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CB6F1-Tg rasH2 Mouse Carrying Human Prototype c-Ha-ras Gene As an Alternative Model For Carcinogenicity Testing For Pharmaceuticals

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ABSTRACT *: The international pharmaceutical and regulatory communities had been recognizing the limited utility of conventional rodent carcinogenicity study particularly on the second species, mouse, after intense investigation of carcinogenicity data base worldwide, and a new scheme for carcinogenicity testing for pharmaceuticals was proposed at the Expert Working Group on Safety in the International Conference on Harmonization (ICH) in 1996. CB6F1-Tg rasH2 mouse carrying human prototype c-Ha-ras gene with its own promoter/enhancer is one of the new carcinogenicity assay model for human cancer risk assessment. Studies have been conducted since 1992 to validate the transgenic (Tg) mice for rapid carcinogenicity testing, short term (26 weeks) studies with genotoxic (by Salmonella), non-genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic non-carcinogens revealed relatively high concordance of the response of the Tg mouse with classical bioassay across classes of carcinogenic agents. Mechanistic basis for carcinogensis in the model are being elucidated in terms of the role of overexpression and/or point mutation of the transgene. This report review the initial studies of validation of the model and preliminary results of ongoing ILSI HESI ACT project will be presented.*

Key Words *: rasH2 mouse, CB6F1-TgrasH2 mouse, Human c-Ha ras proto-oncogene, Short-term carcinogenicity test, Transgenic mouse, Rodent carcinogen, Chemical carcinogenesis*

I. INTRODUCTION

The regulatory requirements for the assessment of carcinogenic potential of pharmaceuticals stipulate chronic (usually 2 year) studies in two rodent species, usually the rat and the mouse for the past 25 years. These rodent bioassays are amongst the most costly elements in preclinical testing in terms of time and resources. Over 1000 animals are required per drug studied and the uncertainty of the outcome for perhaps 3 to 4 years, coupled with increasing doubts over the relevance of the findings for human safety, make these bioassays a severe hurdle in drug development.

The wide spread recognition of the deficiencies and partial redundancy of these bioassays led the ICH Expert Working Group (EWG) to recommend a broader approach to assessing the carcinogenic potential of pharmaceuticals after intensive discussion.

One of the most important changes in the approach offered was the ability to use alternative in vivo models for carcinogenicity assessment as an option to conducting a second 2-year bioassay.

As additional tests for carcinogenic activity in vivo, initiation-promotion models in rodents, transgenic mouse models such as p53 deficient, rasH2, TG.AC and XPA deficient, mouse neonatal model had been proposed. Preliminary evaluation studies of the usefulness of transgenic mice carrying human prototype c-Ha-ras gene (CB6F1-Tg rasH2 mice) as an alternative model for detecting carcinogenic potentials of pharmaceuticals were originally started in Japan in 1992 in collaboration with the National Institute of Health Science (NIHS), the Central Institute for Experimental Animals (CIEA) and several Japanese pharmaceutical companies. US-Japan Joint studies started to conduct validation of the transgenic mice as an animal model for short term carcinogenicity testing in collaboration with the U.S. National Institute of Environmental Health Sciences from August 1995. At the same time, CIEA started a Rapid Carcinogenicty Testing System (RCTS) project in order to test broad

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number of chemicals for the validation of the CB6F1-Tg rasH2 mouse testing system.

In April 1996, in accordance with a request from the Ministry of Health and Welfare(MHW) of Japan, a collaborative study group on the use of transgenic mice as an alternative for carcinogenicity testing was established to step up the S1B draft guideline of the ICH. The reason why the MHW decided to select CB6F1-TgrasH2 mice (RasH2 mice) were as follows: (1) Sufficient genetical control was possible with this transgenic mouse (Saitoh *et al.*, 1990); (2) Sufficient historical control data had already been accumulated; (3) Large number of rasH2 mice could be obtained at one time from the Central Institute for Experimental Animals; (4) This transgenic mouse was considered to be useful for the elucidation of carcinogenic mechanism in man, since the human c-Ha-ras gene was integrated into its genome; (5) Validation studies using around 40 chemicals were being or to be performed in Japan (Mitsumori, 1997).

