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An invited review following *the Soujinkai Young Investigator Award*: Calreticulin Is Highly Expressed in Pancreatic Cancer Stem-Like Cells

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Abstract Pancreatic cancer is an intractable disease with a poor prognosis. Recent research has demonstrated that the resistance of these cancers to conventional treatment, the high rate of local recurrence and distant metastasis may be attributed to a small subset of cells in cancer tissues known as cancer stem-like cells. It is critically important to elucidate the biological properties of cancer stem-like cells and develop strategies targeting these cells to overcome pancreatic cancers. We established a pancreatic cancer stem-like cell induction method from a cancer cell line and identified calreticulin as a highly expressed protein on the surface of these cells using proteomics and flow cytometry. Notably, we demonstrated that calreticulin was expressed on cells with high ATP-binding cassette transporter activity known to mediate drug efflux and related to poor outcomes in pancreatic cancer patients who underwent curative resection. Calreticulin exposure in cancer stem-like cells could be regulated by oxidative stress via hypoxia-inducible factors which induce the expression of CD47 and PD-L1 to evade the immune-surveillance of these cells. Further investigations on calreticulin expression in pancreatic cancer stem-like cells could elucidate the pathophysiology of these cells, leading to the development of novel therapy.

Key words: pancreatic cancer, cancer stem-like cells, calreticulin

Introduction: Pancreatic cancer and cancer stem-like cells

Pancreatic cancer is an intractable disease with a poor prognosis and is the fourth leading cause of cancer-related deaths in Japan (Center for Cancer Control and Information Services, National Cancer Center, Japan). The only curative option is surgical resection; however, most patients present with the locally advanced disease or systemic metastasis at diagnosis, with only 15 to 20% demonstrating

resectable tumors.[1] The major hallmarks of this cancer include resistance to conventional chemotherapy and radiation therapy and a high relapse rate after radical surgery. Recent advancements in chemotherapy, including gemcitabine plus nab-paclitaxel or mFOLFIRINOX (5-FU plus oxaliplatin plus irinotecan plus leucovorin), have improved the prognosis of unresectable pancreatic cancer; however, the median survival has been 8.5-13.5 months.[2-6]

Emerging evidence suggests that the resistance of cancer to conventional treatment and, consequently, the high rate of local recurrence and distant metastasis may be attributed to a small subset of cells in cancer tissues known as cancer stem-like cells (CSLCs).[7-9] CSLCs are defined by their tumor-initiating capacity, unlimited self-renewal, and ability to regenerate the cellular heterogeneity of the parental tumor after implantation into secondary recipients.[10] Moreover, CSLCs have been described to be intrinsically involved in cancer metastasis[11] and resistant to conventional treatment.[12,13] The activation of the epithelial-mesenchymal transition (EMT) program in CSLCs confers metastatic ability to CSLCs.[14,15] Moreover, therapeutic resistance has been attributed to several mechanisms, including the increased levels of ATP-binding cassette (ABC) transporters known to mediate drug efflux[16-19] and elevated antiapoptotic proteins. In addition, recent observations support that the EMT program also contributes to the development of resistance to molecular-targeted drugs and immunotherapy.[20] CSLCs were identified in hematological malignancies at first,[21] followed by solid tumors. Pancreatic CSLCs (P-CSLCs) was first identified by Li et al. in 2007 as tumor-initiating cells characterized with high expression of CD44, CD24, and epithelial-specific antigen (ESA).[22] To increase the efficiency of pancreatic cancer therapy and improve the disease outcome, it is imperative to determine the biological properties of CSLCs and develop CSLC-targeting strategies.

