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研究成果の概要（和文）：悪性軟骨肉腫は再発・転移率が高く、化学・放射線の治療効果が低い軟骨形成腫瘍であり、新治療法の開発が急務である。この研究で我々は悪性軟骨肉腫に放射線抵抗性癌幹細胞の存在を示した。放射線抵抗性癌幹細胞には重粒子線治療やmiR-34 mimic、ラパマイシン投与の併用療法が行われている。ラパマイシンによるmTORの抑制がFOXO3とmiR-34の過剰発現を誘導しKLFを抑制する。我々はラパマイシンとmiR-34によるmTORの抑制が癌幹細胞の放射線抵抗性を阻害することを示唆した。併用療法は薬剤の低用量化、重粒子線治療効果の向上、再発・転移のリスク軽減、正常細胞への影響を減少させることが期待される。

研究成果の学術的意義や社会的意義
Our results suggest that combination approaches might be effective for hard-to-treat, resistant tumors, by targeting treatment-resistant subpopulations of cancer stem cells. This might lead to better outcomes with lower risks of relapse and metastasis.

研究成果の概要（英文）：High-grade chondrosarcomas are chemo- and radio-resistant cartilage-forming tumors of bone that often relapse and metastase. Thus, new therapeutic strategies are urgently needed. In this study, we showed that high-grade chondrosarcoma cells contain a population of radioresistant cancer stem cells that can be targeted by a combination of carbon-ion therapy, miR-34 mimic administration and/or rapamycin treatment that triggers FOXO3 and miR-34 over-expression. mTOR inhibition by rapamycin triggered FOXO3 and miR-34, leading to KLF4 repression. Altogether, our results suggest that mTOR inhibition by rapamycin supplemented with miR-34 mimic treatment may be able to overcome CSC-associated radioresistance in chondrosarcoma during carbon-ion therapy. Combination treatments might also improve the effectiveness of carbon-ion therapies at lower doses, decrease risks of relapse and metastasis, and better preserve surrounding normal tissues against non-targeted effects.

研究分野：Radiation oncology

キーワード：chondrosarcoma　radiation therapy　particle therapy　cancer stem cells　cancer　micro-RNA

１．研究開始当初の背景

Despite striking improvements in the diagnosis and care of human cancer, treatment resistance remains to this day an issue in some hard-to-treat cancers. Chondrosarcomas (CSs) constitute the second most common primary bone tumor in adults. Because these cartilaginous tumors exhibit resistance to chemotherapy and conventional radiation therapy, complete surgical resection still remains the primary treatment, with a 10-year survival rate comprised between 30% and 80% depending on the grade. A significant number of patients experience relapse, metastasis or present unresectable disease with poor clinical outcome and high lethality (grade III). For those reasons, the clinical management of CS is considered to be particularly challenging, and new therapeutic approaches are urgently needed. Some subtypes, such as mesenchymal CS, may be more responsive to chemotherapy, while surgery of dedifferentiated CS may be more successful when combined with chemotherapy. Radiation therapy has been used in skull-base and spinal CS. Recently published molecular therapy targets for CS have included IDH mutations, Hedgehog, Src and PI3K-Akt-mTOR pathways, histone deacetylase inhibitors, angiogenesis or immunotherapy with immune checkpoint inhibition. Some of those targets yielded promising results in preclinical studies, but early phase clinical results were less conclusive.

Cancer stem cells (CSCs) are defined as the subset of dedifferentiated cells within a tumor that possess the ability to self-renew and reconstitute tumor heterogeneity. CSCs are more resistant than their non-CSC counterparts and were suggested to be at least partially responsible for treatment resistance, relapse and metastasis. Cancer treatments that do not effectively target CSCs might ultimately fail, thus it is of paramount importance to develop new treatment strategies that include CSCs. Transformed mesenchymal stem and progenitor cells with multipotent differentiation potential are likely to be cells of origin in CS [8]. CSCs have been characterized in osteosarcomas, but are not well defined in CS.

New high linear energy transfer (LET) radiation therapy modalities (such as heavy-ion particle beams) have emerged, which provide a number of physical and biological advantages over conventional X-ray therapy (including an improved relative biological effectiveness RBE and a lower oxygen enhancement ratio OER) and might finally contribute to overcoming treatment resistance. High LET radiation treatment, in combination with other therapies (for example, the chemotherapeutic agent cisplatin or the PARP inhibitor talazoparib), has shown favourable results in bypassing tumor and CSC radioresistance. Although we have recently shown that low- and high-LET low-dose exposures of CS cells can trigger bystander responses in non-irradiated neighbouring normal chondrocytes, the high RBE of carbon ions might allow lower normal tissue complication probability (NTCP) than protons, for the same local tumor control (TCP), indicating that carbon ion therapy might be an appropriate CS treatment modality.