In 1996, the International Life Science Institute, US formed a committee, the Alternatives to Carcinogenicity Testing Committee (ACT), the objective of the ACT project are to contribute to the development of a sound scientific basis for understanding the value of these assays by expanding the database on these alternative methods, establishing a common experimental approach.

The ILSI/HESI ACT project moved quickly to organize an international collaborative effort with the principal focus on coordination of testing of well-characterized, non-proprietary pharmaceutical compounds in 4 transgenic models (RasH2, p53+/-, Tg.AC and XPA-/-) and in the neonatal mouse model. Standard protocols were developed which included 4-week dose ranging finding studies with toxicokinetics to set dose levels for 26-week (transgenic) 0r 52-week (neonatal mouse) carcinogenicity tests. A total of 21 compounds for which there are reliable 2-year rodent bioassay data and human data are being evaluated. These compounds include known and suspected human carcinogens, rodent carcinogens considered to be non-carcinogenic in humans, and noncarcinogenic agents.

Validation of CB6F1-Tg rasH2 mouse has been conducted to investigate the carcinogenic response of this mouse to more than 50 various carcinogens and non-carcinogens. ILSI international collaborative studies with 21 compounds are on the final stage leaving review of the final draft reports. In this paper, principal study findings of rasH2 mouse are reviewed and preliminary results of ILSI HESI ACT project studies will be presented.

II. MATERIAL AND METHODS

1. Profile of Tg rasH2 mouse

The rasH2 mouse is a hemizygous transgenic mouse carrying the human prototype c-Ha ras gene with its own promoter/enhancer and do not have an activated transgene. Five or six copies of the c-Ha ras gene on a tandem array are integrated into the genome of each transgenic mouse. These mice were originally established by Katsuki *et al.* at the Central Institute for Experimental Animals. No point mutation in the transgenic c-Ha ras gene is found in normal (non-neoplastic) tissue of the transgenic mouse (Saitoh *et al.*, 1990). Expression of transgene has been confirmed by Northern blotting in al tissues of the transgenic mouse (Unpublished data). The ras-encoded p21 protein is shown at 2-3 times normal level in tissues (Unpublished data).

The inbred C57BL/6J Jic and its congenic C57BL/6J-Tg-rasH2 Jic strains, and the inbred BALB/cByJ are maintained for the production of rasH2 mouse (CB6F1Tg-rasH2). Breeding colonies of mice were classified as foundation, expansion and production colonies, and mice were propagated stepwise. The transgenic foundation colony has been maintained by backcrossing to C57BL/6J up to N 23 at present. Mice are subjected to genetic monitoring to confirm the maintenance of the background gene profile in each generation by the ICLAS Monitoring Center located at the Central Institute for Experimental Animals (CIEA). The foundation colonies of each mouse have shown no sign of genetic divergence or alteration to date. The updated results were obtained o April 3, 2000 for C57BL/6J-TgrasH2 Jic with N23.

Final production of CB6F1Tg-rasH2 mouse is achieved by mating hemizygous transgenic C57BL/6J male and non-transgenic BALB/cByJ female because embryos homozygous for the transgene are lethal. The individual genotype is determined by PCR using tail chips of offspring at 5 weeks of age before shipment. Non-

transgenic littermates serve as controls.

To examine genetic stability of transgenes, Southern blot, Northern blot, and FISH (fluorescent in situ hybridization) analysis were performed using the insert 6.8 kb BamH1 fragment) of pSV2Mbras-gpt plasmid constructed to create the original rasH2 mouse as the hybridization probe (Sekiya *et al.*, 1985). Nucleotide sequencing covering exons I and II of the c-Ha-ras gene was also performed to exclude possible mutation in codons 12 and 61 of the transgene. The latest results were obtained in October 1999 for the N 20 generation of rasH2 mice.

