Expression of CD24, a Stem Cell Marker, in Pancreatic and Small Intestinal Neuroendocrine Tumors

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ABSTRACT

Objectives: CD24 has been considered a normal and cancer stem cell marker. Potential intestinal stem cells weakly express CD24. In the pancreas, CD24 is a possible cancer stem cell marker for ductal adenocarcinoma.

Methods: Expression of CD24 in intestinal and pancreatic neuroendocrine tumors (NETs) was examined. Immunohistochemistry was performed on benign duodenum, ileum mucosa, and pancreas, as well as primary duodenal, primary and metastatic ileal, and pancreatic NETs.

Results: Scattered CD24-positive cells were noted in the duodenal and ileal crypts, most of which showed a strong subnuclear labeling pattern. Similar expression was observed in 41 (95%) of 43 primary ileal NETs but in only four (15%) of 26 duodenal NETs (P < .01). In addition, metastatic ileal NETs retained CD24 expression. Pancreatic islets did not express CD24, and only rare cells had subnuclear labeling of CD24 in the pancreatic ducts. Unlike ileal NETs, only five (5%) of 92 pancreatic NETs expressed CD24 in the subnuclear compartment (P < .01). All five NETs showed a unique morphology with prominent stromal fibrosis.

Conclusions: CD24 expression was frequent in primary and metastatic midgut NETs but rare in pancreatic and duodenal NETs. Expression of CD24 in ileal NETs may have future diagnostic and therapeutic implications.

Well-differentiated neuroendocrine tumors (NETs) of the gastroenteropancreatic system comprise approximately two-thirds of total NETs with an annual incidence rate of three to five cases per 100,000 persons.1-4 Based on embryonic source of the organ, they are classified into three groups: foregut, midgut, and hindgut. Foregut NETs include those arising in the stomach, pancreas, duodenum, and proximal jejunum. Midgut NETs are always located in the distal jejunum and ileum. Although rare, small intestinal and pancreatic NETs are the second most common malignant neoplasms in the small intestine and in the pancreas, respectively.5,6 In addition, both tumors are frequently associated with liver metastasis. Most patients die of hepatic failure due to extensive liver involvement by metastases.7-9

Our understanding of gastroenteropancreatic NETs has grown considerably in recent years. The pathogenesis of foregut NETs is distinct from that of midgut NETs. For example, MEN1 mutations are frequently detected in duodenal and pancreatic NETs, but they are rare in midgut NETs. Nevertheless, all NETs are thought to originate from a variety of neuroendocrine cell types or progenitor cells.10 There are more than 10 types of neuroendocrine cells in the gut. Midgut NETs arise from enterochromaffin cells, which are the major neuroendocrine cell type of the small intestine.3 In the duodenum, gastrin-producing and somatostatin-producing cell hyperplasia precedes the development of gastrinoma and somatostatinoma, respectively.4 Pancreatic NETs were thought to derive from islet cells; however, recent studies suggest that they may arise from multipotent pancreatic stem/progenitor cells residing within the ductal epithelium.11
CD24, a heat-stable antigen, has been recognized as both a normal and a malignant stem cell biomarker.\textsuperscript{12,13} Intestinal stem cells weakly express CD24,\textsuperscript{14} while neighboring cells such as Paneth and neuroendocrine cells in the small intestine express higher amounts of CD24.\textsuperscript{12,14-17} In the pancreas, CD24/PDX1-positive progenitor cells were shown to differentiate into insulin-producing cells.\textsuperscript{18} In addition, CD24 may be one marker of cancer stem cells for intestinal and pancreatic ductal adenocarcinomas.\textsuperscript{7,12,19-22} Furthermore, CD24 expression was associated with a poor prognosis and chemotherapy resistance in pancreatic cancer.\textsuperscript{23,24}

Despite extensive studies on CD24 as a stem cell marker, its expression in intestinal and pancreatic NETs remains unknown. In this study, we wanted to determine CD24 expression in pancreatic and small intestinal NETs.

Materials and Methods

CD24 expression was immunohistochemically assessed in five tissue microarrays (TMAs) containing 92 pancreatic NETs (including six cases with both primary and liver metastasis) and 32 midgut (jejunal/ileal) NETs (including seven with both primary and liver metastasis). Immunohistochemical labeling for CD24 was also performed on 26 duodenal NETs, either from endoscopic mucosal resection or Whipple resection. To further examine whether CD24 expression was preserved in liver metastasis, we included an additional 11 jejunal/ileal cases with both resected primary and metastatic tumor in the study. In addition, 13 liver metastases from patients with pancreatic NETs (six wedge resections and seven biopsy specimens) were immunohistochemically labeled with CD24. Among the 13 cases, seven had primary tumor in the TMAs, one had both resected primary and metastasis, and five had liver tumor only. This study was approved by Vanderbilt Institutional Review Board.