２．研究の目的

Due to the lack of effective treatment for advanced chondrosarcoma (CS), new therapeutic approaches are urgently needed. Combination therapies and their efficiency on chondrosarcoma tumors and cancer stem cells (CSC)s have not yet been explored. In this study, we investigated the ability of high LET radiation combined with targeted treatments to target CS cells and CSCs.

３．研究の方法

Human chondrosarcoma cells (CSs) were exposed to X-rays and carbon-ion beams and were transfected with micro-RNA mimics and inhibitors. Cancer stem cells (CSCs) were identified and characterized using flow cytometry-based cell sorting. Cell viability, clonogenic survival, gene and miRNA regulations and well as protein levels were analyzed. CSs were also xenograft in immunodeficient mice and tumor volumes were measured in order to evaluate the efficiency of combination therapies.

４．研究成果

Surgical resection constitutes the cornerstone of treatment for chondrosarcoma (CS), as chemotherapy is most often ineffective. Histologic grade is considered to be the most important indicator of prognosis, and the outcome for grade III CS with surgical resection alone is usually relatively poor. CH-2879, a cell line isolated from recurrent grade III CS, was selected as a suitable model for the development of new therapeutic strategies in hard-to-

treat CSs.

Cancer stem cells (CSCs) have long been presented as an important culprit for treatment resistance. Indeed, different tumors harbor various CSC contents, and the proportion of CSCs may be correlated with radio-resistance. Stem-like properties of CSCs confer them a survival advantage during cancer therapy. Those include higher reactive-oxygen species (ROS) scavenging abilities (resulting in lower radiation-induced ROS) and improved DNA damage repair activation. It is therefore of outmost importance to properly identify and target the stem-like population when establishing new treatment regimen. In CS, a subpopulation of CD133+ cells have been identified that display stem-like characteristics and were capable of inducing and sustain tumor growth in vivo. Significant evidence indicates that enhanced aldehyde dehydrogenase (ALDH) activity is a hallmark of CSCs and is directly involved in CSC-associated resistance. ALDH+ breast cancer cells exhibit increased DNA repair abilities and higher survival in response to radiation exposure, associated with the stimulation of Nanog, BM1, Notch1 and Akt. For these reasons, identification of ALDH+ cells is generally considered to be a reliable marker for stem-like subpopulations. Moreover, the identification of ALDH as a key player in resistance to radiation therapy and tumor recurrence suggest that ALDH may be considered as a potential therapeutic target. Here, sorted ALDH+ CH-2879 cells exhibited a number of CSC distinctive features, such as lower ROS levels, increased self-renewing abilities (as indicated by sphere formation assay), enhanced invasiveness, radioresistance and in vivo tumorigenicity. This suggested that in addition to CD133, ALDH expression should also be an appropriate marker for the identification of stem-like radioresistant subpopulations in CS.

The relative biological efficiency (RBE) of the spread-out Bragg peak (SOBP) carbon-ion beam at the Heavy Ion Medical Accelerator in Chiba (HIMAC), relative to conventional X-rays, was within the previously observed range (1.5-2.5) in other experimental models. It was lower than the RBE of the monoenergetic carbon-ion beam at the Grand Accélérateur National d'Ions Lourds (GANIL). Although ALDH+ cells were more radioresistant than ALDH- cells, their respective RBEs (whether at D10 or at D37) were not significantly different. While carbon-ion beam alone may be more efficient against CSCs in some models, these results demonstrated that the treatment of CS should not rely solely on particle therapy and therefore combination treatments may be needed.

The relationship between CSCs and non-stem cancer cells (NSCCs) has been a matter of enormous attention. CSCs and NSCCs coexist in a highly dynamic, bidirectional equilibrium state, whose maintenance is under the control of a not fully understood molecular crosstalk between CSCs, NSCCs and the tumor microenvironment. microRNAs closely regulate pluripotency and differentiation mechanisms, and a number of CSC-associated microRNA regulations have been described. miR-34 is a well-known tumor-suppressor transcriptionally activated by p53, which has been associated with cancer stem cell homeostasis in several experimental models. mir-34 expression is downregulated in chondrosarcoma cell lines, compared to primary non-tumorous articular chondrocytes. Here, we showed that in CH-2879 CS cell line, administration of a miR-34 mimic was capable of decreasing stem-like radioresistant subpopulations.

