主論文の要旨

BRCA1 haploinsufficiency promotes chromosomal amplification under Fenton reaction-based carcinogenesis through ferroptosis-resistance

（*BRCA1*がん抑制遺伝子のハプロ不全は、
フェントン反応を基盤とする発がん過程において、
フェロトーシス抵抗性を獲得することにより染色体増幅を促進する）

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【Introduction】

Germline-mutation in *BRCA1* tumor suppressor gene is an established risk for carcinogenesis. However, supporting data is insufficient both in the mutation spectra of cancers in the patients with *BRCA1* germline-mutation and in murine models of *Brca1* haploinsufficiency. Furthermore, information on the driving force toward carcinogenesis in *BRCA1* mutation carriers is lacking. Here we applied Fenton reaction-based renal carcinogenesis to a rat heterozygously knockout model of *BRCA1* haploinsufficiency (MUT model; *L63X*/+). It revealed significant promotion of renal cell carcinoma which identified significant increase in chromosomal amplification, including *c-Myc*. Subacute-phase analysis in the MUT model revealed dysregulated iron metabolism with mitochondrial malfunction, leading to renal tubular proliferation with iron overload. In conclusion, we demonstrate that biallelic *BRCA1* provides protection for mitochondrial metabolism under iron-catalyzed oxidative stress, preventing the emergence of neoplastic cells with chromosomal amplification, which can be a target for cancer prevention and therapeutics.

【Methods】

Rat *BRCA1* haploinsufficiency model

A rat *Brca1*-MUT(*L63X*/+) model was established by Prof. Imaoka, and used in the present study. Rats with homozygously MUT alleles (*L63X*/*L63X*) were embryonic lethal. Genotyping was performed with PCR.

Renal carcinogenesis experiments with Fe-NTA

Male rats (*wild-type* and *Brca1*-MUT) were injected ip with Fe-NTA with a dose of 5~10 mg iron/kg for a total of 11 weeks. The rats which were found to have fatal renal cell carcinoma (RCC) by palpation or to be dying were euthanized.

Computed tomography and magnetic resonance imaging were used, if necessary, to confirm the presence of RCC.

Hematoxylin & eosin staining and immunohistochemistry were performed.

Study of chromosomal amplification

Array-based comparative genomic hybridization (aCGH) was performed with genomic DNA from RCC samples (4/4 in wild-type with/without metastasis, 4/4 in Brca1-MUT with/without metastasis) and analyzed.

Fluorescent *in situ* hybridization (FISH) was performed on FFPE RCC sections using *c-Myc*(Cy3)/Ch7CEN(Spectrum Green) dual color FISH probe.

Subacute study

Male rats (wild-type and *Brca1*-MUT) were injected *ip* with Fe-NTA for carcinogenesis

protocol either for 1 or 3 week[s]. The rats were euthanized 48h after the final injection.

Microarray analyses were performed with total RNA from kidney cortex obtained, which were subsequently analyzed (GEO accession: GSE198507). GO term analysis was performed between wild-type and Brca1-MUT groups.

Transmission electron microscopy (TEM) was applied to renal cortical area excised from control and Fe-NTA treated rats of wild-type and Brca1-MUT groups. Immunoblot of Drp1, mTOR, and p62 was examined.

Immunohistochemical analysis of Transferrin and Perl's iron staining were performed to evaluate iron metabolism. Immunoblot of Transferrin, Transferrin Receptor, and Ferritin heavy chain was subsequently applied.

To evaluate nuclear mutagenic and ferroptosis environment, immunohistochemistry of 8-hydroxy-2'-deoxyguanosine (8-OHdG; N45.1), Ki-67, γ-H2AX, and 4-hydroxy-2-nonenal-modified proteins (HNEJ-1) was performed.

【Results】

Brca1 haploinsufficiency significantly promotes Fe-NTA-induced renal carcinogenesis

There was a significant promotional effect of renal carcinogenesis in Brca1 haploinsufficiency in comparison to WT (Fig. 1A). RCCs of MUT rats showed a higher but statistically not significant incidence of peritoneal invasion/dissemination (35.3% and 52.9%, $P = 0.300$; Table 1) with higher Ki-67 cellular proliferation index.

Brca1 haploinsufficiency significantly increases chromosomal amplification in Fe-NTA-induced RCCs

We observed that in comparison to WT, MUT RCCs with/without pulmonary metastasis revealed significantly higher frequency of chromosomal amplification, among which amplification of chromosome 7 was most prominent (Fig. 2A). In WT RCCs, deletions were significantly prevalent in general but only centromeric portion of chromosome 4 showed significantly frequent amplification, where *c-Met* oncogene was located as we previously described (Fig.2A).

