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Detection of hepatocarcinogens by combination of liver micronucleus assay and histopathological examination in 2-week or 4-week repeated dose studies

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Abstract

Background: Currently, revisions to the ICH S1 guidance on rodent carcinogenicity testing are being proposed. Application of this approach would reduce the use of animals in accordance with the 3Rs principles (reduce/refine/replace). The method would also shift resources to focus on more scientific mechanism-based carcinogenicity assessments and promote safe and ethical development of new small molecule pharmaceuticals. In the revised draft, findings such as cellular hypertrophy, diffuse and/or focal cellular hyperplasia, persistent tissue injury and/or chronic inflammation, preneoplastic changes, and tumors are listed as histopathology findings of particular interest for identifying carcinogenic potential. In order to predict hepatocarcinogenicity of test chemicals based on the results from 2- or 4-week repeated dose studies, we retrospectively reanalyzed the results of a previous collaborative study on the liver micronucleus assay. We focused on liver micronucleus induction in combination with histopathological changes including hypertrophy, proliferation of oval cells or bile duct epithelial cells, tissue injuries, regenerative changes, and inflammatory changes as the early responses of hepatocarcinogenesis. For these early responses, A total of 20 carcinogens, including 14 genotoxic hepatocarcinogens (Group A) and 6 non-liver-targeted genotoxic carcinogens (Group B) were evaluated.

Results: In the Group A chemicals, 5 chemicals (NPYR, MDA, NDPA, 2,6-DNT, and NMOR) showed all of the 6 early responses in hepatocarcinogenesis. Five chemicals (DMN, 2,4-DNT, QUN, 2-AAF, and TAA) showed 4 responses, and 4 chemicals (DAB, 2-NP, MCT, and Sudan I) showed 3 responses. All chemicals exhibited at least 3 early responses. Contrarily, in the Group B chemicals (6 chemicals), 3 of the 6 early responses were observed in 1 chemical (MNNG). No more than two responses were observed in 3 chemicals (MMC, MMS, and KA), and no responses were observed in 2 chemicals (CP and KBrO3).

Conclusion: Evaluation of liver micronucleus induction in combination with histopathological examination is useful for detecting hepatocarcinogens. This assay takes much less time than routine long-term carcinogenicity studies.

Keywords: Micronucleus assay, Liver, Hepatocarcinogen, Histopathology, Early responses

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Introduction

The liver is an important tissue not only in general toxicological studies, but also in carcinogenicity studies. About 60% of carcinogens are hepatocarcinogens [1], suggesting that development a new evaluation method targeting the liver is meaningful. In addition to the routinely used erythropoietic micronucleus in rodents, the liver micronucleus assay has been developed to detect genotoxic hepatocarcinogens that require metabolic activation [2–6].

The liver micronucleus assay targets the primary organ for drug metabolism; however, it is not commonly used due to slow hepatocyte proliferation in adult rats. Partial hepatectomy [7–9], mitogen treatment [10, 11], and the use of juvenile rats [12–15] have been introduced to address this drawback. Unfortunately, these methods have disadvantages, including complex surgical procedures and decreased metabolic activity for partial hepatectomy [16], risk of drug interactions for mitogen treatment [17], and a lack of maturation for metabolic activation in juvenile rats [18]. Recently, a repeated-dose liver micronucleus assay (RDLMN) was developed as a new method for evaluating liver micronuclei. The approach used 2- or 4-week repeated-dose treatment for the accumulation of micronucleated hepatocytes (MNHEPs) [19]. This method facilitates the integration of the liver micronucleus assay into repeated-dose general toxicity studies to simultaneously assess genotoxicity and histopathological endpoints with the same animals used for the overall evaluation of chemical risk.

Routine long-term carcinogenicity studies are time consuming and costly and require large numbers of animals. Revision to the ICH S1 guidelines is being discussed to address these issues. In a revised draft, histopathological findings such as cellular hypertrophy, diffuse and/or focal cellular hyperplasia, persistent tissue injury and/or chronic inflammation, preneoplastic changes, and tumors are listed as particular interest for identifying carcinogenic potential [20]. The possibility of predicting hepatocarcinogenicity of test chemicals based on the results of 2- or 4-week repeated-dose studies was assessed using a reanalysis of a previous collaborative study of the liver micronucleus assay [2, 21] in combination with histopathological examination.