Fish analysis showed that the integrated c-Ha-ras gene was stably located in chromosome 15 at generation N 20. Southern blot analysis digested with BamH1 And with several other enzymes expected to cut transgenes at a single site showed a uniform 6.8 kb fragment that is the same size as the introduced human c-Ha-ras gene.

In addition to Southern blotting, the results of inverse PCR analysis confirmed that the transgene was constitutively integrated into the genome of mice in a tandem array.

No mutation was observed in either genomic or cDNA of transgenes of mice not treated with carcinogens. Northern blot analysis showed that the transgene was expressed in all tissues of the mice.

In carcinogenicity testing using rasH2 mice, N-methyl-N-nitrosourea (MNU) at a dose of 75 mg/kg (single intraperitoneal injection at the beginning of the study, at 7 weeks of age) has been used as a standard positive compound. MNU has been used in all ILSI HESI ACT studies of rasH2 mice as a concurrent positive control compound.

The tumor incidences and the type of the target tissues have remained unchanged since establishment of this model in 1992 (Ando *et al.*, 1992), as well as there has been no interlaboratory discrepancy in ILSI HESI ACT studies.

The enzyme activity of cytochrome P450 (CYP or Cyp) isoforms and related enzymes among the gene-manipulated animals are extremely important to determine carcinogenicity because of its ability to activate pro-carcinogens to highly reactive intermediates, the ultimate carcinogens. The content and enzyme activity of cytochrome P450 (CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A),Cytochrome b5, NADPH-cytochrome P450 reductase and Major Phase I and Phase II enzymes have been shown within normal and no discrepancy between wild type and rasH2 transgenic mouse(Kamataki, 2000).

2. Results of initial carcinogenicity Testing using rasH2 mice

Eighteen Salmonella mutagenesis assay-positive trans-species carcinogens, seven Salmonella mutagenesis assay-negative trans-species carcinogens, two Salmonella mutagenesis assay-positive single-species (mouse only) carcinogens, one Salmonella mutagenesis assay-negative single species (mouse only) carcinogen, four Salmonella mutagenesis assay-positive non-carcinogens and four Salmonella mutagenesis assay-negative noncarcinogens were subjected to the rapid carcinogenicity testing. (Yamamoto *et al.*, 1998), 13/17 mutagenic (Salmonella positive) agents produced statistically significant increases in the incidence of at least one tumor type relative to the transgenic control group. And, for 11 of these, the response in the transgenic mouse was significantly greater than in the similarly-treated non-transgenic mice.

Benzene, a genotoxic (clastogenic human carcinogen, was also positive producing a significant increase in lung adenoma and forestomach papillomas.

The human carcinogen cyclosporin, an immunosuppressive agent, and 1,4 dioxane, a non-genotoxic rodent carcinogen did not produce statistically significant effects relative to the vehicle control. 1,1,2-Trichloroethane, a non-mutagenic liver tumorigen in mice, was also negative. A non-genotoxic thyroid tumorigen, which elevates TSH levels, produced thyroid tumors in both transgenic and non-transgenic mice with no significant differences in incidence. The non-concordance of the Tg rasH2 model and conventional rodent results is seemed to be limited to nonmutagenic rodent carcinogens.

Whereas 3 mutagenic non-carcinogens and 2 non-mutagenic carcinogens were negative in rasH2 model indicating that this model do not induce false positive response and the model continue to look promising as a short ñterm alternative test for carcinogenicity.

Additional data from studies performed as part of the ILSI ACT project will provide important additional data on the response of rasH2 transgenic mice

to non-genotoxic rodent carcinogens with a variety of mechanisms.