Specific amplification of *c-Myc* oncogene in the RCCs of Brca1 MUT rats

We found *c-Myc* amplification in 4/8 MUT RCCs examined by aCGH whereas only 1/8 of WT RCCs showed it. FISH analysis confirmed significantly higher incidence of *c-Myc* amplification in the other MUT RCCs in comparison to WT and revealed that *c-Myc* amplification included those in the extrachromosomal DNA (micronuclei) (Fig. 2B).

Brca1 haploinsufficiency causes iron dysregulation in association with mitochondrial malfunction in the subacute phase of Fe-NTA-induced renal carcinogenesis

Microarray analysis between WT and MUT at 3 weeks of carcinogenesis protocol suggested the pathways involved in heme/hemoglobin, oxygen and iron (Fig. 3A), leading to investigation of mitochondria and iron metabolism. We performed TEM analysis, which disclosed that after 3 weeks of Fe-NTA administration, mitochondria became significantly irregular and small-sized with electron-dense deposits and loss of cristae whereas higher lysosomal/autophagosomal fraction was maintained in the *Brca1*-MUT kidney (Fig. 4A-C). This was confirmed by decrease in Drp1, mTOR, and p62 (Figs. 4D). We found significantly higher iron deposition in the MUT kidney at 3 weeks of carcinogenesis protocol than WT with Perl's iron staining and Tf expression (Fig. 5AB), which was consistent with increase in Fth1 and decrease in Tfr1(Fig. 5C). Increase in Tf production in the MUT kidney at 3 weeks of carcinogenesis protocol suggests an establishment of a regulatory system to avoid excess iron.

***Brca1* haploinsufficiency generates carcinogenic environments with ferroptosis-resistance under iron-catalyzed persistent oxidative stress**

At 3 weeks of carcinogenesis protocol, 8-OHdG was significantly increased in the MUT kidney with higher Ki-67 cellular proliferation index in comparison to WT (Fig. 6AB). γ-H2AX, a marker for DNA double-strand breaks, significantly increased in the kidney with WT significantly higher than MUT (Fig. 6C), which may be attributed to Brca1 haploinsufficiency. Immunostaining by HNEJ-1, a novel maker of ferroptosis, showed higher signal in WT than MUT (Fig. 6D), suggesting that MUT kidney is more resistant to ferroptosis under the carcinogenesis protocol.

【Discussion】

Here we demonstrated that *Brca1* haploinsufficiency significantly promotes Fe-NTA-induced renal carcinogenesis, indicating the usefulness of this rat MUT model to clarify the responsible molecular mechanisms in comparison to previously failed murine models. We found that *c-Myc* in chromosome 7 is amplified with a significantly higher probability in the RCCs in *Brca1*-MUT rats. Therefore, BRCA1 prevents or delays extrachromosomal oncogene amplification under oxidative stress. We believe that whole genome sequencing in the future with other studies would clarify why RCCs in the MUT rats show more aggressive behavior. We found that iron metabolism is significantly altered in the renal tubules in response to persistent Fe-NTA-induced oxidative stress. Tf overexpression appears a strategy of tubular cells to regulate intracellular iron concentration either by releasing or preventing uptake of iron to escape ferroptosis, which needs further investigation. After 3 weeks of Fe-NTA administration, ferroptosis markers were significantly lower in the MUT model indicating ferroptosis-resistance. Further microarray data suggested mitochondrial malfunction by the accumulation of iron- and heme-associated genes. Electron microscopic analysis revealed that deformed mitochondria in the MUT kidney was significantly aggravated after 3-week Fe-NTA

administration with smaller mitochondrial mass. Because mitochondria are a central iron metabolism organelle, we assume that mitochondrial dysfunction would dramatically alter the iron distribution intracellularly with *vice versa* and promote the energy acquisition system to be more dependent on glycolytic system as established in cancer. Finally, we interpret the decrease in γ-H2AX in *Brca1*-MUT as an impairment of DNA damage recognition, which needs further investigation. And a decrease in phosphorylated Brca1 may eventually cause failure of cell-cycle checkpoints, leading to genomic amplification.

【Conclusion】

Here we demonstrated that *BRCA1* haploinsufficiency causes mitochondrial dysfunction, leading to cellular iron deposition under Fe-NTA-induced renal carcinogenesis model. This can be a carcinogenic driving force to the early establishment of ferroptosis-resistant target cells (Fig. 7). Further, *BRCA1* haploinsufficiency facilitates Fe-NTA-induced renal carcinogenesis at the promotional phase and allows more chromosomal amplifications, including *c-Myc*. Therefore, manipulating the iron metabolism, especially at the target organs, can be a preventive strategy of various carcinogenesis for the *BRCA1* germline-mutated patients. *Brca1*(L63X/+) haploinsufficient rat provides us with a more plausible model to evaluate possible strategies to increase the quality of life of BRCA1 germline-mutated patients.