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RAPID COMMUNICATION

Novel discovery of PDPN-positive CAFs contributing to tumor-associated lymphangiogenesis through mesenchymal to lymphatic endothelial transition in intrahepatic cholangiocarcinoma

Tumor-associated lymphangiogenesis is thought to be an important metastatic step in intrahepatic cholangiocarcinoma (ICC),[1] while the origin of tumor-associated lymphatic vessels within tumors is little known. Cancer-associated fibroblasts (CAFs) enhance tumor progression through many aspects.[2] CAFs have been discovered exhibiting cellular plasticity of phenotypic transition — such as epithelial–mesenchymal transition (EMT) — which promotes increased migratory and invasive capabilities of cells.[3] However, mesenchymal-to-endothelial (MEndT) transition is yet to be identified in tumor entities. Podoplanin (PDPN), a 38–44 kDa transmembrane glycoprotein, is conventionally expressed in lymphatic endothelial cells. Recently, PDPN has also been discovered as a marker of CAFs, which is associated with cancer cell migration, invasion, and clinical prognosis. In several types of tumors such as breast cancer and lung adenocarcinoma,[4,5] it was demonstrated that PDPN-expressing CAFs are correlated with tumor progression and poor prognosis. Based on abundant CAFs in ICC and PDPN expression in both CAFs and lymphatic endothelial cells, our study aims to investigate whether PDPN-positive CAFs could transform into lymphatic endothelial cells contributing to lymphangiogenesis in ICC.

In clinical data, 106 patients who underwent hepatic resection for ICC at our institution from 2007 to 2015 were retrospectively analyzed (Table S1). In the immunohistochemical staining, ICC cells were surrounded by a large number of fibroblasts which were known as CAFs (Fig. S1A). PDPN expression was detected in both CAFs and lymphatic vessels in ICC (Fig. S1B,C). $PDPN^+$ CAFs were confirmed in 58.5% (62/106) of patients with ICC, while the other 44 patients presented with $PDPN^-$ CAFs (Fig. 1A). Clinical analysis demonstrated that patients with $PDPN^+$ CAFs had a higher level of CA19-9 ($P = 0.007$), larger tumor size ($P = 0.034$), more tumors ($P = 0.029$), more frequent lymph node metastasis ($P < 0.001$), more diffused distribution ($P = 0.044$), and higher frequency of tumor recurrence ($P < 0.001$), which suggests that $PDPN^+$ CAFs are correlated with tumor progression (Fig. S2A and Table S2). Accordingly, patients with $PDPN^+$ CAFs exhibited significantly worse overall and recurrence-free survival rates compared with patients of $PDPN^-$ CAFs ($P < 0.001$) (Fig. 1B). In addition, univariate and multivariate analyses showed that PDPN positivity of CAFs served as one of the independent prognostic factors for overall and recurrence-free survivals (Fig. S2B,C and Table S3,4). To determine whether $PDPN^+$ CAFs translate into tumor-associated lymphangiogenesis, a quantitative analysis of micro-lymphatic vessel density (MLVD) was performed. It is noted that the proportion of MLVD was significantly higher in the cases with $PDPN^+$ CAFs ($P < 0.001$), which reveals that $PDPN^+$ CAFs may contribute to tumor-associated lymphangiogenesis in ICC (Fig. 1C and Table S5). In addition, we stratified the patients into high and low micro-lymphatic vessel density (MLVD) groups according to the Youden index of MLVD ($n = 11.5$) which provides the best prognostic value for survivals (Fig. S3A). Patients in the high MLVD group displayed much worse overall and recurrence-free survival rates as compared with the low MLVD group ($P < 0.001$) (Fig. S3B,C).

Then we isolated CAFs derived from the tumor tissues of different patients. Typical CAFs were characterized by a spindle or satellite shape morphology (Fig. S4A). Diverse

