



Communication

NEAT1 Confers Radioresistance to Hepatocellular Carcinoma Cells by Inducing PINK1/Parkin-Mediated Mitophagy

Hiroyuki Tsuchiya ^{1,*} , Ririko Shinonaga ^{1,†}, Hiromi Sakaguchi ², Yutaka Kitagawa ², Kenji Yoshida ² and Goshi Shiota ¹

¹ Division of Medical Genetics and Regenerative Medicine, Department of Genomic Medicine and Regenerative Therapeutics, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago 683-8503, Japan
² Department of Radiation Oncology, Tottori University Hospital, 86 Nishi-cho, Yonago 683-8503, Japan
* Correspondence: tsuchiyah@tottori-u.ac.jp
† Current address: Cell Fate Dynamics and Therapeutics, Institute for Life and Medical Science (LiMe), Kyoto University, Shogoin Kawaharacho 53, Sakyo, Kyoto 606-8507, Japan.

Abstract: A long noncoding RNA, nuclear paraspeckle assembly transcript 1 (NEAT1) variant 1 (NEAT1v1), confers radioresistance to hepatocellular carcinoma (HCC) cells by inducing autophagy via γ -aminobutyric acid A receptor-associated protein (GABARAP). Radiation induces oxidative stress to damage cellular components and organelles, but it remains unclear how NEAT1v1 protects HCC cells from radiation-induced oxidative stress via autophagy. To address this, we precisely investigated NEAT1v1-induced autophagy in irradiated HCC cell lines. X-ray irradiation significantly increased cellular and mitochondrial oxidative stress and mitochondrial DNA content in HCC cells while NEAT1v1 suppressed them. NEAT1v1 concomitantly induced the phosphatase and tensin homolog-induced kinase 1 (PINK1)/parkin-mediated mitophagy. Interestingly, parkin expression was constitutively upregulated in NEAT1v1-overexpressing HCC cells, leading to increased mitochondrial parkin levels. Superoxide dismutase 2 (SOD2) was also upregulated by NEAT1v1, and GABARAP or SOD2 knockdown in NEAT1v1-overexpressing cells increased mitochondrial oxidative stress and mitochondrial DNA content after irradiation. Moreover, it was suggested that SOD2 was involved in NEAT1v1-induced parkin expression, and that GABARAP promoted parkin degradation via mitophagy. This study highlights the unprecedented roles of NEAT1v1 in connecting radioresistance and mitophagy in HCC.

Keywords: NEAT1; mitophagy; radiation resistance; SOD2; GAPARAP; parkin; PINK1



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1. Introduction

The prognosis of patients with hepatocellular carcinoma (HCC), ~80% of primary liver cancers, is poor because therapeutic options for HCC are limited due to the increased levels of the carcinogenic potential of coexisting chronic liver diseases [1]. Several molecular-targeted drugs and immune checkpoint inhibitors have recently been introduced for treating HCC; however, those agents are expected to prolong patient survival by only several months [2]. Therefore, the continuous challenge to develop more efficient therapeutic options is required to improve the prognosis of patients with HCC.

Radiation therapy is minimally invasive, and targets selective treatment compared to surgery and chemotherapy [3]. Moreover, recent advancements in radiation therapy, such as stereotactic body radiation therapy and heavy ion radiotherapy, efficiently deliver an ablative radiation dose to tumors [3–5]. However, local recurrence remains an important issue of radiation therapy for HCC.

Nuclear paraspeckle assembly transcript 1 (NEAT1) is a long noncoding RNA (lncRNA) expressed in shorter (NEAT1v1) and longer (NEAT1v2) isoforms [6,7]. NEAT1v1 is required for the maintenance and induction of cancer stem cells (CSCs), endowed with a tumor-initiating property, in HCC cells [8,9]. Moreover, consistent with the therapeutic resistant

features of CSCs [9], NEAT1v1 also confers radioresistance to HCC cells [10]. By performing an autophagic flux assay, we previously demonstrated that NEAT1v1 enhances autophagy in irradiated cells via γ -aminobutyric acid A receptor-associated protein (GABARAP): a critical factor for autophagosome-lysosome fusion during starvation-induced autophagy and phosphatase and tensin homolog-induced kinase 1 (PINK1)/parkin-mediated mitophagy [11,12].

Autophagy is a critical process for maintaining cellular homeostasis; it can be tumorigenic and tumor-suppressive depending on the biological context [13–17]. Radiation generates a massive amount of reactive oxygen species (ROS) in cancer cells, which irreversibly damages cellular organelles and biomolecules [18]. In this context, autophagy protects cancer cells from radiation by promoting the regeneration of damaged organelles. Autophagy also controls the mitochondrial quality by degrading mitochondria in nonselective (macroautophagy) and selective (mitophagy) manners. Mitophagy selectively targets damaged mitochondria to maintain mitochondrial homeostasis [19]. Mitophagy induction involves several pathways, including ubiquitin-dependent and -independent receptor pathways [19]. The former is also recognized as the PINK1/parkin pathway. Under normal conditions, PINK1 translocates to the inner membrane of healthy mitochondria and is proteolytically cleaved by mitochondrial proteases [20,21]. Cleaved PINK1 is retrotranslocated to the cytosol and subjected to proteasomal degradation [19]. However, upon mitochondrial depolarization, the translocation is inhibited, and PINK1 is stabilized and exposed to the outer mitochondrial membrane [20,21]. PINK1 on the outer membrane phosphorylates the Ser65 residue of ubiquitin and parkin, thereby activating the E3 ubiquitin ligase activity of parkin [22,23]. Activated parkin accumulates on the outer membrane and induces the K63 ubiquitination of mitochondrial proteins [22,23]. These ubiquitinated proteins are recognized by autophagy cargo receptors, such as optineurin and NDP52, which also bind to GABARAP and LC3 on the autophagosomal membrane, resulting in the engulfment of damaged mitochondria [23,24]. GABARAP and LC3 are members of the ATG8 family and share structural similarities and redundant functions. However, GABARAP is more responsible for the PINK1/parkin-mediated mitophagy than LC3 because the GABARAP knockout markedly impairs the PINK1/parkin-mediated mitophagy, whereas no effect is observed in LC3 knockout cells [12].

