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Plasma membrane expression of ZNF185 is a
prognostic factor in pancreatic ductal
carcinoma.

(ZNF185 の細胞膜への発現は膵管癌の
予後因子である)

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This doctoral thesis was written on the basis of the original article published in “Oncology Letters”.

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Summary

Pancreatic ductal carcinoma (PDC) is one of the most devastating diseases. We examined the hematogenous metastatic potentials of colonic cancer cell lines and PDC cell lines in an in vivo liver metastasis models using severely immune compromised (NOD/Shi-scid/IL-2R^γnull, NOG) mice. Colonic cancer cell line SW48 and PDC cell line BxPC-3 were isolated as weakly metastatic cancer cell lines. Highly metastatic sublines were created through serial xeno-transplantation of the metastatic foci in in vivo the NOG mouse liver metastatic models. To understand the mechanisms of hematogenous metastasis of PDC, we compared the protein expression profiles between weakly metastatic parent cell line (SW48 / BxPC-3) and highly liver-metastatic sublines (SW48LM2 / LM-BxPC-3). We identified ZNF185 as a molecule that was upregulated in both highly liver-metastatic cancer sublines. In prior clinicopathological studies of colon cancer patients, increased expression of Zinc finger protein 185 (ZNF185) was confirmed to be an independent prognostic factor through hepatic metastasis.

The aim of the present study was to evaluate the prognostic

significance of ZNF185 in PDC patient materials. First, we investigated the expression profiles of ZNF185 in PDC-xenografts by immunohistochemistry. Cancer xenografts showed positive features of ZNF185 in the cytoplasm (cZNF185) and on the cytoplasmic membrane (mZNF185), while nuclei (nZNF185) showed no ZNF185. The subcutaneous tumor nest of BxPC-3 expressed less cZNF185 in the central area (4.8%; range: 3.5–9.0%) than in the peripheral area (76.7%; range: 54.3%–97.6%). The subcutaneous xenografts also show decreased expression of mZNF185 with in the central area (0.5%; range: 0.3%–1.6%) compared with the peripheral area (7.5%; range: 2.7%–10.7%). All LM-BxPC-3 cancer cells expressed cZNF185 regardless of the area within the cancer nests. The liver metastatic xenografts showed decreased expression mZNF185 (28.2%; range:8.6%–35.9%) in the central area compared to the peripheral area (51.4%; range: 20.6%–68.5%). ZNF185-positive cells were more common in the peripheral area than the central area. ZNF185 expression was increased in liver-metastatic tumor nests (LM-BxPC-3) compared to BxPC-3 subcutaneous.

We examined the clinical significance of ZNF185 expression on pathological variables, recurrence sites, and prognosis in 182 resected PDC cases. cZNF185 expression were found in 93% of clinical samples, while mZNF185 and nZNF185 were expressed in 39% and 30% of clinical PDC cases, respectively. Venous involvement of PDC was significantly prevalent in mZNF185-positive cases, while cZNF185 and nZNF185

showed no remarkable clinical significance. Median overall survival was 21.3 months (95% confidence interval [CI]; 15.9–26.7) in mZNF185-positive cases, and 30.2 months (95% CI; 12.1–48.4) in mZNF185-negative cases ($P = 0.044$). Median disease-free survival was 10.7 months (95% CI; 7.9–13.4) in mZNF185-positive cases, and 12.9 months (95% CI; 7.9–13.4) in mZNF185-negative cases ($P = 0.038$). Patients with mZNF185-positive PDC experienced significantly shorter overall survival and disease-free survival periods than patients without mZNF185 expression. There were no significant clinical differences in overall survival or disease-free survival between patients with cZNF185- and nZNF185-expressing PDC. Multivariate analyses indicated that expression of mZNF185 was closely associated with shorter overall survival (hazard ratio: 1.51 (95% CI; 1.04–2.19), $P = 0.029$) and disease-free survival periods (hazard ratio: 1.43 (95% CI; 1.003–2.05), $P = 0.048$). Patients with mZNF185-positive cancer cells were more likely to develop hematogenous metastases (odds ratio: 1.82 (95% CI; 0.92–3.60), $P = 0.081$). ZNF185 might play an important role in hematogenous metastasis of PDC and may be a prognostic indicator.

Introduction

Pancreatic ductal carcinoma (PDC) is one of the most devastating types of disease, as early-stage tumors are difficult to detect but have a high potential for dissemination and distant metastasis. A previous autopsy study revealed that 70% of patients succumb to the disease due

to widespread metastasis, most frequently in the liver, peritoneum and lung, and that 30% succumb due to locally destructive disease. The control of hematogenous metastasis has been a long-standing goal for the prevention of cancer-associated mortality, but this has yet to be achieved.