Although vigorous studies evaluating CSLCs have been performed for decades, their origin remains unknown. Recent studies have shown that the tumor microenvironment such as hypoxia, extracellular matrix, and inflammatory surroundings could make it possible to reversibly transit between CSLCs and non-CSLCs.[23]

The population of CSLCs in the tumor tissue is extremely low; therefore, it is difficult to obtain the requisite number of CSLCs necessary for basic research. To eliminate this bottleneck, we induced CSLCs from cancer cell lines by applying tumor microenvironments like nutrient starvation and extracellular matrix. Induced CSLCs expressed CSLC specific molecules, stemness gene, and EMT promoting transcriptional factors, and have demonstrated greater capacities of tumorigenesis and liver metastasis.[24-26]

Calreticulin in pancreatic cancer stem-like cells

In this study, we aimed to identify a molecule that is highly expressed in P-CSLC to be established as a novel therapeutic target. We observed that a chaperone protein calreticulin (CRT) is highly expressed in induced P-CSLCs compared to their parental cells by evaluating the protein expression profile using 2-D electrophoresis and tandem mass spectrometry.[27] CRT is a 46-65-kDa chaperone protein located in the endoplasmic reticulum, with diverse roles in cellular metabolism including Ca^{2+} homeostasis, cell adhesion, and HLA class I assembly. Flow cytometric analysis indicated that the expression of CRT on the surface of induced P-CSLCs was higher than that in the parental cells.[27] In contrast, the cytoplasmic expression of CRT did not differ between induced P-CSLCs and parental cells.[27] Some reports have shown that pre-incubation with N-acetyl-L-cysteine (NAC), a reactive oxygen species (ROS) scavenger, inhibits CRT translocation to the membrane induced by mitoxantrone, oxaliplatin, and ultraviolet C.[28] To examine whether NAC could decrease CRT expression, we treated induced P-CSLCs with 50 mM NAC for 24 h and observed that NAC significantly downregulated the surface expression of CRT, but not that of CD44v9 which is another marker of CSLCs.[27] These results implied that CRT exposure was regulated by ROS and oxidative stress.

Next, we sorted the induced P-CSLCs into three groups including $CRT^{high}/CD44v9^{low}$, $CRT^{low}/CD44v9^{high}$, and $CRT^{high}/CD44v9^{high}$ populations

by fluorescence-activated cell sorting, and analyzed the ABC transporter activity. Increased functional activity of the ABC transporters is characteristic of drug-resistant cancer cells.[29] In this analysis, the ABC transporter activity of $CRT^{high}/CD44v9^{low}$ cells was higher than that in $CRT^{low}/CD44v9^{high}$ cells or $CRT^{high}/CD44v9^{high}$ cells.[27]

Finally, we investigated the correlation between CRT expression in resected tumor samples from 80 patients who underwent curative resection and evaluated the clinical outcomes. Patients with high CRT expression had poorer recurrence-free and overall survival than those with low CRT expression.[27] Furthermore, Cox's regression analysis revealed that CRT expression, age, and postoperative chemotherapy or immunotherapy were independent prognostic factors in this cohort.[27]

Future Perspective

Currently, our research is assessing the stemness and pathophysiology of CRT positive cells in P-CSLCs. Although surface CRT acts as an "eat-me" signal facilitating innate immunity,[30] it has been observed that CRT overexpression is associated with poor survival in patients with several different cancers,[31-33] which is consistent with our results. Two possible explanations could explain this discrepancy.[34] First, cancer cells expressing surface CRT may resist phagocytosis by co-expressing CD47 as an anti-phagocytic signal. Although our results failed to establish a linear relationship between CRT and CD47 expression, conflicting with previous findings, CD47 levels in induced P-CSLCs were similar to those in parental cells.[27] Therefore, the role of CD47 expression in conferring immune privilege to P-CSLCs remains unclear. Second, CRT surface expression could contribute to an aggressive phenotype of cancer cells not associated with their resistance to phagocytosis.

A recent report has indicated that chemotherapy induces the expression of CD47, CD73, and PD-L1 in breast cancer to evade immune-surveillance via hypoxia-inducible factors (HIFs).[35] The expression of HIFs was also increased in our induced CSLCs.[25] Hence, it is possible that immunosuppressive signals like CD47 and PD-L1 could be expressed in CSLCs using the same mechanism.

Conclusion

CRT is highly expressed in P-CSLCs, which is related to poorer survival in pancreatic cancer patients after radical resection. Further investigations evaluating CRT expression on CSLCs could result in the development of novel therapeutic targets aimed at preventing the progression of pancreatic cancer.

Conflict of interest

The authors declare no conflict of interest.

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