This study investigated the effects of NEAT1v1 on mitophagy in irradiated HCC cells and found that NEAT1v1 induces PINK1/parkin-mediated mitophagy via GABARAP and superoxide dismutase 2 (SOD2) to protect HCC cells from radiation.

2. Results

2.1. NEAT1v1 Suppresses Radiation-Induced Mitochondrial Damage

Radiation induces cellular damage by exacerbating oxidative stress. Cellular and mitochondrial oxidative stress were examined with DCFDA and MitoSOX Red, respectively, in irradiated HCC cells. In Figure 1A, cellular and mitochondrial oxidative stress significantly increased by irradiation in a dose-dependent manner. In contrast, oxidative stress was significantly suppressed by NEAT1v1 (Figure 1B). Moreover, the relative copy numbers of mitochondrially encoded genes, *ND1* and *ND5*, to the nuclear-encoded *HBB* gene markedly increased by radiation in control HCC cell lines (Figure 1C). In contrast, this increase was abolished by NEAT1v1 (Figure 1C). The determination of the copy number of *ND1* and *ND5* genes relative to a nuclear gene are an established method to estimate the mitochondrial DNA copy number in a cell [25,26]. These results indicate that radiation induced the accumulation of mitochondrial DNA, while NEAT1v1 suppressed it. In summary, NEAT1v1 protected HCC cells by suppressing radiation-induced mitochondrial damage and the accumulation of damaged mitochondria.

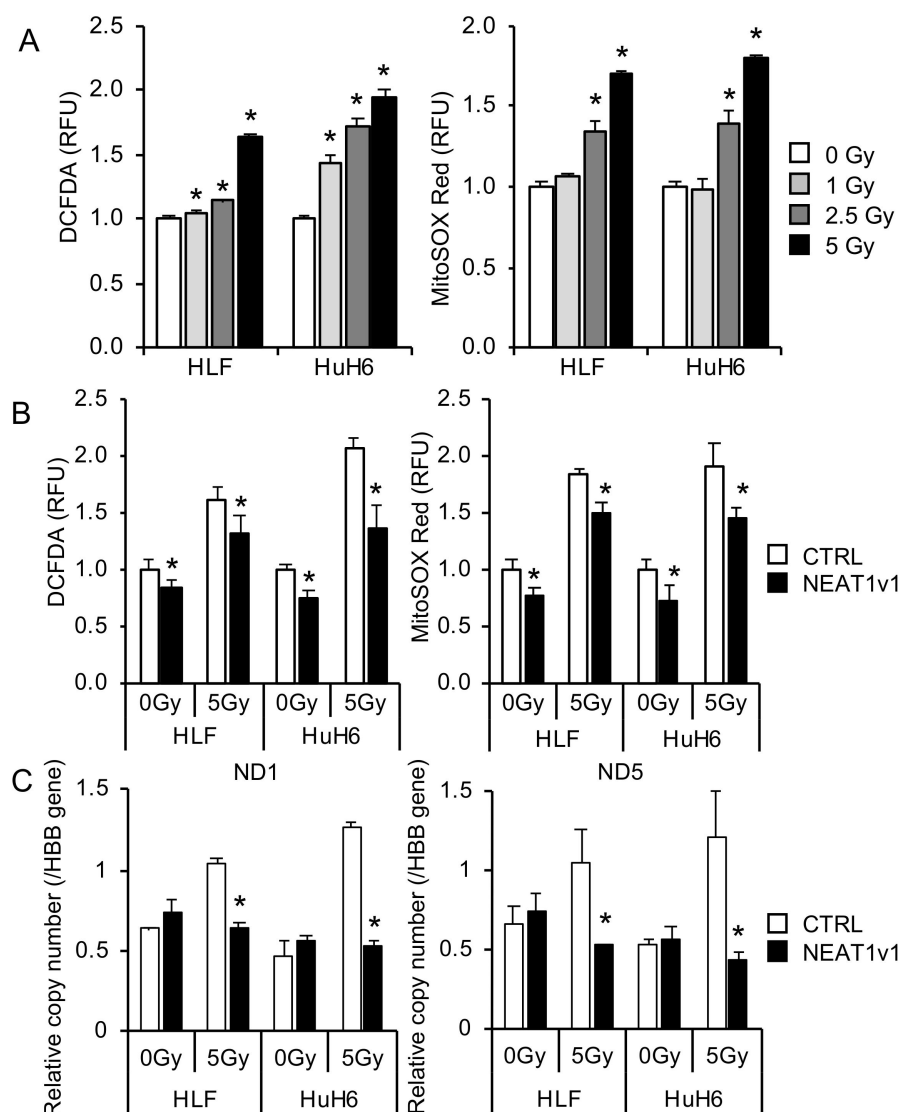


Figure 1. Protective effects of NEAT1v1 on radiation-induced mitochondrial damage. (A) Radiation dose-dependent increases in cellular (left) and mitochondrial (right) oxidative stress. * $p < 0.05$ (Dunnett's test vs. 0 Gy; $n = 4$). (B) Suppression of radiation-induced cellular (left) and mitochondrial (right) oxidative stress. * $p < 0.05$ [Student's t -test, control (CTRL) vs. NEAT1v1-overexpressing cells; $n = 4$]. (C) Relative copy number of mitochondrially encoded genes (*ND1* and *ND5*). * $p < 0.05$ (Student's t -test, CTRL vs. NEAT1v1-overexpressing cells; $n = 3$).

