repair, gene mutation, apoptosis, cell proliferation and immune suppression. If there is a threshold mechanism in some of these steps, it is likely that the carcinogenic compound may have the biological threshold in inducing carcinogenicity. Even in the simplest hypothesis, both genotoxic and non-genotoxic or epigenetic events are required for the completion of carcinogenesis, suggesting a possible threshold to be determined with non-genotoxic events. Finally, statistical or mathematical approaches are concerned with possible practical thresholds even for true genotoxic carcinogens. Taken together, it can be emphasized that the mechanisms of carcinogenicity are crucial to determine as to whether the initiation of carcinogenicity is based on the direct DNA reaction, how much the genotoxicity contributes to the carcinogenicity, or if the carcinogenicity also fits to human risks.

Apart from the threshold issue, another major issue is regarding extrapolation of animal data to human risk. In the IARC evaluation, it has been well documented that the target organs in animals are not always the same as those in human. In International Programme on Chemical Safety (IPCS) as well as International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/ HESI) projects, several chemicals are assessed using weight of evidence (WOE) approach. For example, the MOA of limonene to induce kidney tumors was concluded to be no more qualitatively relevant to human risk because the causal factor α2u-globulin is only present in male rats. Liver tumors induced by some non-genotoxic chemicals are thought to be quantitatively far irrelevant to human risk based on the MOA simply related to transcription factors such as constitutive androstane receptor (CAR), pregnant X receptor (PXR) and peroxisome proliferator-activated receptor-α (PPARα). Possible secondary genotoxicity such as oxidative DNA damage may have a threshold because possible primary events such as inflammation and cytotoxicity followed by oxidative stress would have a threshold. Taken together, it is very important to define what tumor is based on the rodent-specific carcinogenesis mechanisms qualitatively or quantitatively.

Conclusion

Current issues on carcinogenicity assessment for risk factors in food are summarized into two major areas and some minor ones. One of the major issues is regarding the threshold for genotoxic carcinogens. To clarify as to whether DNA damage is really involved in the target organs where neoplastic lesions were developed, transgenic rodents carrying reporter genes such as gpt delta could be useful. Even if positive for in vivo genotoxicity, MOA or statistics might be taken into account for biological or practical threshold. Some key events such as cell proliferation, apoptosis and immunodeficiency for carcinogenicity would tell us something critical as to whether there is any threshold. Then MOE may be applicable because statistical or mathematical evaluation can provide benchmark dose lower confidence limit (BMDL) or MOE even for strictly defined genotoxic carcinogens. Another major issue concerns extrapolation of animal data to human risk. For clarifying species specificity of increased neoplastic lesions, WOE approaches based on MOA could be extremely useful. Transgenic rodents such as p53, nrf2 or CAR knockout mice might be helpful to elucidate the possible mechanisms of carcinogenicity. In the future, in silico and in vitro approaches would be powerful tools for screening genotoxic as well as carcinogenic potentials of a number of chemicals/agents.
References