Chemotherapy regimens for PDC have improved over the past few years, but the prognosis for patients with metastatic pancreatic cancer remains poor, with a median overall survival time of 8–12 months. Surgical resection is the only definitive treatment available, but the cancer recurs postoperatively in 80% of PDC patients. In approximately 20% of patients, recurrence and mortality occur within 1 year, and their survival time differs little from patients who did not undergo surgery. When lesions are identified preoperatively through diagnostic imaging, cases indicative of local disease must be differentiated from cases with occult distant metastases, which cause recurrence after surgery; pancreatectomy may successfully treat local lesions, but offers little benefit to patients with metastatic disease. Local disease and metastatic disease are associated with different treatment regimens and survival times. If identified, a metastasis-specific biomarker may better indicate when resection is appropriate.

In order to understand the underlying mechanisms of hematogenous metastasis in PDC, we compared molecular expression profiles between poorly metastatic subline and their highly liver-metastatic subline. When human colon cancer cell line SW48 and

Among these 8 proteins, we chose ZNF185 as a key protein in the subsequent procedures. First, the expression levels of these 8 proteins were evaluated immunohistochemically in 17 resected colon cancers. HEXA was expressed in 5 of 17 cases (29%), ZNF185 was positive in 12 cases (44%), SUB1 was positive in 7 cases (41%). GTFF1F2, ACTN4, IFL3, Gluco, and CTLC were expressed in all of 17 cases. Next, we evaluated expression levels of HEXA, ZNF185 and SUB1 in 87 resected colon cancers and explored the relationship between the expression of these three proteins and liver metastasis. Liver metastasis tended to occur more often in patients with ZNF185-positive cancer than in those with ZNF185-negative cancer ($P = 0.030$). Expression of HEXA and SUB1 was not correlated with liver metastasis. ZNF185 expression was found to be an independent prognostic indicator in colon cancer.

At present, the biological and clinical significance of ZNF185 in cancer has not been investigated extensively. Only a small number of studies have focused on ZNF185 in the context of cancer. In the present study, the clinicopathological significance of ZNF185 in PDC was investigated.

Discussion

The liver is the most common site for hematogenous metastasis from digestive organ cancer, in particular colon and pancreatic cancer. It is crucial to elucidate the biological mechanisms underlying liver

metastasis to reduce cancer-associated mortality. We identified ZNF185 as a liver metastasis-associated protein by quantitative proteome analysis utilizing poorly and highly liver-metastatic subline of colon and pancreatic cancer. We reported previously that ZNF185 expression was an independent indicator of liver metastasis and prognosis in patients with colon cancer. We reported here that the expression of ZNF185 in PDC was characterized clinicopathologically.

ZNF185 is located on chromosome Xq28 and is expressed in the kidney, prostate, pancreas, blood, placenta and ovary¹⁵. The complete ZNF185 gene was originally cloned from normal human prostate tissue¹⁶. ZNF185 contains two zinc-finger motifs in the C-terminus that fit the consensus pattern of LIM domains. The LIM domain is a cysteine- and histidine-rich double zinc-finger motif named after the three homeodomain proteins: Lin-1, Isl-1 and Mec-3. The LIM domain is present in a wide range of proteins whose functions include a number of fundamental biological processes, such as cytoskeleton organization and organ development. ZNF185 gene expression was shown to be downregulated in matched normal tissues from prostate cancer, craniocervical squamous cell carcinoma, and non-small-cell lung cancer. The ZNF185 gene expression levels in high-grade (Gleason score 9) prostate cancer cells were suppressed more compared to intermediate grade (Gleason score 6) prostate cancer cells. Thus, the dysregulation of ZNF185 gene expression appears to be a frequent event in several cancer types, which suggests its potential role in cancer

development. However, there are currently no published reports on ZNF185 in PDC. The clinical impact of ZNF185 on the prognosis or treatment of various cancer also remains unknown.