2.2. NEAT1v1 Promotes Mitophagy in Irradiated HCC Cells

Because NEAT1v1 protects HCC cells from radiation by promoting autophagy [10], it was postulated that mitophagy might be involved in this phenomenon. Indeed, LC3 localization in mitochondria was observed in irradiated HCC cells (Figure S1). Mitophagy staining showed that similar levels of mitophagy occurred between the control and NEAT1v1-overexpressing cells in a nonirradiated condition (Figure 2A). However, after irradiation, mitophagy in the control cells tended to be impaired, compared with the NEAT1v1-overexpressing cells (Figure 2A and Figure S2).

In nonirradiated cells, NEAT1v1 induced parkin expression in mitochondria and cytosol, whereas mitochondrial localization of PINK1 increased (Figure 2B). Mitochondrial and cytosolic parkin remained high in NEAT1v1-overexpressing cells after irradiation, whereas mitochondrial PINK1 levels were similar between the control and NEAT1v1-overexpressing cells (Figure 2B). These results suggested that NEAT1v1 enhanced mitochon-

drial localization of PINK1 in a nonirradiated condition while constitutively upregulating parkin to promote mitophagy.

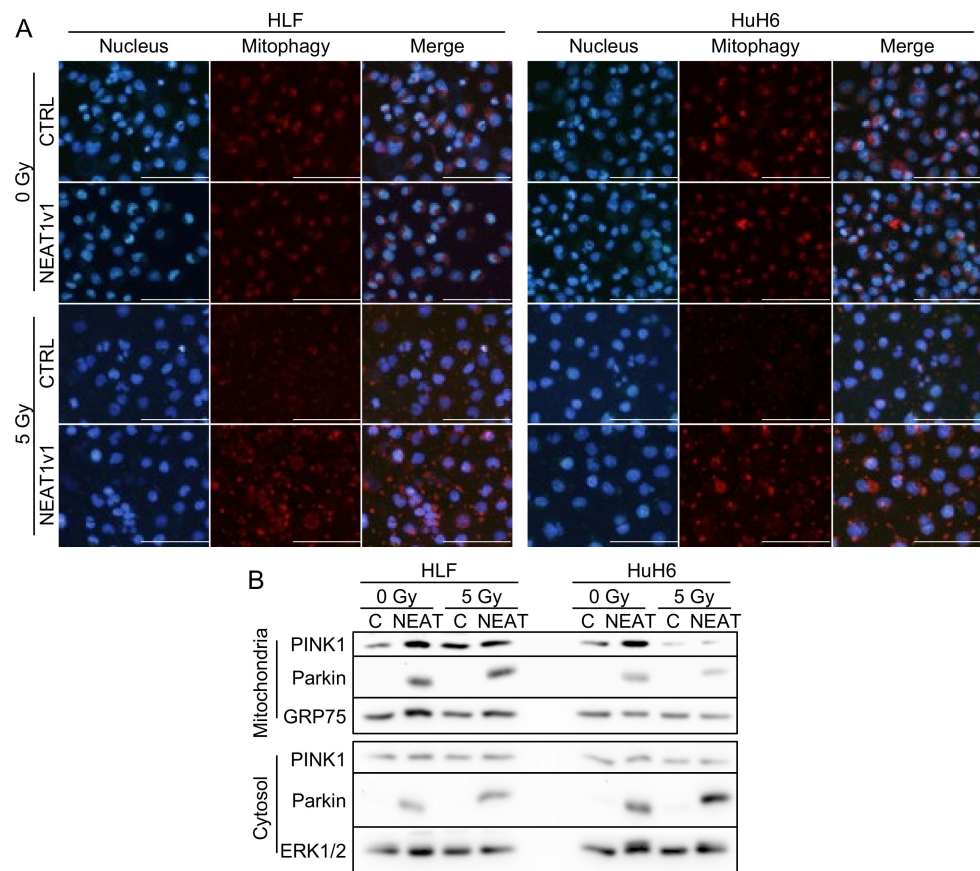


Figure 2. Induction of PINK1/parkin-mediated mitophagy by NEAT1v1 in irradiated cells. **(A)** Representative images of mitophagy staining. Mitophagy was stained with Mitophagy Dye (red). Nuclei were counterstained with Hoechst (blue). Scale bar, 100 μ m. **(B)** Representative Western blot images for mitochondrial and cytosolic PINK1, parkin, GRP75 (mitochondrial marker), and ERK1/2 (cytosolic marker). C, CTRL; NEAT, NEAT1v1-overexpressing cells.