Hundreds of direct miR-34 targets have been identified, with an over-representation of mRNAs involved in cell cycle control, DNA damage response and apoptosis. Notch homolog 1 (NOTCH1), C-MYC, Lemur Tyrosine Kinase 3 (LMTK3) and Krüppel-like factor 4 (KLF4) have all been identified as having a role in maintenance of self-renewal, chemoresistance, invasion and/or stem-like properties in cancer. KLF4, one of the so-called Yamanaka pluripotency factors, was described either as a tumor-suppressor or as an oncogene, depending on the cancer type. In osteosarcoma, KLF4 enhances proliferation and metastasis via alpha-crystallin B chain (CRYAB). In breast cancer, expression of KLF4 is determinant for the maintenance of CSCs and KLF4 seems to play a similar role in CS. Because KLF4 siRNA only partially recapitulated the effect of miR-34 on CSC-like phenotype, we can hypothesize that while KLF4 is a probably a major effector of miR-34 in CSCs, other pathways regulated by miR-34 are expected to be involved. As a matter of fact, miR-34 is able to suppress stem-like characteristics in breast cancer by downregulating Notch pathway. Furthermore, ALDH mRNA levels are reduced in tumor tissues of miR-34-treated mice. Because ALDHs are involved in ROS scavenging, miR-34 effects might rely on ROS accumulation, leading to increased radiosensitivity. Although miR-34 expression levels may not be directly correlated with survival in TCGA data of sarcoma patients, low expression of several miR-34 target genes (C-MYC, Cyclin-dependent kinase 4 – CDK4, Cyclin-dependent kinase 6 – CDK6, E2F Transcription Factor 3 – E2F3) is associated with better sarcoma survival. miR-34 therapy may be effective mainly as a combination with other treatment modalities.

Mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that is regulated in an extensive list of functions, including proliferation, survival, cytoskeleton organization or metabolism. mTOR is the catalytic subunit of two functionally distinct protein complexes: mTOR complex 1 (mTORC1) and mTORC2. The aberrant activation of mTOR activity is observed in multiple cancer types, resulting from phosphoinositide 3-kinase (PI3K) amplification/mutation, phosphatase and tensin homolog (PTEN) loss of function, or from the overexpression of Akt, Ribosomal protein S6 kinase beta-1 (S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1) or eIF4E. For this reason, mTOR pathway inhibition is regarded as an important target for the development of new cancer therapies. Phosphorylation of S6K1 was detected in 69% of conventional CS and 44% of dedifferentiated CS, suggesting that mTOR inhibition may be a good strategy for CS therapy.

Surprisingly, inhibition of mTORC1 by rapamycin lowered the proportion of CSCs. The role of S6K1 and eIF4eBP1 in mTORC1-mediated regulation of translation is well known. Moreover, forkhead box O (FOXO) transcription factors are crucial regulators of cellular homeostasis and are known tumor suppressors in human cancers. The complex interplay between FOXO, mTOR and Akt has been described. FOXOs decrease ROS levels and inhibit mTORC1 via Sestrin3. On the other hand, it was also reported that the mTOR pathway is capable of regulating FOXO3 activity by downregulating glucocorticoid-inducible kinase 1 (SGK1), which is responsible for FOXO3 phosphorylation. The inactivation of mTORC1 induced by p18 depletion led to FOXO3 hypophosphorylation at Ser314. Here, we showed that inhibition of mTORC1 led to increased FOXO3 promoter activity and that it directly led to the reversal of CSC-like phenotype. FOXO3 is a transcriptional regulator of miR-34 and its activation led to the inhibition of miR-34 targets like KLF4.

Therefore, inactivation of mTORC1 by rapamycin has direct effects on miR-34-associated pathways. Rapamycin treatment together with miR-34 mimic administration had a sustain inhibitory effect on CSC-like phenotype. However, the fact that the combination of miR-34 mimic and rapamycin administration is more potent than rapamycin alone suggests that alternative molecular mechanisms are also likely to be involved. In non-treated cells, only higher irradiation doses led to significant effects (such as the induction of cell death pathways). However, high dose exposures can lead to a relative CSC enrichment. By delivering a combination treatment, it was then possible to further decrease irradiation doses while efficiently suppressing CSC-like attributes.

Altogether, our results suggest that mTOR inhibition by rapamycin supplemented with miR-34 mimic treatment may be able to overcome CSC-associated radioresistance in chondrosarcoma during carbon-ion therapy. Combination treatments might also improve the effectiveness of carbon-ion therapies at lower doses, decrease risks of relapse and metastasis, and better preserve surrounding normal tissues against non-targeted effects.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Vares Guillaume、Ahire Vidhula、Sunada Shigeaki、Ho Kim Eun、Sai Sei、Chevalier Francois、Romeo Paul-Henri、Yamamoto Tadashi、Nakajima Tetsuo、Saintigny Yannick	４．巻 150
２．論文標題 A multimodal treatment of carbon ions irradiation, miRNA-34 and mTOR inhibitor specifically control high-grade chondrosarcoma cancer stem cells	５．発行年 2020年
３．雑誌名 Radiotherapy and Oncology	６．最初と最後の頁 253～261
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.radonc.2020.07.034	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Vares Guillaume, Sai Sei, Nenoi Mitsuru, Sugawara Hirotaka, Nakajima Tetsuo, Yamamoto Tadashi, Saintigny Yannick
２．発表標題 Addressing tumor resistance by combining CSC-targeting strategies and high-LET radiation therapy
３．学会等名 Japan Cancer Association Annual Meeting
４．発表年 2019年～2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関			
フランス	CEA	Dr Yannick Saintigny		