別記様式第３号

学　位　論　文　要　旨

氏　　名　　NAKAMURA, Kenji

題　　目　　Studies on the Modes of Action Underlying Acetamide-induced Hepatocarcinogenesis in Rats
（ Acetamide のラット肝発がんメカニズムに関する研究 ）

Acetamide, which was used as a food flavoring agent, has been classified as a Group 2B compound, i.e., a possible human carcinogen, by the International Agency for Research on Cancer based on evidence of its hepatocarcinogenicity in rats. Therefore, the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Addtitives recommended that acetamide should not be used as a food additive. Recent studies have shown that acetamide is naturally found in some foods and products, such as cigarettes, milk and beef. However, many *in vitro* and *in vivo* genotoxicity studies of acetamide have shown negative results. That is, the mechanisms of acetamde-induced hepatocarcinogenesis remain unelucidated. Thus, understanding the mode of action underlying acetamide-induced hepatocarcinogenesis in rats is necessary for the risk assessment of acetamide in humans. In the current study, I evaluated *in vivo* mutagenicity and inducibility of chromosome aberrations of acetamide, and investigated the involvements of them in the hepatocarcinogenesis in rats.

In Chapter 1, I evaluated *in vivo* mutagenicity of acetamide in rat liver, its carcinogenic target organ, using reporter gene transgenic *gpt* delta rats. After 13-week administration of acetamide at the concentrations of 0%, 0.625%, 1.25%, or 2.5% for male F344 *gpt* delta rats (6-week-old, 10 animals per group), I conducted histopathological examination of the liver, immunohistochemical analysis using a preneoplastic marker anti-GST-P antibody, and *gpt* and Spi$^-$ assays. As a result, histopathological examination found that acetamide induced many changes in the liver such as single cell necrosis, karyomegaly, and vacuolation of hepatocytes at 1.25% and higher. In addition, basophilic cytoplasmic inclusion, which was positive for Feulgen staining, was frequently observed at 1.25% and higher. The immunohistochemical analysis showed that the number and area of GST-P-positive foci were significantly increased at the carcinogenic doses of 1.25% and higher. On the other hand, acetamide did not induce significant changes in *gpt* or Spi$^-$ mutant frequencies in the liver at any doses. Therefore, it was revealed that acetamide did not show any mutagenicity in rat liver even at the carcinogenic doses. In contrast, acetamide induced high-frequently the cytoplasmic inclusion derived from nuclear components, assuming that acetamide might induce chromosome aberrations in the rat liver.

Next, in Chapter 2, I evaluated the inducibility of chromosome aberrations and DNA damages of acetamide using *in vivo* micronucleus assay and liver comet assay in rats. After 4-week administration of acetamide at the same concentrations in Chapter 1 for male F344 rats (6-week-old, 5 animals per group), I conducted micronucleus assay in liver and bone marrow. Also, after 3-day oral administration of acetamide by gavage at 0, 200, 600, or 2000 mg/kg for male F344 rats (6-week-old, 5 animals per group), I conducted liver comet assay. As a result, whereas acetamide showed negative result in the bone marrow micronucleus assay, micronucleated hepatocytes were significant increased at 1.25% and higher of acetamide in the liver. In addition, large micronucleated hepatocytes were significant increased at 0.625% and higher, and they corresponded to the cytoplasmic inclusion morphologically. On the other hand, in the liver comet assay, acetamide did not induce DNA damages at any doses. Taken together, I revealed that acetamide induced chromosome aberrations specifically in rat liver without DNA damages. Thus, it was suggested that chromosome aberrations induced by acetamide may involve in hepatocarcinogenesis. Moreover, the large micronuclei were found to be detected histopathologically as the cytoplasmic inclusion.

