FOOD SAFETY


doi: 10.14252/foodsafetyfscj.2013001

Review (Invited)

# Carcinogenicity Assessment for Risk Factors in Food:

## Current Issues and a Proposal

Akiyoshi Nishikawa

Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501

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-

Received: 15 June 2013; Accepted: 26 June 2013
* Corresponding author: E-Mail: nishikaw@nihs.go.jp
The contents of this article reflect solely the view of the author(s).
**Abbreviations:** ADI, acceptable daily intake; ALARA, as low as reasonably achievable; BMDL, benchmark dose lower confidence limit; CAR, constitutive androstane receptor; IARC, International Agency for Research on Cancer; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; IPCS, International Programme on Chemical Safety; ILSI/HESI, International Life Sciences Institute/ Health and Environmental Sciences Institute; MOA, mode of action; MOE, margin of exposure; NOAEL, no-observed-adverse-effect-level; NOGEL, no-observed-genotoxic-effect-level; OECD, Organisation for Economic Co-operation and Development; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; SAR, structure-activity relationship; TTC, threshold of toxicological concern; WOE, weight of evidence

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 1.** Known Genotoxic Carcinogens in Food

| Chemical | Source | Rodent Carcinogenicity | IARC |
|---|---|---|---|
| Aflatoxin $B_1$ | Fungus | Liver | 1 |
| Acrylamide | Cooked food | Thyroid, Breast *etc.* | 2A |
| Heterocyclic amines | Cooked food | Liver, Colon *etc.* | 2A-2B |
| Nitrosamines | *Sec* Amine + Nitrite | Liver, Kidney *etc.* | 2A-2B |
| Safrole | Cinnamon *etc.* | Liver | 2B |
| Hydrazine | Mushroom | Lung, Liver *etc.* | 2B |
| Ochratoxin A | Fungus | Kidney | 2B |
| Potassium bromate | Bread making process | Kidney *etc.* | 2B |
| Maddor color | Maddor root | Kidney, Liver | 3 |

### 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 $B_1$, 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 *in vivo h*as been recently detected in the dissected kidney of *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 *gpt* delta rats[23].

### Food Additives

Madder color extracted from the roots of *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 $\alpha_{2u}$-globulin-associated kidney tumors in male rats.

**Table 2.** Risk Assessment Methodologies for Carcinogens

| Method | Advantages | Disadvantages |
|---|---|---|
| ALARA | Very simple to use | No risk prioritization used |
| BMDL | Quantitative risk characterization | At least 3 dose levels required |
| MOE | Risk estimate provided | Quantitative data required |
| NOAEL | Simple to use | Dose-response ignored |
| SAR | No experimental data needed | Limited use |
| T25 | Simple to use | Sensitive to experimental design |
| TTC | Simple to use | Worst case assumed |

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[48]. 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[49,50], *in vivo* Comet assay[51] and transgenic rodent assay carrying reporter genes[52,53] have been developed very recently. Such genotoxicity assays *in vivo* could be promising test methods to evaluate genotoxicity in the target tissues and therefore have been extensively validated[54].

A threshold is suggested *in vivo* even in the genotoxicity, especially for chromosomal aberration including micronucleus tests[14–17]. 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[11].

## Proposed Test Methods

### Transgenic Rodents Carrying Reporter Genes

Recently, a new guideline on transgenic rodent mutation assays has been released from the OECD[52], in which Muta™Mouse, Big Blue®, *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[53]. 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)[55]. Recently, *gpt* delta rats have been similarly developed in Sprague-Dawley rats[56] and backcrossed to F344[57] 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[56]. 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[58].

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[19,23,59–76]

because these rodent models would be powerful tools for the evaluation of both genotoxicity and carcinogenicity at the same organ level[59,60]. Some genotoxic carcinogens showed significantly increased genotoxicity in the liver of *gpt* delta rats, the target organ for carcinogenicity[75]. In contrast, a genotoxic chlorinated water by-product, MX, failed to exert the *in vivo* genotoxicity and carcinogenicity in *gpt* delta mice[74]. On the other hand, a known non-genotoxic carcinogen dicyclanil increased the *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[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 *gpt* delta rats treated with ochratoxin A[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/*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 *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[77].