2.3. SOD2 Is Involved in NEAT1v1-Induced Radioresistance

The suppression of oxidative stress by NEAT1 suggests an enhanced removal of ROS by antioxidative enzymes; thus, the expression of antioxidative enzymes was examined in NEAT1v1-overexpressing HCC cells. Both cell lines showed a significant increase in SOD2 expression by NEAT1v1, whereas other antioxidative enzymes showed no difference or significant changes in either cell line (Figure 3A). Because superoxide anion (O_2^-) is constitutively produced by the electron transfer reaction, mitochondria are one of the major sources of ROS, and SOD2 is a mitochondrial antioxidative enzyme that detoxifies O_2^- . Therefore, this result suggested that SOD2 was responsible for suppressing radiation-induced mitochondrial oxidative stress by NEAT1v1.

NEAT1 knockdown significantly downregulated SOD2 expression in the control and NEAT1v1-overexpressing HCC cells (Figure 3B and Figure S3), suggesting that NEAT1v1 directly regulated SOD2 mRNA expression. SOD2 protein was also upregulated by NEAT1v1 overexpression regardless of irradiation (Figure 3C). Because NEAT1v1 enhances radioresistance in HCC cells via GABARAP [10], the effects of SOD2 knockdown on NEAT1v1-induced radioresistance were further examined. ShRNAs targeting SOD2 downregulated SOD2 protein expression in NEAT1v1-overexpressing HCC cells but did not affect SOD1 expression (Figure 3D). The colony formation assay revealed that SOD2 knockdown significantly decreased the radioresistance of NEAT1v1-overexpressing HCC cells (Figure 3E).

These results suggested that NEAT1v1 conferred radioresistance to HCC cells via SOD2 in addition to GABARAP.

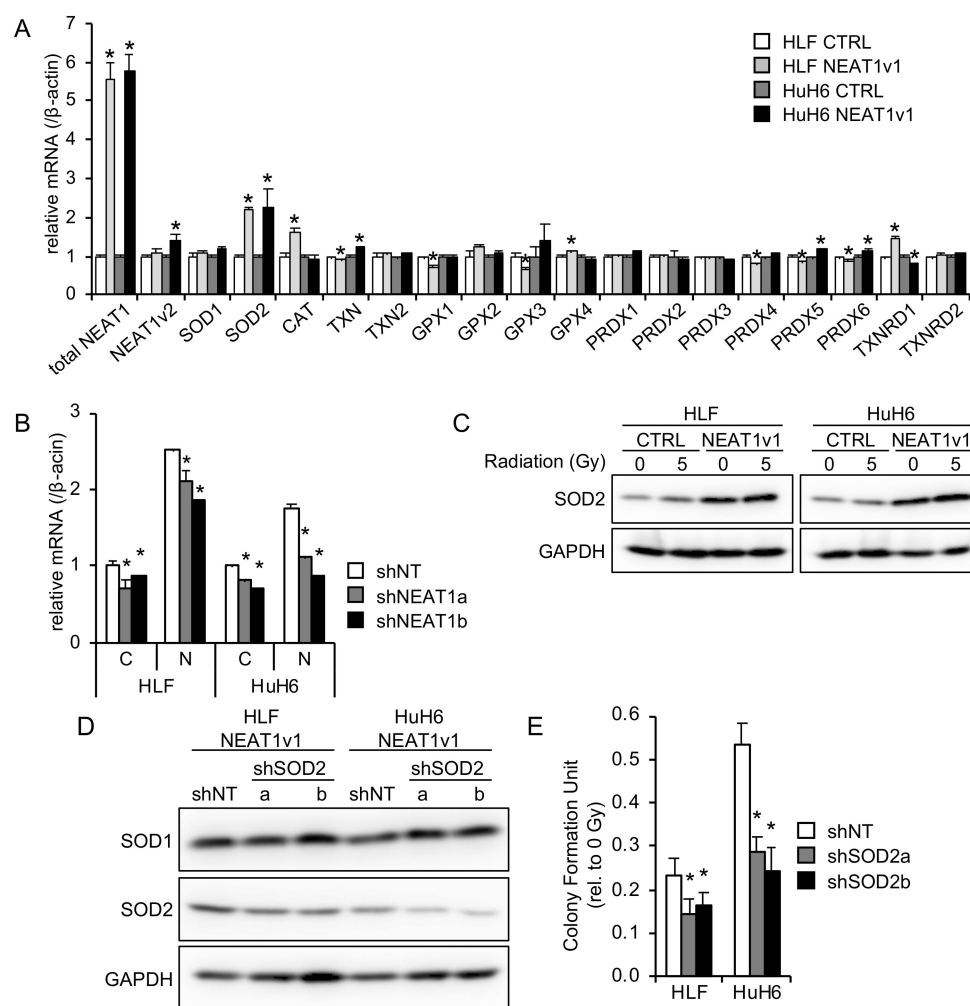


Figure 3. Involvement of SOD2 in NEAT1v1-induced radioresistance. (A) MRNA expression levels of genes encoding antioxidative enzymes in NEAT1v1-overexpressing cells. * $p < 0.05$ [Student's *t*-test, control (CTRL) vs. NEAT1v1-overexpressing cells; $n = 3$]. (B) Expression levels of SOD2 mRNA in control (C) or NEAT1v1-overexpressing cells (N) transduced with adenoviruses expressing nontarget shRNA (shNT) or NEAT1-specific shRNAs (shNEAT1a and shNEAT1b). * $p < 0.05$ (Dunnett's test vs. shNT; $n = 3$). (C) Representative Western blot images for SOD2 and GAPDH (internal control) using whole-cell lysates after 0 or 5 Gy irradiation. (D) Representative Western blot images for SOD1, SOD2, and GAPDH (internal control) using whole-cell lysates of cells transduced with adenoviruses expressing shNT or SOD2-specific shRNAs (shSOD2a and shSOD2b). (E) Colony formation abilities of NEAT1v1-overexpressing cells knocked down for SOD2 after 2.5 Gy irradiation. * $p < 0.05$ (Dunnett's test vs. shNT; $n = 6$).