In Chapter 3, focusing on the histopathologically-detectable large micronucleus, I investigated the relationship between chromosome aberrations and hepatocarcinogenesis induced by acetamide. For the livers obtained from the animals administered with acetamide at 2000 mg/kg for 3 days (day 3) or the animals administered with acetamide at 1.25% for 4 weeks (week 4) in Chapter 2, I conducted immunofluorescent analyses of nuclear envelope-associated proteins such as lamin B1, lamin A/C, emerin, and BAF (Barrier-to-autointegration factor), chromatin markers such as H3K9me3 and H4K8ac, and DNA damage markers such as γ-H2AX and 53BP1, as well as electron microscopic examination. I found that the large micronucleus showed lack of lamin B1 and lamin A/C, and overexpressions of emerin and BAF, as well as strong fluorescence intensity of a DNA-specific probe DAPI with the regions showing abnormal nuclear envelope morphology. The large micronucleus also showed high-electron density nuclear structures demonstrated by electron microscopy, which corresponded to the overexpression of H3K9me3 and lack of H4K8ac expression. In addition, the double immunofluorescent staining of γ-H2AX and 53BP1 showed that the large micronucleus co-expressed them. These molecular pathological features in the large micronucleus were progressed from day 3 to week 4. Overall, the large micronucleus induced by acetamide showed abnormal expressions of nuclear envelope proteins, increased heterochromatinization, and massive DNA damage, and these changes were progressed with time-dependency on duration of acetamide-treatment. Since the large micronucleus showing such morphological features seemed to be related to following chromoanagenesis, a clustered chromosome rearrangements, it was implied that the chromosome rearrangements through micronucleus formation might involve in acetamide-induced hepatocarcinogenesis

In Chapter 4, I investigated the involvement of chromosome rearrangements in hepatocarcinogenesis using mutational analyses for acetamide-induced hepatic tumors in rats. After 26-30-week administration of acetamide at 2.5% for male F344 rats (6-week-old, 25 animals), I conducted necropsy and histopathological examination of liver. After DNA

extraction from hepatic tumors, I conducted whole-genome sequencing analyses using Illumina Novaseq 6000 and analyzed gene mutations such as copy number variants (CNVs) and structural variants (SVs). For comparison, hepatic tumors in DEN and phenobarbital (PB)-treated two-stage hepatocarcinogenesis model rats were also applied for the whole-genome analyses. In the necropsy and histopathological examination, hepatic tumors were observed in 22/25 animals, and these tumors were hepatocellular adenoma or carcinoma. The average of multiplicity of tumors was 3.7 per animal, and the multiplicity of hepatocellular adenoma was significantly higher than that of hepatocellular carcinoma. The whole-genome analyses of acetamide-induced and DEN/PB-induced hepatic tumors revealed that wide regions of CNVs were observed in various and different chromosomes in the acetamide-induced tumors, whereas these were almost lacking in the DEN/PB-induced hepatic tumors. In addition, the patterns of CNVs were quite different among tumors in the acetamide-treated group. With regard to SVs, numbers of insertion and translocation in the acetamide-induced tumors were higher than those in the DEN/PB-induced tumors. Overall data strongly suggested the occurrence of chromoanagenesis-like chromosome rearrangements in acetamide-induced hepatic tumors. Therefore, chromosome aberrations induced by acetamide seemed to contribute to tumorigenesis via chromosome rearrangements.

In the current study, I revealed that acetamide induced chromosome aberrations specifically in rat liver, and chromosome rearrangements-like CNVs through micronucleus formation induced by chromosome aberrations involved in acetamide-induced hepatocarcinogenesis. This study would be very important in clarifying the involvement of chromosome aberrations in the process of *in vivo* chemical carcinogenesis, in addition to providing the data that contribute to the risk assessment of a food contaminant acetamide.

（備考）１　学位論文題目が日本語の場合は英訳を、英語の場合は和訳を（　　）内に記入すること。（英語の表記は，論文目録と同じ。）

２　日本語の場合は、２，０００字程度とする。

３　英語の場合は、１，２００語程度とする。

４　図表は、この要旨には記載しないこと。

５　枚数は１枚を超えても差し支えありません。