## *In Silico* Tools

Several *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[78–85]. To overview how such *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[78]. As a result, the available *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 *in silico* tools for screening non-genotoxic carcinogenicity have also been designed, besides genotoxicity[86].

## *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 v-Ha-*ras* gene[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[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[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[87].

## Toxicogenomics-based Systems

Gene-based prediction models for early assessment of potential carcinogenicity of chemicals have been developed in rats by using toxicogenomics database[93–97]. It has been reported that prediction accuracies for carcinogenicity were high enough, and false positive predictions were almost completely eliminated[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[95]. Such toxicogenomic models might be useful for the prospective screening of carcinogenicity of compounds and prioritization of compounds for carcinogenicity testing[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[98]. 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[1]. 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 $\alpha_{2u}$-globulin is only present in male rats[99,100]. 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), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor-α (PPARα)[101,102]. 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[14,103,104]. 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


1. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. online (PDF) versions of IARC Monographs. 2013. Available at: http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php.
2. Merrill RA. Food safety regulation: reforming the Delaney Clause. Annu Rev Public Health. 1997; **18**: 313–340.
3. Kroes R, Renwick AG, Feron V, et al. Application of the threshold of toxicological concern (TTC) to the safety evaluation of cosmetic ingredients. Food Chem Toxicol. 2007; **45**(12): 2533–2562.
4. Kroes R, Kleiner J, Renwick A. The threshold of toxicological concern concept in risk assessment. Toxicol Sci. 2005; **86**(2): 226–230.
5. Kroes R, Renwick AG, Cheeseman M, et al. European branch of the International Life Sciences Institute. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. Food Chem Toxicol. 2004; **42**(1): 65–83.
6. Kroes R, Kozianowski G. Threshold of toxicological concern (TTC) in food safety assessment. Toxicol Lett. 2002; **127**(1-3): 43–46.
7. Kroes R, Galli C, Munro I, et al. Threshold of toxicological concern for chemical substances present in the diet: a practical tool for assessing the need for toxicity testing. Food Chem Toxicol. 2000; **38**(2-3): 255–312.
8. Barlow S, Renwick AG, Kleiner J, et al. Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. Food Chem Toxicol. 2006; **44**(10): 1636–1650.
9. Dybing E, O'Brien J, Renwick AG, Sanner T. Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic—an overview. Toxicol Lett. 2008; **180**(2): 110–117.
10. O'Brien J, Renwick AG, Constable A, et al. Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal. Food Chem Toxicol. 2006; **44**(10): 1613–1635.