2.4. GABARAP and SOD2 Suppress Radiation-Induced Mitochondrial Oxidative Stress

The effects of the GABARAP and SOD2 knockdown on oxidative stress in HCC cells overexpressing NEAT1v1 after irradiation were investigated. In Figure 4A, cellular and mitochondrial oxidative stress after irradiation significantly increased by the GABARAP and SOD2 knockdown. These results suggested that GABARAP and SOD2 were involved in the NEAT1v1-mediated protection of HCC cells from radiation-induced oxidative stress. Concomitantly, the knockdown significantly increased the mitochondrial DNA copy number (Figure 4B).

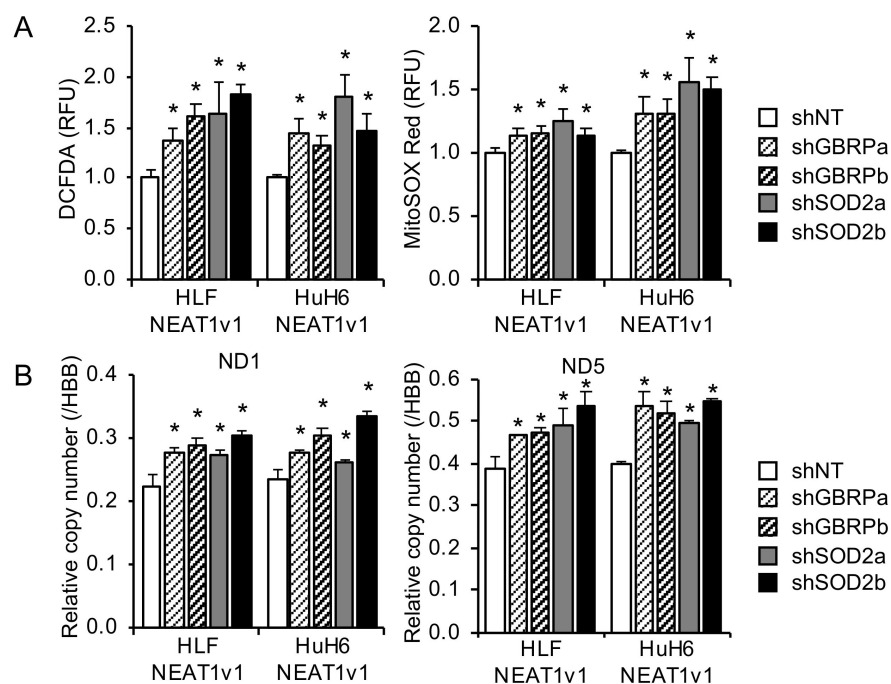


Figure 4. Involvement of GABARAP and SOD2 in the suppressive effects of NEAT1v1 on radiation-induced mitochondrial damage. **(A,B)** Cellular (left) and mitochondrial (right) oxidative stress **(A)** and relative copy number of mitochondrially encoded genes (ND1 and ND5); **(B)** in NEAT1v1-overexpressing HLF and HuH6 cells knocked down for GABARAP (shGBRPa and shGBRPb) or SOD2 (shSOD2a and shSOD2b) after 5 Gy irradiation. * $p < 0.05$ [Dunnett's test vs. shNT; $n = 6$ **(A)** and 3 **(B)**].

2.5. GABARAP and SOD2 Are Involved in NEAT1v1-Induced Mitophagy

GABARAP is a critical factor for mitophagy [11,12], and SOD2 is a mitochondria-specific antioxidative enzyme. Moreover, their knockdown induced the accumulation of damaged mitochondria (Figure 4), suggesting that these two factors played a role in NEAT1v1-induced mitophagy. Whereas the GABARAP and SOD2 knockdown in NEAT1v1-overexpressing cells did not affect PINK1 expression, parkin expression markedly increased by the GABARAP knockdown (Figure 5A), consistent with a previous report, in which the GABARAP knockdown perturbed autophagy [10]. In contrast, the SOD2 knockdown resulted in parkin downregulation in the cytosol and mitochondria (Figure 5A), suggesting that SOD2 was involved in the constitutive upregulation of parkin by NEAT1v1.