Table 1 Liver MN assay results in the collaborative study by CSGMT/JEMS MMS and rat carcinogenicity data for the test chemicals

Group	Chemical	Abbreviation	CAS no.	In vivo MN assay (Liver)				Rat carcinogenicity		
				2 weeks	Ref.	4 weeks	Ref.	Liver	Other sites	Ref.
Group A	Dimethylnitrosamine	DMN	62–75-9	+	[2]	+	[2]	+	kid, lun, vsc, tes	[22]
	N-Nitrosopyrrolidine	NPYR	930–55-2	+	[2]	+	[2]	+	kid, vsc, tes	[22, 23]
	4,4′-Methylenedianiline	MDA	101–77-9	+	[2]	+	[2]	+	thy	[24]
	N-Nitrosodipropylamine	NDPA	621–64-7	+	[2]	ND		+	eso, nas	[22]
	2,4-Dinitrotoluene	2,4-DNT	121–14-2	+	[2]	+	[2]	+	ski, mgl	[22]
	2,6-Dinitrotoluene	2,6-DNT	606–20-2	+	[2]	+	[2]	+	–	[22]
	Quinoline	QUN	91–22-5	+	[2]	+	[2]	+	–	[25]
	p-Dimethylaminoazobenzene	DAB	60–11-7	+	[2]	+	[2]	+	–	[22]
	2-Nitropropane	2-NP	79–46-9	+	[2]	+	[2]	+	–	[26]
	Monocrotaline	MCT	315–22-0	+	[2]	+	[2]	+	–	[22]
	N-Nitrosomorpholine	NMOR	59–89-2	+	[2]	ND		+	vsc	[22]
	2-Acetylaminofluorene	2-AAF	53–96-3	+	[2]	+	[2]	+	ski, mgl	[22]
	Sudan I (C.I.solvent yellow 14)	Sudan I	842–07-9	+	[21]	ND		+	–	[22]
	Thioacetamide	TAA	62–55-5	+	[21]	+	[21]	+	–	[22]
Group B	Mitomycin C	MMC	50–07-7	+	[2]	+	[2]	–	per	[22]
	Cyclophosphamide H2O	CP	6055-19-2	–	[2]	ND		–	ub, lym, ner	[27]
	Potassium bromate	KBrO3	7758-01-2	–	[2]	–	[2]	–	kid, per, thy	[22]
	N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	MNNG	70–25-7	–	[2]	–	[2]	–	eso, smi, sto	[22]
	Methyl methanesulfonate	MMS	66–27-3	+	[2]	–	[2]	–	hmo, lun, ner	[22, 26]
	Kojic acid	KA	501–30-4	–	[2]	–	[2]	–	thy (mouse)	[22]

MN assay: micronucleus assay
+: positive; –: negative; ND: no data;
kid: kidney; lun: lung; vsc: vascular system; tes: testes; thy: thyroid gland; eso: esophagus; nas: nasal cavity; ski: skin; mgl: mammary gland; per: peritoneal cavity; ub: urinary bladder; lym: lymphocyte; ner: nervous system; smi: small intestine; sto: stomach; hmo: hematopoietic system; pan: pancreas
Group A, Genotoxic hepatocarcinogens;
Group B, Genotoxic carcinogens but non-liver-targeted

Materials and methods

Classification of chemicals and previous collaborative study by CSGMT/JEMS MMS

Twenty genotoxic carcinogens examined in a previous collaborative study by CSGMT/JEMS MMS were classified into two groups: Group A consisted of 14 genotoxic hepatocarcinogens and Group B consisted of 6 non-liver-targeted genotoxic carcinogens. Liver micronucleus assay data were then integrated (Table 1).

Male Crl:CD (SD) rats used in the previous report [28–44] were purchased from Charles River Japan Inc. (Atsugi, Hino or Tsukuba, Japan) and used at the age of 6 weeks. The animals were housed in an air-conditioned room with a 12-h light/dark cycle and allowed free access to food and water. The animal experiments were approved by the Institutional Animal Care and Use Committee of each testing facility in advance. The rats (5/group) were given each chemical repeatedly by oral gavage for 14 or 28 consecutive days. Twenty-four hours after the last administration, the rats were euthanized under thiopental anesthesia. Livers were removed and a part of each liver (left lateral lobe) was used for the liver micronucleus assay [28–44]. The remaining tissue was fixed with 10% phosphate-buffered formalin, embedded in paraffin, thin-sectioned, and stained with hematoxylin and eosin according to standard protocols. Histopathological examination was performed by a pathologist using light microscopy.