11. European Commission risk assessment methodologies and approaches for genotoxic and carcinogenic substances. Health and Consumer Protection. 2009; 1-47. Available at: http://ec.europa.eu/health/ph_risk/committees/04_scher/docs/scher_o_113.pdf.
12. Boobis A, Flari V, Gosling JP, et al. Interpretation of the margin of exposure for genotoxic carcinogens - Elicitation of expert knowledge about the form of the dose response curve at human relevant exposures. Food Chem Toxicol. 2013; **57**: 106–118.
13. Benford D, Bolger PM, Carthew P, et al. Application of the Margin of Exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. Food Chem Toxicol. 2010; **48** (Suppl 1): S2–S24.
14. Platel A, Nesslany F, Gervais V, Claude N, Marzin D. Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: determination of No-Observed-Genotoxic-Effect-Levels. Mutat Res. 2011; **726**(2): 151–159.
15. Elhajouji A, Lukamowicz M, Cammerer Z, Kirsch-Volders M. Potential thresholds for genotoxic effects by micronucleus scoring. Mutagenesis. 2011; **26**(1): 199–204.
16. Johnson GE, Doak SH, Griffiths SM, et al. Non-linear dose-response of DNA-reactive genotoxins: recommendations for data analysis. Mutat Res. 2009; **678**(2): 95–100.
17. Gocke E, Müller L. *In vivo* studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. Mutat Res. 2009; **678**(2): 101–107.
18. Hibi D, Kijima A, Kuroda K, et al. Molecular mechanisms underlying ochratoxin A-induced genotoxicity: global gene expression analysis suggests induction of DNA double-strand breaks and cell cycle progression. J Toxicol Sci. 2013; **38**(1): 57–69.
19. Hibi D, Suzuki Y, Ishii Y, et al. Site-specific *in vivo* mutagenicity in the kidney of *gpt* delta rats given a carcinogenic dose of ochratoxin A. Toxicol Sci. 2011; **122**(2): 406–414.
20. Wislocki PG, Miller EC, Miller JA, McCoy EC, Rosenkranz HS. Carcinogenic and mutagenic activities of safrole, 1'-hydroxysafrole, and some known or possible metabolites. Cancer Res. 1977; **37**(6): 1883–1891.
21. Roe FJ, Grant GA, Millican DM. Carcinogenicity of hydrazine and 1,1-dimethylhydrazine for mouse lung. Nature. 1967; **216**(5113): 375–376.
22. Epstein SS, Andrea J, Jaffe H, Joshi S, Falk H, Mantel N. Carcinogenicity of the herbicide maleic hydrazide. Nature. 1967; **215**(5108): 1388–1390.
23. Jin M, Kijima A, Suzuki Y, et al. Comprehensive toxicity study of safrole using a medium-term animal model with *gpt* delta rats. Toxicology. 2011; **290**(2-3): 312–321.
24. Inoue K, Yoshida M, Takahashi M, et al. Carcinogenic potential of alizarin and rubiadin, components of madder color, in a rat medium-term multi-organ bioassay. Cancer Sci. 2009; **100**(12): 2261–2267.
25. Inoue K, Yoshida M, Takahashi M, et al. Induction of kidney and liver cancers by the natural food additive madder color in a two-year rat carcinogenicity study. Food Chem Toxicol. 2009; **47**(1): 184–191.
26. DeAngelo AB, George MH, Kilburn SR, Moore TM, Wolf DC. Carcinogenicity of potassium bromate administered in the drinking water to male B6C3F1 mice and F344/N rats. Toxicol Pathol. 1998; **26**(5): 587–594.
27. Kurokawa Y, Takayama S, Konishi Y, et al. Long-term in vivo carcinogenicity tests of potassium bromate, sodium hypochlorite, and sodium chlorite conducted in Japan. Environ Health Perspect. 1986; **69**: 221–235.
28. Kurokawa Y, Aoki S, Matsushima Y, et al. Dose-response studies on the carcinogenicity of potassium bromate in F344 rats after long-term oral administration. J Natl Cancer Inst. 1986; **77**(4): 977–982.