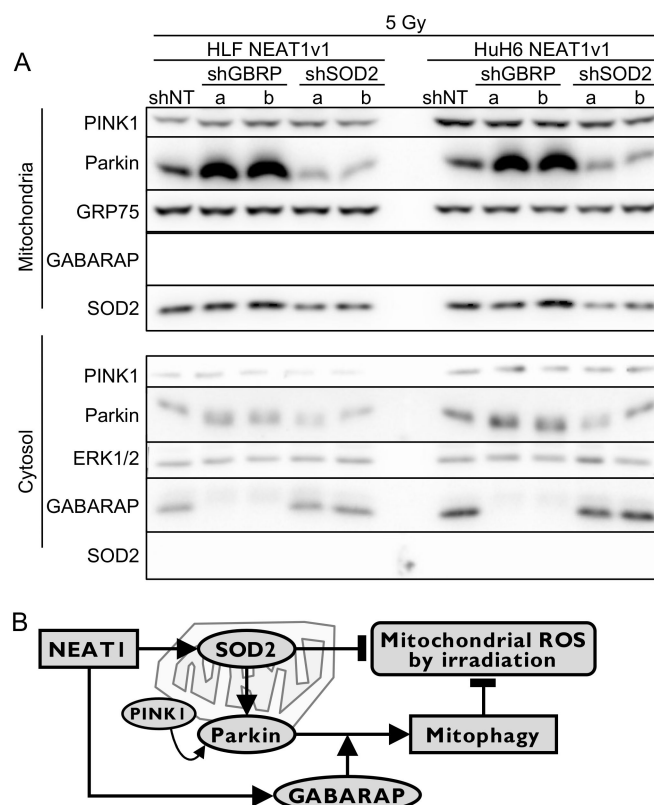


Figure 5. Involvement of GABARAP and SOD2 in NEAT1v1-induced mitophagy. **(A)** Representative Western blot images for mitochondrial and cytosolic PINK1, parkin, SOD2, GABARAP, GRP75 (mitochondrial marker), and ERK1/2 (cytosolic marker). C, CTRL, control cells; NEAT, NEAT1v1-overexpressing cells. **(B)** Schematic representation of NEAT1v1-induced radioresistance via the PINK1/parkin-mediated mitophagy. NEAT1v1 upregulates GABARAP and SOD2 in HCC cells. GABARAP is a critical factor for mitophagy, whereas SOD2 reduces oxidative stress by its antioxidative activity and induces parkin expression.

3. Discussion

This study demonstrated that radiation increases mitochondrial oxidative stress, whereas NEAT1v1 suppresses it by enhancing PINK1/parkin-mediated mitophagy through GABARAP and SOD2 (Figure 5B). Considering that NEAT1v1 confers radioresistance to HCC cells [10], NEAT1v1 promotes the regeneration of healthy mitochondria by removing damaged mitochondria through mitophagy. Moreover, it was shown that NEAT1v1 significantly suppressed oxidative stress (Figure 1B), possibly due to the increased expression of SOD2, and increased the expression and mitochondrial localization of parkin (Figure 2B) even in nonirradiated cells. These findings suggest that NEAT1v1 regulates the basal levels of mitophagy in HCC cells under a physiological condition. This is in agreement with our previous report [10], in which NEAT1v1 was shown to promote autophagy in nonirradiated HCC cells. However, mitophagy staining using Mitophagy Dye showed no difference between the control and NEAT1v1-overexpressing cells without irradiation (Figure 2A). Although other mitophagy assessments, e.g., those using mito-OC [27] or mito-Keima [28], would more accurately quantify their difference, the contribution of NEAT1v1 to basal mitophagy may not be so significant, at least, to the extent that Mitophagy Dye can detect. Moreover, GABARAP or SOD2 knockdown resulted in a marginal increase in mitochondrial DNA, compared with the suppressive effect of NEAT1v1 on the radiation-induced accumulation of mitochondrial DNA (Figures 1C and 4B). This might be due to the insufficient knockdown efficiency, in particular, of SOD2; however, it is known that the quality of mitochondria is controlled by several pathways, including

ubiquitin-independent mitophagy and fission/fusion [29]. Therefore, it is possible that NEAT1v1 might also control the quality of mitochondria via such mechanisms other than the PINK1/parkin-mediated mitophagy. The comprehensive understanding of molecular mechanisms underlying NEAT1v1-mediated mitochondrial quality control should be addressed in future studies.

It was previously demonstrated that radiation suppressed autophagy [10]. This study also revealed increased mitochondrial DNA content and oxidative stress, suggesting the accumulation of damaged mitochondria in control cells after irradiation at a dose of 5 Gy. Consistently, a low dose (0.5 Gy) of carbon ions modestly damaged mitochondria and induced mitophagy in cervical and breast cancer cells [30]. However, when treated with a high dose (3 Gy), apoptosis was preferentially induced rather than mitophagy [30]. Because the content of mitochondrial DNA after irradiation was not increased in NEAT1v1-overexpressing cells, NEAT1v1 can promote the removal of damaged mitochondria through mitophagy even at the lethal irradiation dose. In the present study, we assessed mitophagy at 48 h after irradiation based on our preliminary experiments, in which an apparent difference in mitophagy staining was observed at 48 h, but not at 24 h after irradiation. Radiation-induced cell death via mitotic catastrophe requires a relatively long period because it must be preceded by several attempted divisions to accumulate sufficient genetic damage for mitotic death [31]. Therefore, it is worth investigating whether mitophagy induced by NEAT1v1 could contribute to cell survival at longer periods of culture time after irradiation.

NEAT1v1-induced autophagy has a cytoprotective effect on cancer cells [10]. Consistently, NEAT1 also induces autophagy by targeting miR-34a and miR-204 as a competing endogenous RNA (ceRNA) in colorectal cancer and HCC, respectively, resulting in the upregulation of autophagy-related proteins ATG9A, ATG4A, and ATG3 [32,33]. Eventually, NEAT1-induced autophagy leads to chemoresistance to 5-fluorouracil and sorafenib [32,33]. Moreover, NEAT1 also increases PINK1 and parkin expression, thereby enhancing lung mitophagy in chronic obstructive pulmonary disease [34]. However, the mitophagy induction by NEAT1 in cancer cells has not been studied. In contrast, NEAT1 suppresses mitophagy in neuron and renal epithelial cells and exaggerates the pathogenesis of Alzheimer's disease and diabetic nephropathy [35,36]. This inconsistency might arise from the difference between nontransformed and transformed cells. However, in mitophagy suppression, NEAT1 plays a role as a ceRNA against miR-150-5p [36]. Therefore, NEAT1v1 might also regulate GABARAP and SOD2 expression as a ceRNA.