Reanalysis of pathological findings and application to the hepatocarcinogenesis process

Common markers for a precancerous stage in hepatocarcinogenesis include (i) transformation of normal hepatocytes into preneoplastic hepatocytes, (ii) selection of preneoplastic hepatocytes for growth, and (iii) isolation of preneoplastic hepatocytes from normal hepatic tissue. Transformation, selection, and isolation are thus general processes for the progression of preneoplastic hepatocytes into malignant cells [45]. With references to this report and the histopathology findings of particular interest for identifying carcinogenic potential pointed out in the draft S1 guidelines [20], changes in each carcinogenic process were roughly divided into 10 categories: mutation (including liver micronucleus induction), hypertrophy, tissue injuries, proliferation of oval cells or bile duct epithelial cells, regenerative changes, inflammatory changes, focus of altered hepatocytes, non-regenerative or regenerative hyperplasia, adenoma, and liver cancer (Fig.1).

We used the above information to reanalyze the presence of 9 liver pathological responses based on the findings from the previous collaborative study. Each of the 20

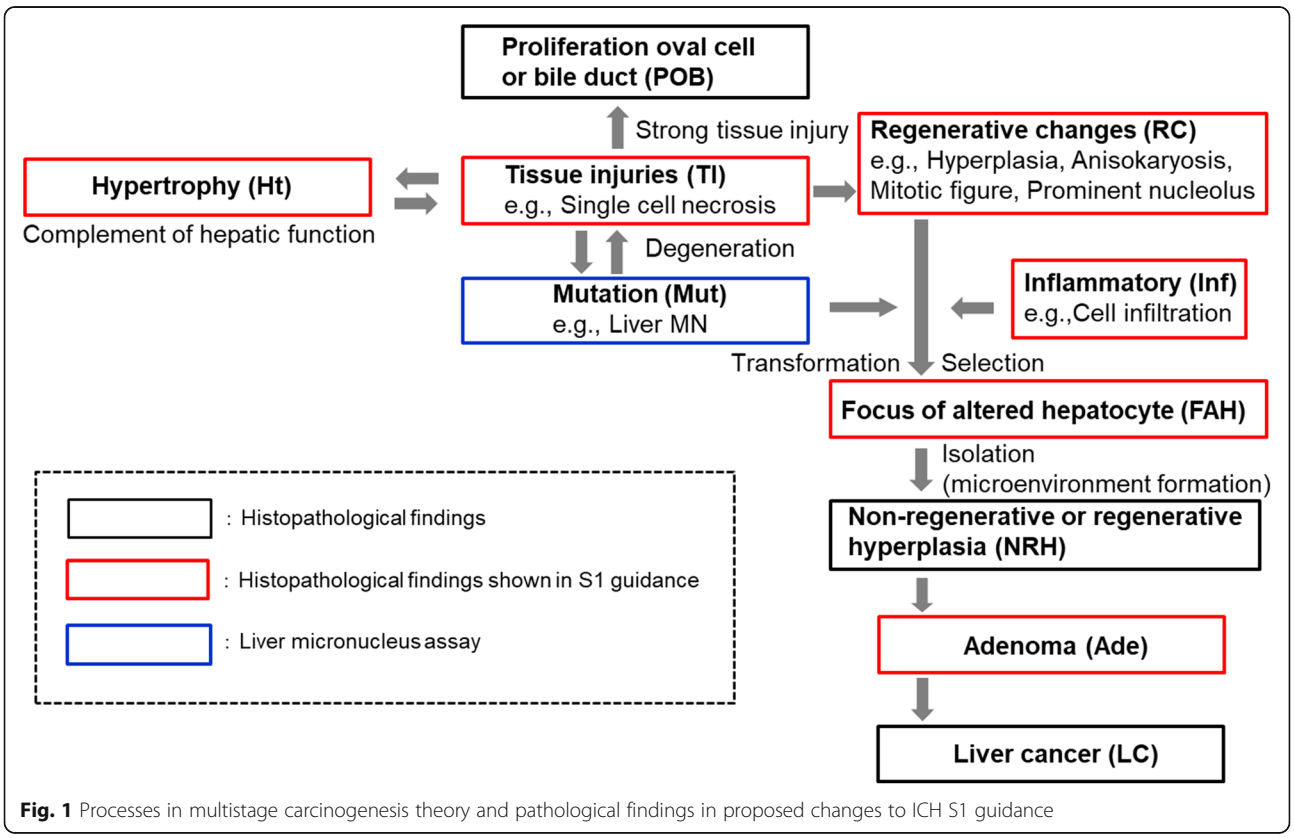

Fig. 1 Processes in multistage carcinogenesis theory and pathological findings in proposed changes to ICH S1 guidance

