主論文の要旨

Fenton reaction-induced renal carcinogenesis in *Mutyh*-deficient mice exhibits less chromosomal aberrations than the rat model

（*Mutyh* 欠損マウスにおけるフェントン反応誘発腎がんは同ラットモデルほど多数の染色体異常を呈さない）

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Introduction

Oxidative stress is caused by a variety of chemical, physical and biological agents and is associated with carcinogenesis. Iron excess may cause Fenton reaction-induced oxidative stress, and this is recognized as one of the causes of carcinogenesis based on human epidemiological data. The level of 8-oxoguanine, a major oxidatively modified base in DNA, is maintained very low by three distinct enzymes, encoded by *OGG1*, *MUTYH* and *MTH1*. Germline biallelic inactivation of MUTYH represents a familial cancer syndrome called MUTYH-associated polyposis.

We have used an iron-mediated renal carcinogenesis model. Ferric nitrilotriacetate (Fe-NTA) is an iron chelate that induces oxidative renal tubular damage via Fenton-like reaction. Repetitive intraperitoneal Fe-NTA administration raises renal cell carcinoma in mice or rats. In this study, we applied the carcinogenesis model to MUTYH deficient mice.

Methods

Animal experiments

There were four groups of 20 male mice at the start of carcinogenesis study: *Mutyh* +/+ and Fe-NTA treatment; *Mutyh* -/- and Fe-NTA treatment; *Mutyh* +/+ with no treatment, and *Mutyh* -/- with no treatment. We used 8 to 9-week-old male mice that each weighed 22-24 g. Fe-NTA was injected intraperitoneally (ip) at a dose of 3 mg iron/kg five times a week for the first week, which was thereafter increased to 5 mg iron/kg 5 times a week during the next 11 weeks. The mice were euthanized when they appeared ill or showed >5% weight reduction in a week. Finally, at ~120 weeks, the final effective numbers of mice in each group were 14, 15, 16 and 19, respectively.

For the subacute experiments, 11 male *Mutyh* +/+ and 12 male *Mutyh* -/- mice were used. The mice were euthanized 48 h after a week of Fe-NTA administration.

Array-based CGH (aCGH) analysis

The aCGH analysis was performed with a Mouse Genome CGH Microarray 4x180K (Agilent Technologies). Genomic DNA from a normal wild-type mouse kidney was labeled with Cy3 as the reference. Selected tumor samples, including 4 RCCs and 8 lymphomas, were labeled with Cy5.

We also used the aCGH data from Fe-NTA-induced rat RCCs we previously published (GEO accession: GSE36101) to compare with the mouse data in the present study.

Methylation analysis

The Genomic DNA (1 μg) was modified with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen), and after which, target segments in the genome were amplified with specific PCR primer pairs. Pyrosequencing was then performed using a PSQ96 system (Qiagen).

Mutational analysis of *Cdkn2a* gene

All the 4 exons in *Cdkn2a* gene were amplified by PCR from the genomic DNA of 4 RCCs. Direct DNA sequencing was performed, using Applied Biosystems 3100 Genetic Analyzer.

Results

Subacute study on Fe-NTA-induced renal carcinogenesis exhibits more oxidative stress with increased proliferation in the proximal tubular cells of *Mutyh*-deficient mice (Fig. 1). Although the *C57BL/6* background is cancer-resistant, a repeated intraperitoneal administration of Fe-NTA induced a high incidence of renal cell carcinoma (RCC; 26.7%) in *Mutyh*-deficient mice in comparison to wild-type mice (7.1%) (Table 1). Fe-NTA treatment also induced renal malignant lymphoma, which did not occur without the Fe-NTA treatment in both the genotypes (Fig. 3a). Renal tumor-free survival after Fe-NTA treatment was marginally different ($P = 0.157$) between the two genotypes (Fig. 2a). Array-based comparative genome hybridization analyses revealed, in RCC, the loss of heterozygosity in chromosomes 4 and 12 without $p16^{INK4A}$ inactivation (Fig. 2i); these results were confirmed by a methylation and mutation analyses and showed no significant difference between the genotypes. Lymphomas showed a preference for genomic amplifications (Fig. 3h and i). *Dlk1* inactivation by promoter methylation may be involved in carcinogenesis in both tumors (Table 2). Fe-NTA-induced murine RCCs revealed significantly less genomic aberrations than those in rats, demonstrating a marked species difference (Table 3).

Discussion

We obtained a higher incidence of Fe-NTA-induced RCC in the *Mutyh*-deficient mice in comparison to the wild-type counterparts in a carcinogenesis study of > 2 y. This confirms a role of MUTYH in preventing oxidative stress-induced carcinogenesis, despite the relatively small study. The data in the subacute phase was consistent with the final carcinogenesis results in that 8-oxoG and the mitotic index by Ki67 were significantly higher in the Mutyh-deficient mice.

We also performed aCGH analyses on Fe-NTA-induced RCC, for the first time,

in mice. There was no major distinction in the aCGH results of RCC between the two genotypes, and basically, deletions were observed with a common region of hemiallelic loss in chromosomes 4 and 12 but with no common amplifications. Surprisingly, we found no homozygous deletion of $p16^{INK4A}/p15^{INK4B}$, which we previously hypothesized to be specific for iron-induced carcinogenesis, based on the data of rats and humans. Furthermore, the remaining allele of $p16^{INK4A}/p19^{ARF}$ was not inactivated in the RCCs with additional epigenetic and mutation analyses. In Fe-NTA-induced RCCs in rats, we observed numerous genomic locations of amplifications, and among which, a *c-Met* amplification was the most common. These results, using the same model in different species, suggest that murine carcinogenesis is different from those in rats and is much farther from those in humans.

In addition, we compared the frequency of the chromosomal aberrations in Fe-NTA-induced RCC between mice and rats. The chromosomal aberrations in mice were significantly less than those in rat RCC. This may suggest that mice obtain cancer in fewer steps than rats. In this sense, the rat model may be more similar to human counterparts that occur sporadically. The mice model appears to be more similar to those of familial cancer syndromes or childhood cancers. At the same time, we are warned by the results that murine carcinogenesis might be very different from those of humans. Carcinogenesis models with rats should be revalued in that regard.

We observed, for the first time, malignant lymphoma of the kidney after Fe-NTA treatment in both the genotypes but none in the mice without Fe-NTA treatment. A repeated Fenton reaction causes chronic inflammation in the renal cortex. Thus, the occurrence of malignant B-cell type lymphoma might be similar to human inflammation-associated lymphomas, such as *Helicobacter pylori*-associated gastric lymphoma or pyothorax-associated lymphoma. In contrast to murine RCC, lymphomas of both the genotypes revealed both amplifications and a hemiallelic loss.

Finally, we performed analyses of the methylation of the CpG islands in selected genes and found that *Dlk1* is a good candidate for a target tumor suppressor gene, which is suggested also in human RCC. Of note, the inactivation of *Dlk1* was observed not only in RCC but also in all the lymphomas examined.

Conclusion

We obtained a high incidence of murine RCC by repeated ip administration of Fe-NTA even in the cancer-resistant *C57BL/6* background when *Mutyh*-

deficient mice were used. However, the chromosomal aberrations in RCC were much less than those in rats. The results not only confirm the role of *Mutyh* in preventing Fenton reaction-induced carcinogenesis but also suggest that a murine carcinogenesis model might be more distant from human counterparts than we believed.