29. Kurokawa Y, Hayashi Y, Maekawa A, Takahashi M, Kokubo T, Odashima S. Carcinogenicity of potassium bromate administered orally to F344 rats. J Natl Cancer Inst. 1983; **71**(5): 965–972.
30. Sai K, Uchiyama S, Ohno Y, Hasegawa R, Kurokawa Y. Generation of active oxygen species *in vitro* by the interaction of potassium bromate with rat kidney cell. Carcinogenesis. 1992; **13**(3): 333–339.
31. George J, Shukla Y. Pesticides and cancer: insights into toxicoproteomic-based findings. J Proteomics. 2011; **74**(12): 2713–2722.
32. Mitsumori K. Evaluation on carcinogenicity of chemicals using transgenic mice. Toxicology. 2002; **181-182**: 241–244.
33. Storer RD, French JE, Donehower LA, et al. Transgenic tumor models for carcinogen identification: the heterozygous Trp53-deficient and RasH2 mouse lines. Mutat Res. 2003; **540**(2): 165–176.
34. Mitsumori K. Possible mechanism on enhanced carcinogenesis of genotoxic carcinogens and unsolved mechanisms on lesser carcinogenic susceptibility to some carcinogens in *ras*H2 mice. J Toxicol Sci. 2003; **28**(5): 371–383.
35. Maruyama C, Tomisawa M, Wakana S, et al. Overexpression of human H-*ras* transgene is responsible for tumors induced by chemical carcinogens in mice. Oncol Rep. 2001; **8**(2): 233–237.
36. Maronpot RR, Mitsumori K, Mann P, et al. Interlaboratory comparison of the CB6F1-Tg rasH2 rapid carcinogenicity testing model. Toxicology. 2000; **146**(2-3): 149–159.
37. Mitsumori K, Koizumi H, Nomura T, Yamamoto S. Pathological features of spontaneous and induced tumors in transgenic mice carrying a human prototype c-Ha-*ras* gene used for six-month carcinogenicity studies. Toxicol Pathol. 1998; **26**(4): 520–531.
38. Yamamoto S, Urano K, Koizumi H, et al. Validation of transgenic mice carrying the human prototype c-Ha-*ras* gene as a bioassay model for rapid carcinogenicity testing. Environ Health Perspect. 1998; **106** (Suppl 1): 57–69.
39. Yamamoto S, Hayashi Y, Mitsumori K, Nomura T. Rapid carcinogenicity testing system with transgenic mice harboring human prototype c-HRAS gene. Lab Anim Sci. 1997; **47**(2): 121–126.
40. Yamamoto S, Mitsumori K, Kodama Y, et al. Rapid induction of more malignant tumors by various genotoxic carcinogens in transgenic mice harboring a human prototype c-Ha-*ras* gene than in control non-transgenic mice. Carcinogenesis. 1996; **17**(11): 2455–2461.
41. Tsuda H, Futakuchi M, Fukamachi K, et al. A medium-term, rapid rat bioassay model for the detection of carcinogenic potential of chemicals. Toxicol Pathol. 2010; **38**(1): 182–187.
42. Ito N, Tamano S, Shirai T. A medium-term rat liver bioassay for rapid in vivo detection of carcinogenic potential of chemicals. Cancer Sci. 2003; **94**(1): 3–8.
43. Shirai T, Hirose M, Ito N. Medium-term bioassays in rats for rapid detection of the carcinogenic potential of chemicals. IARC Sci Publ. 1999; (146): 251–272.
44. Ito N, Imaida K, Tamano S, Hagiwara A, Shirai T. Medium-term bioassays as alternative carcinogenicity test. J Toxicol Sci. 1998; **23** (Suppl 2): 103–106.
45. Harmonised Tripartite Guideline ICH. S1B Testing for carcinogenicity of pharmaceuticals, 1997. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm074916.pdf.
46. Alison RH, Capen CC, Prentice DE. Neoplastic lesions of questionable significance to humans. Toxicol Pathol. 1994; **22**(2): 179–186.
47. Gopinath C. The predictive value of pathological findings in animal toxicity studies. J Toxicol Pathol. 1995; **8**: 89–100.
48. OECD 2007. OECD Guidelines for the Testing of Chemicals, Section 4 Health Effects. Available at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.
49. Suzuki H, Ikeda N, Kobayashi K, et al. Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). Mutat Res. 2005; **583**(2): 133–145.
50. Suzuki H, Shirotori T, Hayashi M. A liver micronucleus assay using young rats exposed to diethylnitrosamine: methodological establishment and evaluation. Cytogenet Genome Res. 2004; **104**(1-4): 299–303.
51. Ding W, Petibone DM, Latendresse JR, et al. *In vivo* genotoxicity of furan in F344 rats at cancer bioassay doses. Toxicol Appl Pharmacol. 2012; **261**(2): 164–171.
52. OECD 2011. OECD Guidelines for the Testing of Chemicals. Transgenic rodent somatic and germ cell gene mutation assays (TG488). Available at: http://www.oecd-ilibrary.org/environment/test-no-488-transgenic-rodent-somatic-and-germ-cell-gene-mutation-assays_9789264122819-en.
53. Nohmi T, Katoh M, Suzuki H, et al. A new transgenic mouse mutagenesis test system using Spi$^-$ and 6- thioguanine selections. Environ Mol Mutagen. 1996; **28**: 465–470.
54. EFSA Scientific Committee Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, EFSA Journal 2011; 9(9): 2379 [69 pp.]. Available at: http://www.sftox.com/actualites/EFSA_Genotox.pdf.
55. Masumura K, Matsui M, Katoh M, et al. Spectra of *gpt* mutations in ethylnitrosourea-treated and untreated transgenic mice. Environ Mol Mutagen. 1999; **34**: 1–8.
56. Hayashi H, Kondo H, Masumura K, Shindo Y, Nohmi T. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi$^-$ selection. Environ Mol Mutagen. 2003; **41**: 253–259.
57. Umemura T, Kanki K, Kuroiwa Y, et al. *In vivo* mutagenicity and initiation following oxidative DNA lesion in the kidneys of rats given potassium bromate. Cancer Sci. 2006; **97**: 829–835.
58. Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; **455**: 191–215.