3. Preliminary results of ILSI HESI ACT project

The selection process for compounds to be studied in the HESI ACT program targeted compounds for which there has been extensive human exposure and experience and which have also been well characterized in pre-clinical in vivo and in vitro studies. Compounds selected were generally non-proprietary, available from several sources, and represented a broad array of mechanisms. In the category of compounds with evidence of tumorigenic activity in humans, 3 genotoxic carcinogens (phenacetin, cyclophosphamide, and melphalan), 2 hormones (diethylstilbestrol, and estradiol) and an immunosuppressive drug (cyclosporin A) were selected for testing. A second category of non-genotoxic rodent carcinogens which are putatively non-carcinogenic in human includes 12 compounds for which there is either epidemiologic or mechanistic data which indicate that the compounds are not tumorigenic in human. The 12 compounds in this category include an enzyme inducer (Phenobarbital), peroxisome proliferaters (Clofibrate, diethylhexylphthalate, and WY-14643), thyroid tumorigen (sulfamethoxazole), agents that can induce increases in serum prolactin levels and stimulate mammary tumorigenesis (reserpine, haloperidol, chlorpromazine), a cytotoxic rodent kidney tumorigen (chloroform), a bete adrenergic agonist from a class of compounds that induce leiomyomas (metaproterenol), an antihistamine that induces rodent liver tumors (methapyrilene), and a pesticide which also induces rodent liver tumors (dieldrin). A large number of compounds were selected for testing in this category since the majority of new pharmaceutical compounds brought forward for development and testing are non-genotoxic. Finally 3 non-genotoxic, non-carcinogens (ampicillin, mannitol and sulfisoxazole) were also selected for testing.

Those studies with rasH2 model are now almost completed, except one study of WY-14643 due to delay of start. All other studies are now at the final stage for auditing the final draft reports. For most compounds, dose range finding studies are conducted as well as concurrent positive control, MNU, groups are included. Laboratory who participated the studies are as followings in random order; Teikoku Hormone, Novo Nordisk, Kyowa Hakko, Novartis, Mitsubishi-Tokyo, Janssen, Monsanto/Searle, Aventis, Tanabe, Nipponshinyaku, Eisai, Glaxo Welcome, Daiichi, Wyeth Lederle, Yamanouchi, Sankyo, Takeda, Dainippon, Fujisawa, Shionogi and CIEA. Detail of the studies will be published by each sponsor in near future.

Cyclophosphamide induced statistically significant transitional cell carcinoma in the bladder and Harderian gland adenoma and adenocarcinoma. Melphalan showed equivocal results, Phenacetin induced spleen hemangiosarcoma, forestomach papilloma and lung adenoma statistically significantly. Cccyclosporin A was negative, Diethylstilbestrol induced testicular Leidig cell tumor I the Tg male mice. Estradiol was negative. Phenobarbital, rodent hepatocarcinogen, did not induce any tumor in the Tg rasH2 mice. Clofibrate induced hepatcelluler adenoma in male Tg mice without clear dose responding manner, reserpine was negative, dieldrin was also negative, methapyrilene did not induce tumors, chloroform was negative, metaproterenol induced hepatocelluar adenoma in male high dose group but no statistical significance due to one adenoma bearing mouse in the vehicle control group, DEHP induced hepatocellular adenoma in male Tg mice with dose responding manner, chlorpromazine was negative, sulfamethoxazole was negative. 3 non-carcinogens (sulfisoxazole, Mannitol, ampicillin) were all negative.

III. CONCLUSION

RasH2 model was clearly shown that the response of the rasH2 to carcinogens, particularly to genotoxic human carcinogens have yielded promising results for carcinogenic hazard identification for human risk assessment.

The testing of non-genotoxic rodent carcinogens, putative human non-carcinogens suggests that the rasH2 model does not readily produce false positive results for human carcinogenic risk assessment.

Peroxisome proliferator (Clofibrate, DEHP, possibly WY-14643), rodent carcinogens, induced receptor (peroxisome proliferator activated receptor alpha) mediated hepatocellular tumor.

The testing of non-carcinogens suggests that the rasH2 model does not produce false positive results.

For compounds with diverse mechanisms, such as immunossupressant, synthetic estrogens, modified protocol (in utero exposure, extending the duration of the study) might be considered.

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