主論文の要旨

Ferroptosis resistance determines high susceptibility of murine A/J strain to iron-induced renal carcinogenesis

（A/J 系統マウスはフェロトーシス抵抗性により
鉄誘発発がんモデルにおいて高感受性を示す）

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【Introduction】

The trait of cancer susceptibility is important in human carcinogenesis. This study was aimed to investigate the susceptibility to renal carcinogenesis using different mouse strains. We previously observed a low incidence in the ferric nitrilotriacetate (Fe-NTA) induced renal carcinogenesis model with *C57BL/6J* strain. In this study, we chose *A/J* strain expecting a higher susceptibility. After confirming the high susceptibility in *A/J* strain, we explored the mechanism for the different susceptibilities to the carcinogenesis between *A/J* and *C57BL/6J*.

【Methods】

The mice were given repeated intraperitoneal administration with Fe-NTA for 3 months for carcinogenesis. We utilized array-based comparative genetic hybridization to examine the genetic alteration in the obtained renal cell carcinomas (RCCs). Three-week repeated Fe-NTA injection and single injection were performed for subacute and acute renal injury experiments. We investigated the difference in iron metabolism via western blotting, immunohistochemistry, real-time quantitative PCR.

【Results】

Incidence of Fe-NTA-induced RCC is significantly higher in A/J mice than in C57BL/6J mice

After repeated intraperitoneal Fe-NTA administration of 12 weeks, long-term survival rate in *A/J* mice was consistently lower than that in *C57BL/6J* mice (Figure 1A). A significantly higher incidence of RCC was observed in *A/J* mice (Figure 1B). Proliferation of atypical glandular cells with CD10-immunopositivity were observed presenting either papillary, irregularly tubular or solid structures with loss of normal renal tubular organization (Figure 1C, S1). Ki-67 immunostaining showed increased positivity in *A/J* RCCs (Figure 1D). We performed the array-based CGH analysis on Fe-NTA-induced *A/J* mice RCCs (GEO accession: GSE183173) (Figure 1E, S2). In this study, we firstly found *Cdkn2a/2b* homozygous loss in 1 case and hemizygous loss in 3 RCC cases of *A/J* mice. (Figure 1F).

Ferroptosis is repressed in A/J mice with decreased lipid peroxidation after subacute renal injury

Histologically, we observed significantly less damage in renal tubular cells of *A/J* mice than *C57BL/6J* mice 3 weeks after repeated administration of Fe-NTA (Figure 2A). We found that 4-HNE was highly abundant in *C57BL/6J* mice but not in *A/J* mice (Figure 2B). High Ki-67 positivity suggests simultaneous regenerative changes in *C57BL/6J* mice (Figure 2C). Persistently high expression of GPX4 in *A/J* mice was observed (Figure 3A). Immunoblot showed that *A/J* mice but not *C57BL/6J* mice can sustain high expression of

GPX4 after 3-week Fe-NTA treatment (Figure 3B). The high expression of xCT was observed in *A/J* mice after 3-week Fe-NTA treatment but not in *C57BL/6J* mice (Figure 3C).

Transferrin receptor is downregulated in A/J mice after subacute renal injury

TfR1 expression decreased in both the strains after 3-week Fe-NTA administration with significantly lower expression in *A/J* mice than in *C57BL/6J* mice (Figure 4A). This was confirmed both by mRNA measurement (Figure 4B) and immunohistochemistry (Figure 4C). We observed the higher retained expression of IRP1 and IRP2 in *A/J* mice than in *C57BL/6J* mice (Figure 4D, 4E). mRNA levels of megalin and cubilin were significantly downregulated in *C57BL/6J* mice in the 3-week group (Figure 4F, 4G). High expression of transferrin was observed in *C57BL/6J* mice in the 3-week group (Figure 4H).

Higher induction of ferritin in A/J mice after subacute renal injury

Both ferritin light chain (FTL) and heavy chain (FTH) were more significantly increased after subacute renal injury in *A/J* mice than in *C57BL/6J* mice (Figure 5A, 5B), which was consistent with immunohistochemical analysis (Figure 5C). Iron staining with Prussian blue was more prominent in *C57BL/6J* mice especially in the stroma area than in *A/J* mice (Figure 5D).