We deparaffinized by routine methods 4-\mu mol/L unstained slides from the TMAs and formalin-fixed, paraffin-embedded resection/biopsy specimens. For antigen retrieval, the sections were heated to 105°C for 20 minutes in a pH 9.0 EDTA buffer and then allowed to cool to room temperature. After the retrieval, the tissue sections were quenched with 3% H\textsubscript{2}O\textsubscript{2} in sodium azide for 5 minutes at room temperature. Anti-CD24 antibody at 1:100 dilution (ab118070; Abcam, Cambridge, MA) was then incubated with the tissue sections, followed by antibody localization using the DAKO Envision\textsuperscript{+} HRP-labeled polymer (DAKO, Carpenteria, CA). Staining was visualized by 5-minute incubation with diaminobenzidine.

Tumors with greater than 5% of the tumor cells demonstrating subnuclear concentrated cytoplasmic CD24 labeling were considered positive. The immunohistochemical stains were read by two pathologists (S.S. and C.S.).

Results

Expression of CD24 in the Small Intestine and Small Intestinal NETs

Consistent with observations by others,\textsuperscript{14} we found CD24 expression by normal small intestine epithelial cells. Scattered CD24-positive cells were seen in normal intestinal crypts but rare in the intestinal villi. Scattered CD24-positive cells were seen in normal intestinal crypts but rare in the intestinal villi. Figure 1A. Several labeling patterns were found, including weak membrane and supranuclear, subnuclear, and diffuse cytoplasmic staining; however, most of the CD24-positive cells displayed strong subnuclear cytoplasmic labeling Figure 1B.

Next, we immunohistochemically labeled small intestinal NETs with anti-CD24 antibodies. Forty-one (95%) of 43 midgut NETs showed moderate to strong CD24 expression (Table 1). The CD24 labeling was characteristically located in the subnuclear compartment Figure 2A, and the staining pattern was similar to that observed in some CD24-positive cells in normal small intestine, suggesting that these CD24-positive cells in normal intestinal epithelium initiated cells for midgut NETs. It appeared that the liver metastases retained CD24 expression Figure 2B. Only one (6%) of 18 liver metastases had negative CD24 labeling; in this case, the corresponding primary tumor also lacked CD24 expression. Primary tumors and their respective metastases showed CD24 positivity in more than 50% of tumor cells, with moderate to strong subnuclear labeling. This pattern was observed in all but two of the cases. The liver metastases demonstrated CD24 labeling that was always stronger at the periphery than in the center of the lesion.

The adjacent benign intestinal mucosa was also evaluated for CD24 expression. With the exception of one case, there was no evident increase in CD24-expressing cells. In this one case, there was a clinical history of Crohn disease, and the NET appeared to arise in a background of inflammatory bowel disease. The adjacent mucosa showed a prominent increase in CD24-positive cells; however, the unaffected normal mucosa showed only scattered CD24-positive cells Figure 2C and 2D.

CD24 expression in duodenal NETs was also examined. Twenty-six duodenal NETs included 14 from the duodenal bulb, four from the second portion, five from the ampulla/papilla, two from the distal duodenum, and one from an unknown segment. Although the duodenal crypts had CD24-positive cells, duodenal NETs infrequently expressed CD24 compared with midgut NETs (P < .01). Only four (15%) of the 26 tumors expressed CD24 Figure 3A and 3B. (Table 1). Of the four tumors, two were from the ampulla and two from the duodenal bulb. There were no morphologic differences between CD24-positive tumors and those that lacked expression.
Expression of CD24 in the Pancreas and Pancreatic NETs

CD24-positive cells were not observed in the islets of Langerhans. Previous studies reported frequent CD24 expression in pancreatic ductal adenocarcinoma; however, CD24-positive cells were rare in benign pancreatic ducts.\textsuperscript{23,25} Immunohistochemical labeling of 20 pancreatic sections detected only two cells in the pancreatic ducts showing subnuclear CD24 expression \textbf{Image 4A}.

Only five (5\%) of 92 pancreatic NETs demonstrated moderate to strong CD24 expression (Table 1). The labeling was also predominantly located in the subnuclear region of the tumor cells, as seen in the small intestine NETs. A group of pancreatic NETs (9\%3 [10\%]) displayed weak and diffuse cytoplasmic staining \textbf{Image 4B}. When we examined benign acinar and islet cells in some cases, we also found a similar pattern of weak cytoplasm staining. Therefore, this pattern of labeling was considered nonspecific. None of the positive cases had corresponding liver metastasis. However, 18 liver metastases, including eight with CD24-negative primary tumor, showed no CD24 expression.