59. Nishikawa A, Umemura T, Ishii Y, et al. *In vivo* approaches to study mechanism of action of genotoxic carcinogens. Genes Environ. 2008; **30**: 120–124.
60. Nishikawa A, Suzuki T, Masumura K, et al. Reporter gene transgenic mice as a tool for analyzing the molecular mechanisms underlying experimental carcinogenesis. J Exp Clin Cancer Res. 2001; **20**(1): 111–115.
61. Jin M, Kijima A, Hibi D, et al. *In vivo* genotoxicity of methyleugenol in *gpt* delta transgenic rats following medium-term exposure. Toxicol Sci. 2013; **131**(2): 387–394.
62. Tasaki M, Kuroiwa Y, Inoue T, et al. Oxidative DNA damage and *in vivo* mutagenicity caused by reactive oxygen species generated in the livers of *p53*-proficient or -deficient *gpt* delta mice treated with non-genotoxic hepatocarcinogens. J Appl Toxicol. 2012; doi:10.1002/jat.2807. [Epub ahead of print].
63. Jin M, Kijima A, Suzuki Y, et al. *In vivo* genotoxicity of 1-methylnaphthalene from comprehensive toxicity studies with B6C3F1 *gpt* delta mice. J Toxicol Sci. 2012; **37**(4): 711–721.
64. Suzuki Y, Umemura T, Hibi D, et al. Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats. Arch Toxicol. 2012; **86**(10): 1593–1601.
65. Okamura T, Ishii Y, Suzuki Y, et al. Effects of co-treatment of dextran sulfate sodium and MeIQx on genotoxicity and possible carcinogenicity in the colon of *p53*-deficient mice. J Toxicol Sci. 2010; **35**(5): 731–741.
66. Okamura T, Ishii Y, Suzuki Y, et al. Enhancing effects of carbon tetrachloride on *in vivo* mutagenicity in the liver of mice fed 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). J Toxicol Sci. 2010; **35**(5): 709–720.
67. Masumura K, Sakamoto Y, Ikeda M, et al. Antigenotoxic effects of *p53* on spontaneous and ultraviolet light B—induced deletions in the epidermis of *gpt* delta transgenic mice. Environ Mol Mutagen. 2011; **52**(3): 244–252.
68. Tasaki M, Umemura T, Suzuki Y, et al. Oxidative DNA damage and reporter gene mutation in the livers of *gpt* delta rats given non-genotoxic hepatocarcinogens with cytochrome P450-inducible potency. Cancer Sci. 2010; **101**(12): 2525–2530.
69. Toyoda-Hokaiwado N, Inoue T, Masumura K, et al. Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. Toxicol Sci. 2010; **114**(1): 71–78.
70. Totsuka Y, Higuchi T, Imai T, et al. Genotoxicity of nano/microparticles in *in vitro* micronuclei, *in vivo* comet and mutation assay systems. Part Fibre Toxicol. 2009; **6**: 23.
71. Umemura T, Tasaki M, Kijima A, et al. Possible participation of oxidative stress in causation of cell proliferation and *in vivo* mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate. Toxicology. 2009; **257**(1-2): 46–52.
72. Umemura T, Kuroiwa Y, Tasaki M, et al. Detection of oxidative DNA damage, cell proliferation and *in vivo* mutagenicity induced by dicyclanil, a non-genotoxic carcinogen, using *gpt* delta mice. Mutat Res. 2007; **633**(1): 46–54.