In the xenograft models, we observed a unique distribution of the ZNF185 molecule in cancer cells. ZNF185 expression was increased in liver metastatic tumor nests (LM-BxPC-3) compared with the subcutaneous nests of BxPC-3. ZNF185 positive cells were more common in the peripheral area than in the central area. It is suggested that both the amount and subcellular location of ZNF185 are correlated with the position of the cancer cells expressing it within cell nests. The periphery of cancer cell nests directly contacts the surrounding environment. Cancer cells change their highly differentiated and epithelial morphology to migratory and invasive phenotype in the peripheral area of cancer nest. The multistep process of cancer metastasis consists of proliferation, angiogenesis, invasion, detachment, circulation, adherence to vessel wall, extravasation, establishment of a microenvironment, and proliferation at the target organ. The epithelial-mesenchymal transition (EMT) is a key step in the progression of various cancers. The EMT is involved in the early steps of the metastatic cascade, where this process enables the invasion of carcinoma cells into the stroma and their translocation. EMT is reported to occur at the invasive front of several carcinomas, such as colorectal carcinoma and esophageal squamous carcinoma.

During the EMT process, cells downregulate their apical and

basolateral epithelial-specific tight and adhesion junction proteins such as E-cadherin and cytokeratin. Furthermore, cells re-express mesenchymal molecules such as vimentin and N-cadherin. The decreased expression of E-cadherin is a poor prognostic factor in pancreatic cancer and colorectal cancer. In this study, membranous ZNF185 (mZNF185) expression was more common in the peripheral area than the central area, as well as at liver metastatic nests versus parental subcutaneous tumor nests. These data suggest that mZNF185 may play a role in tumor behavior, including motility.

In clinical samples of PDC, patients with mZNF185 expression showed significantly shorter survival than patients without mZNF185 expression. Additionally, mZNF185-positive cancer cells were more likely to develop hematogenous metastases. It has been suggested that, in prostate cancer cells, ZNF185 is co-localized with F-actin and partially with paxillin and vinculin. F-actin, paxillin, and vinculin line the plasma membrane. In cancer cells undergoing on EMT, globular-actin (G-actin) polymerize and filamentous actin (F-actin) is formed. Newly formed actin filaments subsequently interact with binding proteins leading to the movement of actin fibers. This dynamic reorganization of the actin cytoskeleton is a prerequisite for an EMT. Both paxillin and vinculin are associated focal adhesion composed of several kinases, adaptors, and cytoskeletal proteins which couple the cell membrane to the actin cytoskeleton and extracellular matrix. The dynamics of focal adhesion turnover is a key determinant for the

regulation of cancer cell migration. Paxillin binds to numerous proteins involved in the organization of the actin cytoskeleton, which is necessary for motility events associated with tumor metastasis. The over expression of paxillin was related with distant metastases in colorectal cancer, salivary adenoid cystic carcinoma and hepatocellular carcinoma. Vinculin has also been implicated in cell invasion and metastasis. Vinculin was significantly downregulated in highly metastatic colorectal cancer cells. We speculate that ZNF185 serves a role in the acceleration of cancer invasion and metastasis, as well as in focal adhesion. To the best of our knowledge, only one previous study has indicated that ZNF185 functions as a tumor-suppressive protein through an association with actin cytoskeletal dynamics in prostate cancer. Further prospective studies must additionally investigate the involvement of mZNF185 in hematogenous metastasis in PDC.

In clinical samples of PDC, venous involvement was significantly prevalent in mZNF185-positive cases. Venous invasion of tumor cells is important for hematogenous metastasis. The liver is the most common site for hematogenous metastasis from colorectal and pancreatic cancer. Venous invasion was a significant risk factor for liver metastasis in colorectal cancer. Venous invasion was also reported to be a poor prognostic factor for pancreatic cancer. In this study, patients with mZNF185-positive cancer cells were more likely to develop hematogenous metastases. The association of mZNF185 expression with venous invasion and hematogenous metastases in

clinical samples supported the quantitative promote analysis data suggested that ZNF185 may be a liver metastasis-associated protein.

Both cytoplasmic ZNF185 (cZNF185) and mZNF185 was expressed more often in liver metastatic foci of LM-BxPC-3 than in the subcutaneous tumors of BxPC-3. We are interested in ZNF185 expression in liver metastatic foci of clinical sample. However, we could not obtain surgical specimens of liver metastatic tumor of PDC because liver metastasis of PDC is not an indication for resection. Therefore, we could not evaluate ZNF185 expression of liver-metastatic PDC in clinical sample.

Conclusion

The expression of membranous ZNF185 (mZNF185) is an independent indicator of unfavorable prognosis in patients with pancreatic ductal cancer (PDC), being particularly evident in highly liver-metastatic cancer cells located at the periphery of cancer nests in xenograft models. It is also suggested that mZNF185 may be involved in cancer cell motility, particularly hematogenous metastasis, and that the amount and the subcellular location of ZNF185 are each correlated with the location of the PDC cell nests.