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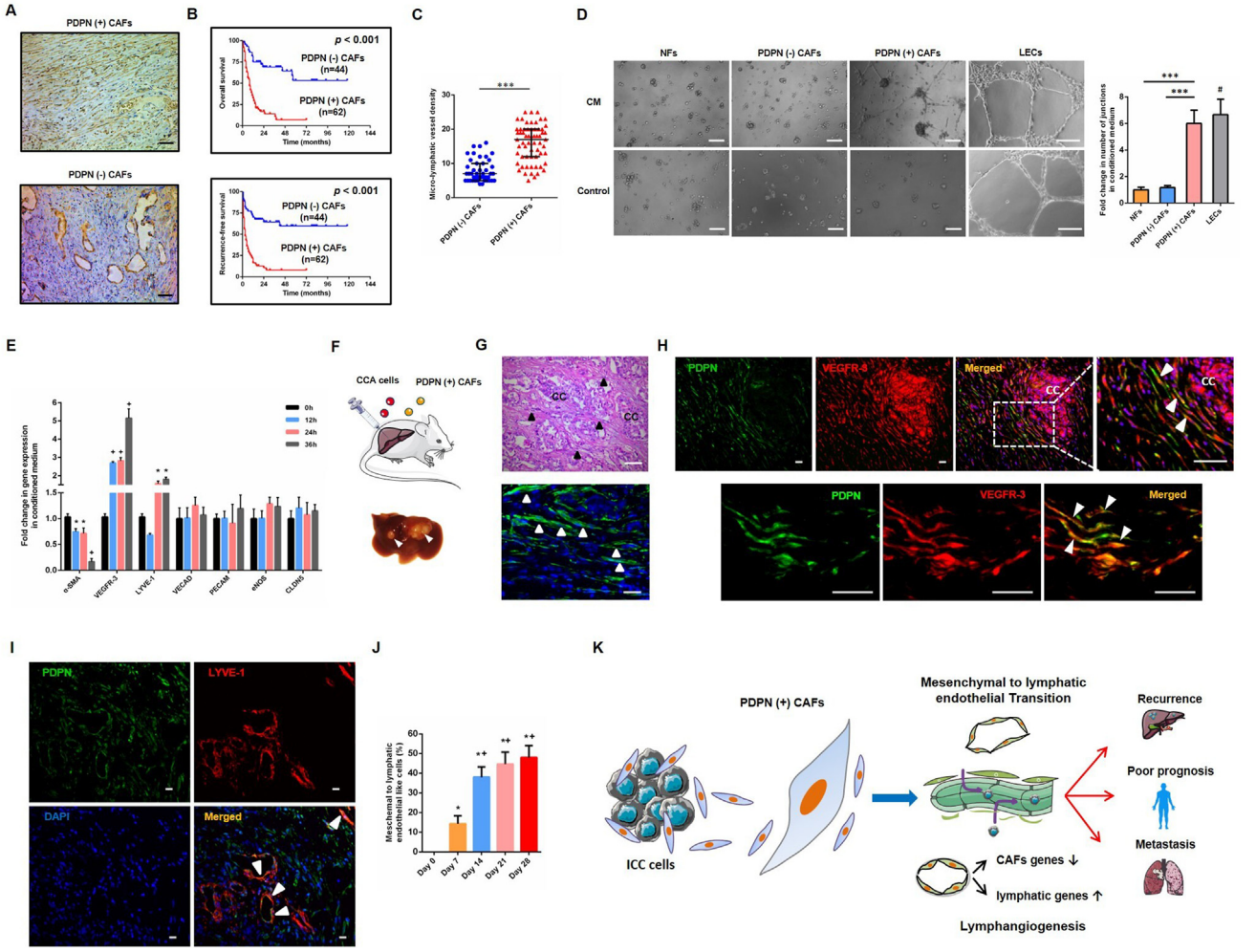

Figure 1 PDPN$^+$ CAFs transform into lymphatic endothelial cells through mesenchymal-to-endothelial (MEndT) contributing to lymphangiogenesis in ICC. **(A)** PDPN$^+$ CAFs (≥30%) were confirmed in 58.5% (62/106) of ICC patients, while the other 44 patients presented with PDPN-negative CAFs (<30%). **(B)** Patients with PDPN$^+$ CAFs exhibited significantly shorter overall and recurrence-free survival rates compared with patients of negative CAFs ($P < 0.001$). **(C)** The proportion of micro-lymphatic vessel density (MLVD) was significantly higher in the cases with PDPN$^+$ CAFs ($P < 0.001$). **(D)** PDPN$^+$ CAFs grown in a conditioned medium (CM) during the tube formation assay showed robust tube formation, while PDPN$^-$ CAFs and NFs did not form tubes. Normal lymphatic endothelial cells (LECs) cultured in CM and controlled medium showed comparable tube formation. Scale bar = 10 μm. A significant increase in the number of tube junctions was observed in PDPN$^+$ CAFs ($^{***}P < 0.001$) in CM. No significant difference in tube junctions was observed between LECs and PDPN$^+$ CAFs cultured in CM ($^{\#}P > 0.05$). **(E)** PDPN$^+$ CAFs cultured in CM exhibited decreased α-SMA mRNA levels and increased levels of PDPN and LYVE-1 as time increased ($^*P < 0.05$ versus 0 h, $^+P < 0.01$ versus 0 h). While vascular endothelial gene expression (VECAD, PECAM, eNOS, and CLDN5) had no significant change. **(F)** Mixed cells of PDPN$^+$ CAFs and cholangiocarcinoma cells were injected into the livers of mice to establish the orthotopic tumor model. **(G)** The harvested tumors were characterized by abundant CAFs surrounding cancer cells. Scale bar = 10 μm. **(H)** On the 20th day since implantation, PDPN$^+$ CAFs labeling lymphatic endothelial marker (VEGFR-3) were found surrounding cancer cells. Scale bar = 10 μm. **(I)** Double-positive cells expressing PDPN and LYVE-1 demonstrating the shape and characteristics of lymphatic vessels were discovered on the 30th day after implantation. Scale bar = 10 μm. **(J)** A significantly increased proportion of PDPN$^+$ CAFs in tumor stroma evolved into lymphatic endothelial cells as time increased ($^*P < 0.05$ versus 0 h). **(K)** Schematic illustration of MEndT in ICC, which involves a progressive loss of fibroblast markers and gain of lymphatic endothelial cell markers, contributing to tumor-associated lymphangiogenesis and poor prognosis.