73. Kuroiwa Y, Umemura T, Nishikawa A, et al. Lack of *in vivo* mutagenicity and oxidative DNA damage by flumequine in the livers of *gpt* delta mice. Arch Toxicol. 2007; **81**(1): 63–69.
74. Nishikawa A, Sai K, Okazaki K, et al. MX, a by-product of water chlorination, lacks *in vivo* genotoxicity in *gpt* delta mice but inhibits gap junctional intercellular communication in rat WB cells. Environ Mol Mutagen. 2006; **47**(1): 48–55.
75. Kanki K, Nishikawa A, Masumura K, et al. *In vivo* mutational analysis of liver DNA in *gpt* delta transgenic rats treated with the hepatocarcinogens *N*-nitrosopyrrolidine, 2-amino-3-methylimidazo[4,5-*f*]quinoline, and di(2-ethylhexyl)phthalate. Mol Carcinog. 2005; **42**(1): 9–17.
76. Masumura K, Horiguchi M, Nishikawa A, et al. Low dose genotoxicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in *gpt* delta transgenic mice. Mutat Res. 2003; **541**(1-2): 91–102.
77. Cohen SM. Human carcinogenic risk evaluation: an alternative approach to the two-year rodent bioassay. Toxicol Sci. 2004; **80**: 225–229.
78. Fioravanzo E, Bassan A, Pavan M, Mostrag-Szlichtyng A, Worth AP. Role of *in silico* genotoxicity tools in the regulatory assessment of pharmaceutical impurities. SAR QSAR Environ Res. 2012; **23**(3-4): 257–277.
79. Blake BW, Enslein K, Gombar VK, Borgstedt HH. *Salmonella* mutagenicity and rodent carcinogenicity: quantitative structure-activity relationships. Mutat Res. 1990; **241**(3): 261–271.
80. Ashby J. Aspects of database construction and interrogation of relevance to the accurate prediction of rodent carcinogenicity and mutagenicity. Environ Health Perspect. 1991; **96**: 97–100.
81. Klopman G, Rosenkranz HS. International Commission for Protection Against Environmental Mutagens and Carcinogens. Approaches to SAR in carcinogenesis and mutagenesis. Prediction of carcinogenicity/mutagenicity using MULTI-CASE. Mutat Res. 1994; **305**(1): 33–46.
82. Benigni R, Bossa C, Worth A. Structural analysis and predictive value of the rodent *in vivo* micronucleus assay results. Mutagenesis. 2010; **25**(4): 335–341.
83. Sutter A, Amberg A, Boyer S, et al. Use of *in silico* systems and expert knowledge for structure-based assessment of potentially mutagenic impurities. Regul Toxicol Pharmacol. 2013; **67**(1): 39–52. Available at http://www.sciencedirect.com/science/article/pii/S0273230013000688.
84. Worth AP, Lapenna S, Serafimova R. QSAR and metabolic assessment tools in the assessment of genotoxicity. Methods Mol Biol. 2013; **930**: 125–162.
85. Mekenyan OG, Petkov PI, Kotov SV, et al. Investigating the relationship between *in vitro-in vivo* genotoxicity: derivation of mechanistic QSAR models for *in vivo* liver genotoxicity and *in vivo* bone marrow micronucleus formation which encompass metabolism. Chem Res Toxicol. 2012; **25**(2): 277–296.
86. Carrasquer CA, Malik N, States G, Qamar S, Cunningham SL, Cunningham AR. Chemical structure determines target organ carcinogenesis in rats. SAR QSAR Environ Res. 2012; **23**(7-8): 775–795.