chemicals was reassessed. The grades of findings and frequency of appearance were disregarded to simplify the evaluation. Judgment was used only for the presence or absence determination. Except for accidental findings, findings judged to result from toxic insult were comprehensively evaluated. Mutation was identified via induction of liver micronuclei. Chemicals evaluated in 14- and 28-day repeated dose studies were judged to be "with findings" if chemical-related toxicity was observed in either time frame. Chemicals without findings in either time frame were judged to be "without findings".

Results

Group A chemicals (genotoxic hepatocarcinogens)

We evaluated 14 Group A chemicals for 10 markers of the carcinogenic pathways (9 liver pathological responses and liver micronucleus induction) (Fig.2). The liver micronucleus induction was most frequently observed (100% [14/14]) followed by hypertrophy (93% [13/14]), tissue injuries (79% [11/14]), proliferation of oval cells or bile duct epithelial cells (50% [7/14]), regenerative changes (71% [10/14]), inflammatory changes (50% [7/14]), focus of altered hepatocytes (21% [3/14]), and adenomas (7% [1/14]). Non-regenerative or regenerative hyperplasia and liver cancer were not observed.

One chemical (2,6-DNT) demonstrated 7 of the 10 aforementioned responses. Five chemicals (NPYR, MDA, NDPA, NMOR, and 2-AAF) displayed 6 responses, 5 chemicals (DMN, 2,4-DNT, QUN, 2-NP, and TAA) exhibited 4 responses, and 3 chemicals (DAB, MCT, and Sudan I) showed 3 responses. No chemical showed fewer than three responses.

Group B chemicals (genotoxic carcinogens but not liver targeted)

We evaluated 6 Group B chemicals (Fig.3). The response frequencies for these chemicals were liver micronucleus induction (33% [2/6]), hypertrophy (33% [2/6]), tissue injuries (17% [1/6]), regenerative changes (17% [1/6]), and inflammatory changes (17% [1/6]).