levels of PDPN were expressed in CAFs by real-time PCR (Fig. S4B), which was consistent with flow cytometric analysis, with a range of 4.5%—92% positivity (Fig. S4C). In addition, co-localization of immunofluorescence staining also showed isolated CAFs were partly stained for PDPN, confirming the heterogeneous expression of PDPN in CAFs (Fig. S4D). To determine whether CAFs could transform into lymphatic endothelial-like cells *ex vivo*, we isolated PDPN$^+$ CAFs from primary cells by immunoreactivity. We then seeded different subgroups of CAFs on matrigel and subjected them to a conditioned medium (CM). Normal lymphatic endothelial cells (LECs) served as a positive

control. In contrast to NFs and PDPN$^-$ CAFs, PDPN$^+$ CAFs cultured in CM formed tube-like structures. The cultured positive cells showed a significantly increased number of tube junctions ($P < 0.001$). While LECs cultured in CM and controlled medium presented comparable tube formation. No significant difference in tube junctions was observed between LECs and PDPN$^+$ CAFs cultured in CM (Fig. 1D). To further evaluate whether the tube-like cells were vascular or lymphatic endothelial cells, specific markers were tested. It turned out that tube formation was associated with decreased α-SMA mRNA levels and increased levels of lymphatic markers (VEGFR-3 and LYVE-1) as time increased, while vascular endothelial gene expression (VECAD, PECAM, eNOS, and CLDN5) had no significant change (Fig. 1E). The results provide evidence for lymphatic endothelial cells derived from PDPN $^+$ CAFs *in vitro*. Then mixed cells of PDPN $^+$ CAFs and cholangiocarcinoma cells were injected into the livers of mice to establish the orthotopic tumor model (Fig. 1F). Tumors were harvested every 10 days for triple mice since implantation. The harvested tumors were characterized by abundant CAFs surrounding cancer cells (Fig. 1G). On the 20th day, PDPN$^+$ CAFs labeling the lymphatic endothelial marker VEGFR-3 were found surrounding cancer cells (Fig. 1H). Furthermore, double-positive cells expressing PDPN and LYVE-1 demonstrating the shape and characteristics of lymphatic vessels were discovered on the 30th day after transplantation (Fig. 1I). Eventually, a significantly increased proportion of PDPN-positive CAFs in tumor stroma evolved into lymphatic endothelial cells as time increased ($P < 0.01$) (Fig. 1J). Thus, we provide a dynamic process for lymphatic vessels derived from CAFs in *in vivo* experiments.

In this study, we demonstrated that highly expressed PDPN$^+$ CAFs were correlated with increased lymphangiogenesis within tumors and significantly worse prognosis in ICC. Then we showed that isolated PDPN$^+$ CAFs adopt a phenotype of lymphatic-like cells with increased levels of lymphatic markers and a decreased level of CAF marker *in vitro*. Further, in the orthotopic tumor model, PDPN$^+$ CAFs demonstrating the shape of lymphatic vessels and labeling lymphatic endothelial markers were discovered in tumors. Collectively, our results provided evidence that PDPN$^+$ CAFs may transform into lymphatic endothelial cells through MEndT contributing to lymphangiogenesis in ICC (Fig. 1K).

Author contributions

MS and JC designed the research; MS and SJ performed research; NX and CC analyzed data; MS, HLH, and CS wrote the paper; YT and JC reviewed the research.

Conflict of interests

All authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.023.

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