87. Sakai A, Sasaki K, Hayashi K, et al. An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. Mutat Res. 2011; **725**(1-2): 57–77.
88. Sakai A, Sasaki K, Muramatsu D, et al. A Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity. Mutat Res. 2010; **702**(1): 100–122.
89. Muramatsu D, Sasaki K, Kuroda S, Hayashi K, Tanaka N, Sakai A. Comparison of sensitivity to arsenic compounds between a Bhas 42 cell transformation assay and a BALB/c 3T3 cell transformation assay. Mutat Res. 2009; **675**(1-2): 66–70.
90. Ohmori K, Umeda M, Tanaka N, et al. An inter-laboratory collaborative study by the Non-Genotoxic Carcinogen Study Group in Japan, on a cell transformation assay for tumour promoters using Bhas 42 cells. Altern Lab Anim. 2005; **33**(6): 619–639.
91. Asada S, Sasaki K, Tanaka N, Takeda K, Hayashi M, Umeda M. Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells). Mutat Res. 2005; **588**(1): 7–21.
92. Ohmori K, Sasaki K, Asada S, Tanaka N, Umeda M. An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells. Mutat Res. 2004; **557**(2): 191–202.
93. Yamada F, Sumida K, Uehara T, et al. Toxicogenomics discrimination of potential hepatocarcinogenicity of non-genotoxic compounds in rat liver. J Appl Toxicol. 2012; doi:10.1002/jat.2790. [Epub ahead of print].
94. Matsumoto H, Yakabe Y, Saito F, et al. New short term prediction method for chemical carcinogenicity by hepatic transcript profiling following 28-day toxicity tests in rats. Cancer Inform. 2011; **10**: 259–271.
95. Uehara T, Minowa Y, Morikawa Y, et al. Prediction model of potential hepatocarcinogenicity of rat hepatocarcinogens using a large-scale toxicogenomics database. Toxicol Appl Pharmacol. 2011; **255**(3): 297–306.
96. Matsumoto H, Yakabe Y, Saito K, et al. Discrimination of carcinogens by hepatic transcript profiling in rats following 28-day administration. Cancer Inform. 2009; **7**: 253–269.
97. Uehara T, Hirode M, Ono A, et al. A toxicogenomics approach for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals in rats. Toxicology. 2008; **250**(1): 15–26.
98. Armitage P, Doll R. The age distribution of cancer and a multi-stage theory of carcinogenesis. Br J Cancer. 1954; **8**(1): 1–12.
99. Fenner-Crisp PA, Mayes ME, David RM. Assessing the human carcinogenic potential of tetrahydrofuran: I. Mode of action and human relevance analysis of the male rat kidney tumor. Regul Toxicol Pharmacol. 2011; **60**(1): 20–39.
100. Cruzan G, Borghoff SJ, de Peyster A, et al. Methyl tertiary-butyl ether mode of action for cancer endpoints in rodents. Regul Toxicol Pharmacol. 2007; **47**(2): 156–165.
101. Hall AP, Elcombe CR, Foster JR, et al. Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes—conclusions from the 3rd International ESTP Expert Workshop. Toxicol Pathol. 2012; **40**(7): 971–994.
102. Ross J, Plummer SM, Rode A, et al. Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane *in vivo*. Toxicol Sci. 2010; **116**(2): 452–466.
103. Platel A, Nesslany F, Gervais V, Marzin D. Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the *in vitro* micronucleus test: Determination of No-Observed-Effect Levels. Mutat Res. 2009; **678**(1): 30–37.
104. Fukushima S, Kinoshita A, Puatanachokchai R, Kushida M, Wanibuchi H, Morimura K. Hormesis and dose-response-mediated mechanisms in carcinogenesis: evidence for a threshold in carcinogenicity of non-genotoxic carcinogens. Carcinogenesis. 2005; **26**(11): 1835–1845.