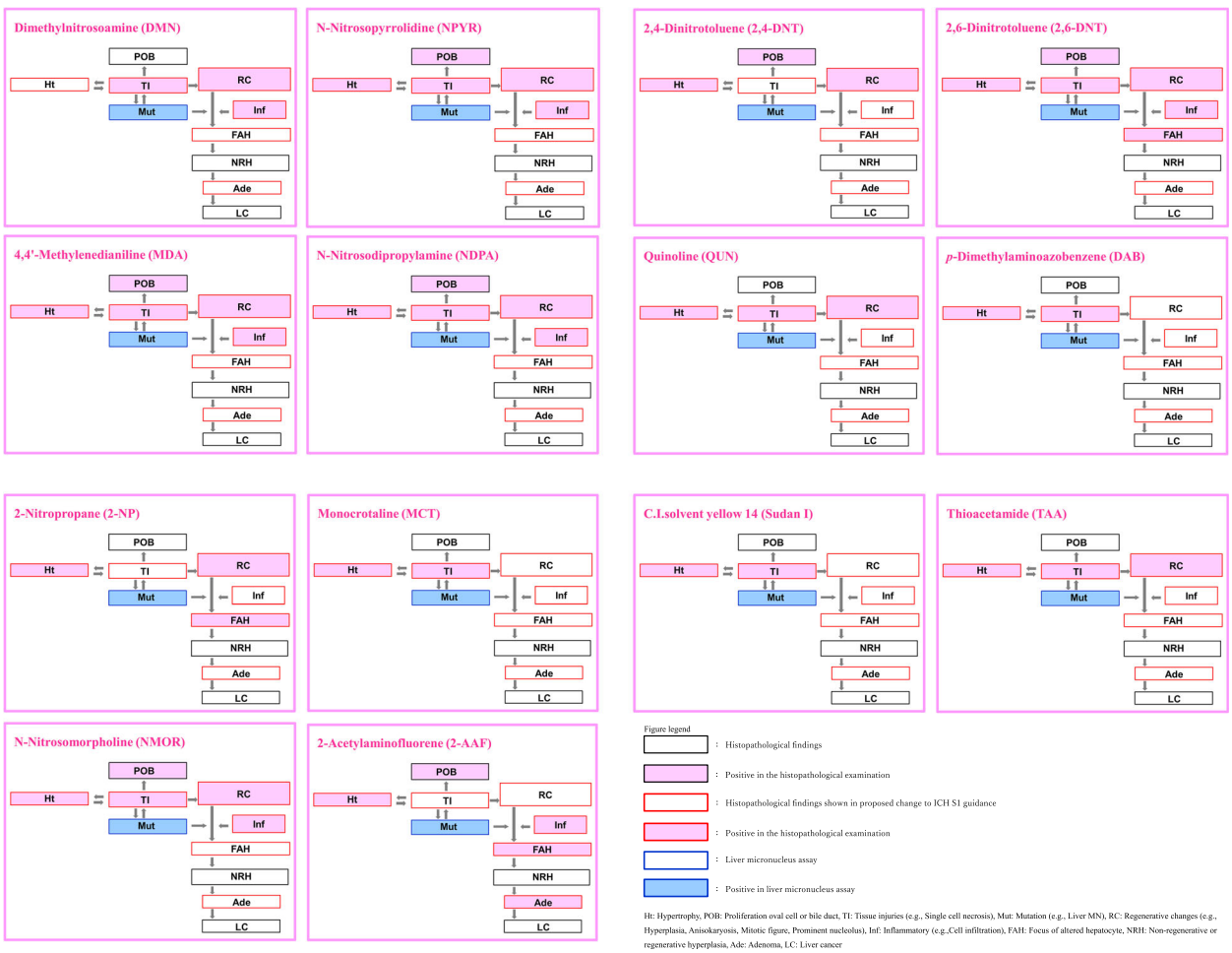

Fig. 2 Liver micronucleus induction and histopathological changes observed in 14-day and/or 28-day repeated-dose studies – genotoxic hepatocarcinogens