It is worth noting that mRNA expression levels of other GABARAP subfamily members, GABARAPL1 and GABARAPL2, did not significantly change in NEAT1v1-overexpressing cells. Therefore, among the GABARAP subfamily, GABARAP plays a central role in NEAT1v1-induced mitophagy. However, GABARAP has been suggested as a tumor promoter and suppressor. Carcinogen-induced tumor incidence was significantly reduced in GABARAP-deficient mice [37]. Moreover, high GABARAP expression in tumor tissues was significantly associated with poor prognosis of patients with colorectal carcinoma and breast cancer [38,39]. Likewise, GABARAP was upregulated in tumor necrosis factor- α -resistant breast cancer cells, concomitant with several other autophagy-related genes [40]. In contrast, GABARAP suppressed breast cancer progression through the AKT/mTOR signaling pathway [41]; however, how GABARAP suppressed the signaling pathway remains unclear. This study is the first report demonstrating the involvement of GABARAP-induced mitophagy in the radioresistance of cancer cells. Further studies must be undertaken to clarify the clinical significance of GABARAP-induced mitophagy in tumors and radiotherapy.

The mitochondrial electron transfer chain generates O_2^- , which is catabolized to less toxic hydrogen peroxide by the well-known mitochondrial antioxidative enzyme, SOD2. Thus, SOD2 upregulation by NEAT1v1 protects HCC cells from mitochondrial oxidative stress. However, because mitophagy is induced by depolarization of the mitochondrial membrane potential [42–45], it is suggested that SOD2 inhibits mitophagy [46–48], although limited information is available on their direct relationship. In contrast, SOD2

activity was significantly reduced in the myocardium of aged mice, whereas mitophagy was concomitantly impaired [49]. Likewise, SOD2 activity and PINK1 expression concomitantly increased in the liver of Per-Arnt-Sim kinase-deficient mice under fasting conditions, in which increased mitophagy was suggested by a characteristic mitochondria morphology [50]. A similar result was also observed in human umbilical vein endothelial cells treated with a plant-derived substance, scutellarin, by which SOD2, parkin, and PINK1 expression were induced, concomitant with increased mitophagy [51]. Moreover, nuclear factor erythroid 2-related factor 2 (NRF2) regulates the expression of antioxidative enzymes, including SOD2, and mitophagy-related proteins, including p62 and PINK1, whereas parkin overexpression activates NRF2, leading to the upregulation of antioxidative enzymes, including SOD2 [52–54]. These observations suggest that SOD2 can be involved in mitophagy induction depending on a cellular context. It remains yet to be clarified how NEAT1v1 induces parkin expression via SOD2; however, direct evidence was provided for the involvement of SOD2 in mitophagy induction, as SOD2 knockdown significantly impaired NEAT1v1-induced mitophagy and downregulated parkin expression. These findings indicate that SOD2 is involved in the mitophagy induction in HCC cells by inducing parkin expression. In the future, it is necessary to clarify how SOD2 induces parkin expression and mitophagy in irradiated HCC cells.

The present study based on *in vitro* experiments demonstrated the protumor roles of SOD2 in HCC. Moreover, we recently found that SOD2 is involved in NEAT1v1-induced chemoresistance in HCC cells. Nonetheless, the pathological significance of SOD2 in HCC is under debate, as its expression showed different expression patterns between cohorts [55]. Consistently, it has been suggested that SOD2 has dual roles in cancer including HCC [56]. Therefore, it is of interest to investigate *in vivo* whether an SOD2-targeting therapy is a promising strategy to potentiate the therapeutic efficacy of chemo/radiotherapy against HCC.

Several mechanisms have been suggested to underlie the radiosensitivity/radioresistance of HCC, including ferroptosis [57], CD133+CSCs [58], autophagy induction [10], suppression of DNA repair by melatonin-induced lncRNA RAD51-AS1 [59], miR320b/RAD21 axis [60], etc. However, the involvement of mitophagy in radioresistance in HCC has not been reported; thus, this report highlights mitophagy as an important therapeutic target for HCC radiotherapy. Several studies also have suggested that mitophagy is a critical cellular process for determining the radiosensitivity of cancer cells other than HCC. Increased mitochondrial oxidative stress by radiation induced BNIP3- and BNIP3L-mediated mitophagy to protect colorectal cancer cells from radiation-induced cytotoxicity [61]. Moreover, a newly synthesized anticancer compound, temozolomide-perillyl alcohol conjugate, was shown to inhibit mitophagy, thereby sensitizing non-small lung cancer cell lines to radiation [62]. Therefore, the suppression of mitophagy is a promising strategy to improve the clinical efficacy of radiotherapy.

4. Materials and Methods

4.1. Cell Culture

HCC cell lines (HLF and HuH6) and those overexpressing human NEAT1v1 and their control cells have been reported previously [10]. At 24 h after seeding, cells were irradiated (0, 1, 2.5, or 5 Gy) using an X-ray generator (MX-160Labo; mediXtec Japan, Chiba, Japan).

4.2. Adenovirus Vectors

Adenovirus vectors expressing nontargeting (NT) short hairpin RNA (shRNA; shNT), NEAT1-targeting shRNAs (shNEAT1a/b), and GABARAP-targeting shRNAs (shGBRPa/b) have been reported previously [10].