Ferroptosis is suppressed in A/J mice during acute renal injury

Abundant tubular necrosis with nuclear pyknosis as ferroptosis was observed in *C57BL/6J* mice (Figure 6A). TfR1 expression was more significantly decreased in *A/J* mice (Figure 6B). Catalytic Fe (II) is significantly higher in *C57BL/6J* mice than in *A/J* mice 6 h after Fe-NTA administration. (Figure 6C). Acute renal tubular injury was significantly reversed in *C57BL/6J Lcn2* (-/-) mice (Figure 7A), which was confirmed with 4-HNE and 8-OHdG immunostaining (Figure 7B, 7C). Immunohistochemical staining of LCN2 in proximal tubules in *C57BL/6J* mice was more prominent than in *A/J* mice (Figure 7D). mRNA levels of *Lcn2* in *C57BL/6J* were 50-fold higher than in *A/J* mice, indicating higher acute tubular injury (Figure 7E).

【Discussion】

Fe-NTA-induced renal carcinogenesis model is distinctive in that wild-type rats acquire somatic mutations frequently found in human cancers, such as *Cdkn2a/2b* homozygous deletion. Notably, the association of excess iron as carcinogenic milieu and *Cdkn2a/2b* homozygous deletion has been well known. This deletion-type mutation permanently provides cells with apoptosis inhibition and removal of cell-cycle brakes. In this study, we have established a murine strain difference of cancer susceptibility that the incidence of Fe-NTA-induced RCC is markedly higher in *A/J* mice than in *C57BL/6J* mice, which was associated with an additional mechanism, ferroptosis-resistance.

We hypothesized that cancer susceptibility may be associated with ferroptosis, a

catalytic Fe(II)-dependent regulated necrosis accompanying lipid peroxidation, in which a major product is 4-HNE. Our results indicate that ferroptosis-resistance by counteracting oxidative stress was inherently observed in *A/J* strain. TfR1 expression was decreased in the subacute renal injury, where the decrease was more significant in *A/J* strain. Contrary to TfR1, mRNA levels of cubilin and megalin were higher in *A/J* mice than in *C57BL/6J* mice. Megalin and cubilin are multiligand endocytic receptors which can absorb transferrin-iron by endocytosis in kidney proximal tubule epithelial cells.

A/J mice may have a more efficient mechanism to control iron import to prevent excess iron-mediated renal tubular damage. Increased ferritin can store insoluble Fe (III) by oxidizing catalytic Fe (II) to protect host from excess iron, thus preserving iron safely in ferritin. Further, Prussian blue staining, detecting mainly insoluble Fe (III) in lysosomes, including hemosiderin, is increased in *C57BL/6J* mice, indicating a higher level of iron overload in *C57BL/6J* mice than in *A/J* mice. Thus, high expression of ferritin under less iron overload is another ferroptosis-resistance mechanism in *A/J* mice. Acute study of 3-h provided us with similar results. More severe renal tubular damage and relatively high expression of TfR1 were observed in *C57BL/6J* mice in the 3-h group. Reportedly, LCN2 increases intracellular iron accumulation and promote the cellular level of ROS via mammalian siderophores. We observed a lower level of catalytic Fe(II) in *A/J* mice expressing lower levels of LCN2 and TfR1 in comparison to *C57BL/6J* mice. We confirmed that higher LCN2 expression correlated with oxidative stress. In *Lcn2* (-/-) mice, the damage of renal tubules and high lipid peroxidation were reversed, indicating that LCN2 deficiency can alleviate Fe-NTA induced-oxidative renal tubular injury.

In conclusion, ferroptosis-resistance is a key factor to determine susceptibility to iron-induced renal carcinogenesis. *A/J* mice after Fe-NTA exposure can maintain an appropriate level of oxidative stress to induce oxidative DNA damage without excessive ferroptosis at an early stage, eventually leading to a high incidence of RCCs (Figure 8). In this sense, *A/J* mice is a better model for genetic research of iron- induced renal carcinogenesis than *C57BL/6J* mice. Further studies are warranted on the application of the present murine data to human cancer prevention, where carcinogenic exposure is much longer but presumably lighter. We also have to consider the possibility that cancer-resistance may a trade-off effect on other pathologies, such as infection susceptibility.

【Conclusions】

Ferroptosis resistance may promote renal carcinogenesis in *A/J* mouse strain.

Ferroptosis resistance may be associated with *Cdkn2a/2b* deletions

A/J mice is a better model for iron-induced renal carcinogenesis than *C57BL/6J* mice