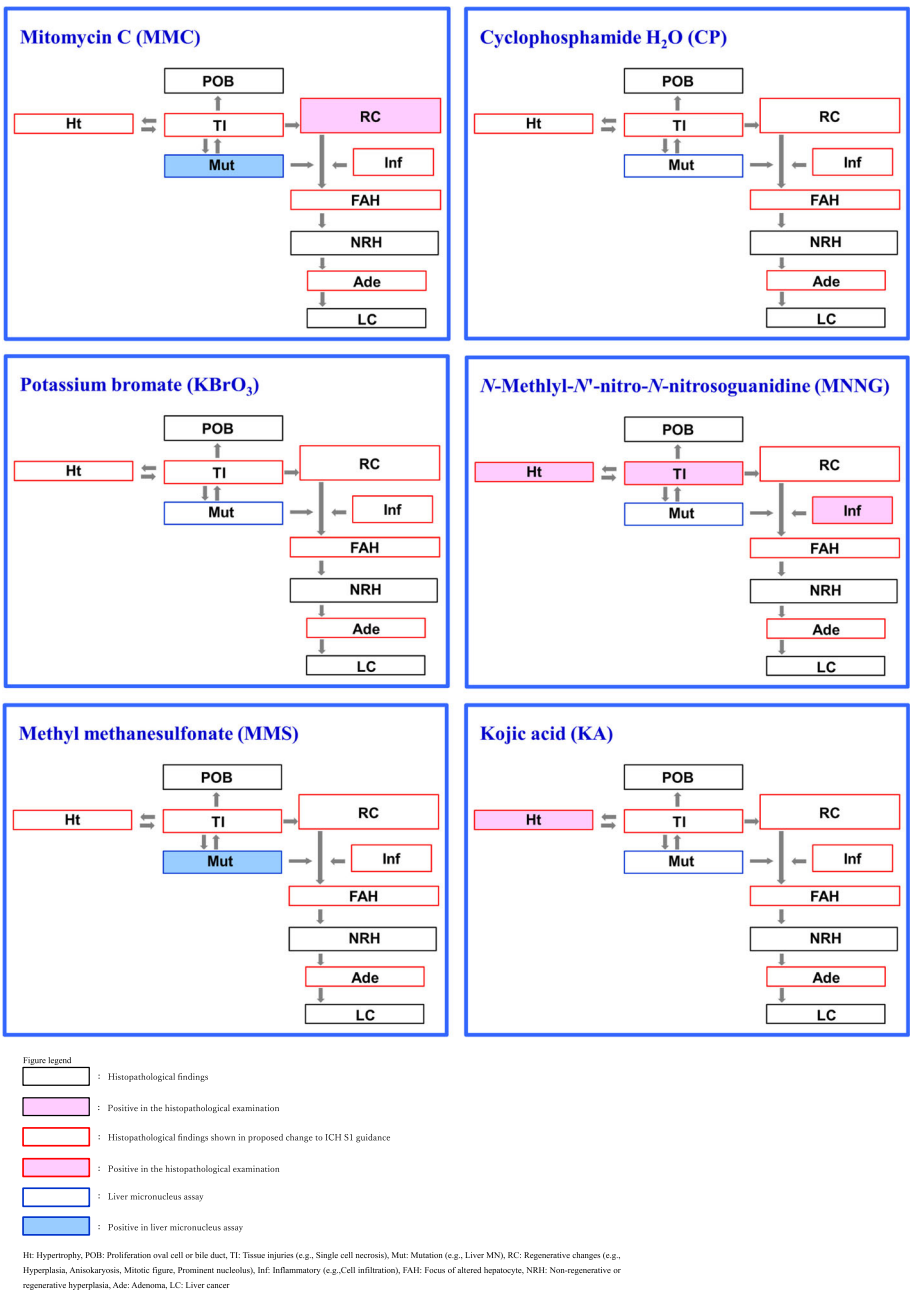

Fig. 3 Liver micronucleus induction and histopathological changes observed in 14-day and/or 28-day repeated-dose studies – genotoxic carcinogens but not liver targeted

Group B chemicals did not cause proliferation of oval cells or bile duct epithelial cells, focus of altered hepatocytes, non-regenerative or regenerative hyperplasia, adenoma, or liver cancer. MNNG showed 3 responses, but 3 chemicals (MMC, MMS, and KA) showed only one or two responses. CP and $KBrO_3$ did not show any targeted responses.

Discussion

Few Group A chemicals caused the focus of altered hepatocytes (21% [3/14]) or adenoma (7% [1/14]). No chemical Group A or B exhibited non-regenerative or regenerative hyperplasia or liver cancer. The latter parameters are recognized as the most credible indicators of hepatocarcinogenesis [46–48]. The present study was a retrospective survey of short-term study with 14- or 28-day repeated dose design, and such findings are not expected. Thus, we selected 6 responses that expected to occur very early in the process of carcinogenesis, including hypertrophy, proliferation of oval cells or bile duct epithelial cells, tissue injuries, mutation (including liver

micronucleus induction), regenerative changes, and inflammatory changes (Table 2).

All 14 Group A chemicals were positive for liver micronucleus assay; only 2 of 6 Group B chemicals induced micronuclei. These 2 chemicals, namely, MMC and MMS, are carcinogens but are not liver-targeted. Both are direct-acting genotoxic chemicals that are used as positive controls in genotoxicity tests and induce micronuclei in various tissues, including the liver [2]. Therefore, liver micronucleus induction was considered to be a useful indicator for possible hepatocarcinogenesis. Speculatively, the chromothripsis could involve fragmentation and subsequent reassembly of a single chromatid from a micronucleus [49, 50]. Chromothripsis is a new concept for mutational process; it involves genome reorganization associated with micronuclei. This process might elucidate the mechanisms for the production of micronuclei and genome instability and cellular evolution essential in complex diseases such as cancer [50].

In addition to liver micronucleus induction, many Group A chemicals exhibited two or more of the other five responses assumed to be early predictors of carcinogenesis. Contrarily, in Group B chemicals, liver micronucleus induction was not observed in 4 out of 6 chemicals. These chemicals demonstrated varying responses, including hypertrophy (50% [2/4]), proliferation oval cells or bile duct epithelial cells (0% [0/4]), tissue injuries (25% [1/4]), regenerative changes (0% [0/4]), and inflammatory changes (25% [1/4]). Further, hypertrophy, proliferation oval cells or bile duct, tissue injuries, and inflammatory were not observed in the two chemicals that were positive for liver micronucleus induction. Only regenerative changes were observed for one of these chemicals. Thus, even if a chemical is found to be positive for liver micronucleus induction, negative results for all other pathological findings indicative of early stages of carcinogenesis suggest a low probability of cancer development in the liver.