Adenovirus vectors expressing SOD2-targeting shRNAs (shSOD2a/b) were constructed as reported previously [8]. Briefly, oligo DNAs (Table S1) were ligated into BsaI-digested pENTR/U6-AmCyan1 with Ligation High version 2 (Toyobo, Osaka, Japan). Then, shRNA and AmCyan1-expressing cassettes were transferred by the LR reaction to

pAd/BLOCK-iT-DEST (Thermo Fisher Scientific, Waltham, MA, USA). Adenovirus vectors were constructed by transfection of PacI-digested adenovirus plasmid DNA with LipofectAMINE2000 into 293A cells (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Adenovirus titer was determined by the infectious genome titration protocol [63]. When knocking down genes in irradiated cells, these adenoviruses were transduced immediately after irradiation.

4.3. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) and Western Blot Analysis

An RT-qPCR and Western blot analysis were performed as reported previously [8,10]. mRNA and protein samples were prepared 48 h after seeding, adenovirus transduction, or irradiation. The primers used for the RT-qPCR are summarized in Table S1. β -Actin was used as an internal control for calculating the relative mRNA expression levels. Antibodies against 75-kDa glucose-regulated protein (GRP75; sc-133137), extracellular signal-regulated kinases (ERK) 1/2 (sc-514302), GABARAP (sc-377300), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-365062), parkin (sc-32282), PINK1 (sc-518052), SOD1 (sc-101523), and SOD2 (sc-133134) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.4. Preparation of Cytosolic and Mitochondrial Fractions

Cytosol and mitochondria were fractionated by the Cell Fractionation Kit-Standard (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol with a slight modification. In brief, 2×10^6 cells at 48 h after irradiation were suspended in 300 μ L of buffer A, and were combined with an equal volume of buffer B. The cells were rotated at room temperature for 7 min. Following centrifugation at $5000 \times g$ for 2 min, supernatants were transferred in new tubes, and were centrifuged again at $10,000 \times g$ for 2 min. The supernatants (cytosolic fractions) were recovered and stored -80°C until use, while the pellets of two centrifugation steps were combined, and resuspended in 300 μ L of buffer A. An equal volume of buffer C was added and rotated at room temperature for 10 min. Following centrifugation at $5000 \times g$ for 2 min, supernatants were transferred in new tubes, and were centrifuged again at $10,000 \times g$ for 2 min. The supernatants (mitochondrial fractions) were recovered and stored -80°C until use.

4.5. Determination of Mitochondrial DNA Content

Cells were recovered 48 h after irradiation and lysed in proteinase K buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.0), 1% Tween 20, and 1 mg/mL proteinase K] for 3 h at 55°C . After phenol/ CHCl_3 extraction, cellular DNA was precipitated with isopropanol and dissolved in H_2O . A qPCR was performed to obtain the Ct values of mitochondrially encoded *NADH dehydrogenase subunit 1 (ND1)* and *5 (ND5)* genes and *nuclear-encoded hemoglobin subunit β (HBB)* gene with the primers in Table S1. Relative copy numbers were calculated from a standard curve created using serially diluted samples, and those of *ND1* and *ND5* genes were normalized by that of the *HBB* gene.

4.6. Measurement of Oxidative Stress

Cells were recovered and counted 48 h after irradiation and incubated with 20 μM 2',7'-dichlorofluorescein diacetate (DCFDA; DCFDA/H2DCFDA-Cellular ROS Assay Kit; Abcam, Cambridge, MA, USA) or 5 μM MitoSOX Red (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37°C . Cells were washed once with phosphate-buffered saline and plated on 96-well black plates at 2×10^4 cells/well. Fluorescence was measured by Infinite F500 (Tecan, Männedorf, Switzerland) using excitation filters 485/20 (DCFDA) or 535/25 (MitoSOX Red) and emission filters 535/25 (DCFDA) or 590/20 (MitoSOX Red).

4.7. Mitophagy Detection

Mitophagy staining was performed by incubating the cells at 48 h after irradiation with 100 nM Mitophagy Dye (Mitophagy Detection Kit, Dojindo, Kuma-moto, Japan) for 30 min. Nuclei were concomitantly counterstained with 5 µg/mL Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA). Images were obtained with a fluorescent microscope (Olympus, Tokyo, Japan).

LC3 colocalization in mitochondria was visualized by MitoTracker Deep Red (200 nM for 30 min; Thermo Fisher Scientific, Waltham, MA, USA) staining of cells transfected with pmCherry-LC3 [64] by Viofectin (Viogen, New Taipei City, Taiwan). Images were obtained with a confocal microscope (Olympus, Tokyo, Japan) 48 h after irradiation.

4.8. Statistical Analysis

Three or more independent samples for each experiment were analyzed, and all experimental values were expressed as the mean ± standard deviation. The differences between the two groups were assessed by Student's *t*-test. Multiple comparisons were made by Dunnett's and Tukey's tests, as indicated. $p < 0.05$ was considered statistically significant.

5. Conclusions

The lncRNA NEAT1v1 confers radioresistance to HCC cells by inducing PINK1/parkin-mediated mitophagy, in which SOD2 and GABARAP are involved (Figure 5B). This study highlights the unprecedented roles of NEAT1v1 in connecting radioresistance and mitophagy in HCC. This finding deepens the understanding of the radioresistance mechanism and provides new insights for developing and improving HCC radiotherapy.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms232214397/s1>.

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