Much debate has occurred over the issue of whether hypertrophy is a key early response in hepatotoxicity or hepatocarcinogenicity in rodent toxicity studies [51–54].

We suggest that hypertrophy in the liver without micronucleus induction does not predict future hepatocarcinogenesis. Hypertrophy with micronucleus induction is, however, closely related to hepatocarcinogenesis. Clofibrate is a typical non-genotoxic hepatocarcinogen that

Table 2 Histopathological changes and induction of liver micronuclei seen as very early responses of hepatocarcinogenesis

Group	Chemical	Mut	Ht	Pob	TI	RC	Inf
Group A	DMN	■			■	■	■
	NPYR	■	■	■	■	■	■
	MDA	■	■	■	■	■	■
	NDPA	■	■	■	■	■	■
	2,4-DNT	■	■	■		■	
	2,6-DNT	■	■	■	■	■	■
	QUN	■	■		■	■	
	DAB	■	■		■		
	2-NP	■	■			■	
	MCT	■	■		■		
	NMOR	■	■	■	■	■	■
	2-AAF	■	■	■			■
	Sudan I	■	■		■		
	TAA	■	■		■	■	
Group B	MMC	■				■	
	CP						
	$KBrO_3$						
	MNNG		■		■		■
	MMS	■					
	KA		■				

Group A: Genotoxic hepatocarcinogens, Group B: Genotoxic carcinogens but not liver targeted
Mut: Liver MN induction, Ht: Hypertrophy, Pob: Proliferation oval cell or bile duct, TI: Tissue injuries, RC: Regenerative change, Inf: Inflammatory

induces hepatocyte hypertrophy and liver micronuclei [2].

A recently developed formalin fixation method for the liver micronucleus assay [21, 55] enables retrospective evaluation using formalin-fixed liver samples from general toxicity and carcinogenicity studies completed in the past. With this method, the prediction of hepatocarcinogenicity of a test substance with accuracy is possible using data from 2- and 4-week repeated-dose toxicity studies, including previously published work.

Conclusion

Liver micronucleus induction can be employed to predict hepatocarcinogenesis. The combination of this assay with histopathological findings observed in the early stages of the carcinogenic process (hypertrophy, proliferation of oval cells or bile duct epithelial cells, tissue injuries, regenerative changes, and inflammatory changes) can increase the accuracy of the prediction even in a short-term repeated dose study of 2 or 4 weeks.

Abbreviations

DMN: Dimethylnitrosamine; NPYR: *N*-Nitrosopyrrolidine; MDA: 4,4′-Methylenedianiline; NDPA: *N*-Nitrosodipropylamine; 2,4-DNT: 2,4-Dinitrotoluene; 2,6-DNT: 2,6-Dinitrotoluene; QUN: Quinoline; DAB: *p*-Dimethylaminoazobenzene; 2-NP: 2-Nitropropane; MCT: Monocrotaline; NMOR: *N*-Nitrosomorpholine; 2-AAF: 2-Acetylaminofluorene; Sudan I: C.I.solvent yellow 14; TAA: Thioacetamide; MMC: Mitomycin C; CP: Cyclophosphamide H_2O; $KBrO_3$: Potassium bromate; MNNG: *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine; MMS: Methyl methanesulfonate; KA: Kojic acid; ICH: The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IARC: International Agency for Research on Cancer; JEMS: The Japanese Environmental Mutagen Society; MMS: Mammalian Mutagenicity Study Group; CSGMT: The Collaborative Study Group for the Micronucleus Test

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Authors' contributions

SH, MS, and WO performed liver micronucleus assay of compounds and statistical analysis of the results obtained in the assay. YW and KK performed histopathological examination. SH, WO, TM1, and MH performed comprehensive evaluation of all laboratory data. SH, KS, TM2 and TF created table, fig, and manuscript. All authors have read and approved the final manuscript. TM1: Takeshi Morita, TM2: Tatsuya Mitsumoto.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval

The animal experiments were approved by the Institutional Animal Care and Use Committee of each testing facility prior to conducting the experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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