The histology of the five CD24-positive pancreatic NETs was further examined. Interestingly, these tumors all shared unique morphologic features, including dense fibrosis, small nests/tubules, and an infiltrative growth pattern. In addition, they frequently surrounded a large pancreatic duct, causing pancreatic duct stenosis and chronic pancreatitis in the surrounding pancreas \textbf{Image 4C} and \textbf{Image 4D}. This variant of pancreatic NET has been described in previous studies. They shared some features of midgut NETs, including serotonin and CDX2 expression.\textsuperscript{26-28}

\textbf{Discussion}

CD24 expression has been studied in small intestinal crypts and pancreatic ductal adenocarcinomas. It has been identified as a marker of intestinal stem cells. CD44+/CD24 low/CD166+ cells were identified as putative intestinal stem cells in both mouse and human, and single CD44+/CD24 low/CD166+ cells were shown to give rise to endoenteroids containing multiple intestinal epithelial lineages, including neuroendocrine cells, Paneth, and goblet cells.\textsuperscript{14} CD44+/CD24 high/CD166+ cells isolated from mouse intestine were predominantly nonproliferative secretory cells displaying markers of enteroendocrine, Paneth, and goblet cells. The mature neuroendocrine cells derived from the CD44+/CD24 low/CD166+ cell strongly expressed CD24 in mouse models. In humans, scattered
CD24-positive cells are present in both duodenal and ileal epithelium. Some have been identified as neuroendocrine cells by previous studies.\textsuperscript{14,17} Interestingly, we observed a similar pattern of CD24 expression in a majority of midgut NETs, but this was not a frequent observation in duodenal NETs (95% vs 14%). Midgut NETs demonstrated a predominantly strong subnuclear CD24 labeling pattern. This unique immunohistochemical labeling was also observed in liver metastases from the primary CD24-positive midgut NETs (94%). These data suggest that CD24-expressing cells in the intestinal crypts are the precursor cells of most intestinal NETs.

In all but one case, the adjacent mucosa showed no increase in CD24-expressing cells; however, this case in a patient with inflammatory bowel disease showed prominent CD24-positive cells. These data confirm that most small intestinal NETs are not initiated by way of the hyperplasia and dysplasia pathway.

The cell of origin of pancreatic NETs is unclear. A small subpopulation of human PDX-1 and nestin-expressing pancreatic stem/progenitor cells isolated from an adult human pancreatic duct were shown to be capable of differentiating into insulin-, glucagon-, and somatostatin-positive cells in vitro in the presence of specific growth factors.\textsuperscript{25} In addition, the human CXCR4-positive cells expressing different stem cell markers isolated from the islet-depleted pancreas were able to form islet-like structures.\textsuperscript{29} These observations suggest that adult stem/
progenitor cells residing in the adult pancreatic duct give rise to pancreatic endocrine cells, and genetic and/or epigenetic alterations in these cells may potentially transform them into pancreatic NETs.

In adult mice, CD24-negative pancreatic ductal cells differentiated into insulin-secreting cells; however, CD24 expression in human adult pancreatic ducts had not been explored previously. We noted that CD24-expressing cells were extremely rare in the pancreatic duct. In addition, islet cells do not express CD24. These findings may help to explain why most pancreatic NETs (95%) did not demonstrate any CD24 expression. Consequently, a lack of CD24 expression was also noted in all the liver metastases from the pancreatic NETs.

In particular, those pancreatic NETs demonstrating CD24 expression were morphologically distinct from CD24-negative pancreatic NETs. While the CD24-negative pancreatic NETs were hypercellular with minimal stromal fibrosis, CD24-positive pancreatic NETs showed histomorphologic overlap with small intestine NETs. These tumors were often adjacent to large ducts and demonstrated dense fibrotic bands with infiltrative nests and tubules of neoplastic cells. Although it remains unclear if the CD24-positive cells in the pancreas are indeed neuroendocrine cells, the possibility that these tumors may derive from the rare CD24-positive cells seen in the pancreatic duct should be considered. Their rarity precludes a definitive determination of origin by immunofluorescence double staining.

CD24 expression can be either cytoplasmic or membranous in malignancies. In pancreatic ductal adenocarcinoma, CD24 and CD44 expression has been associated with poor prognosis in patients, although the effect was small and was lost when analyzing CD44, CD24, and EpCAM triple-positive cells. Cytoplasmic CD24 expression was associated with shortened patient survival in colorectal cancers. All CD24-expressing NETs in this series had cytoplasmic labeling in the subnuclear compartment. Recently, intracellular CD24 expression was shown to inhibit the specific endoribonuclease activity of G3BP, a phosphorylation-dependent endoribonuclease. In addition, the study showed that intracellular CD24 inhibits cell invasion by posttranscriptional regulation of BART through interaction with G3BP. However, whether cytoplasmic expression of CD24 in small intestine NETs confers an indolent clinical course requires further study.

Immunohistochemical studies for CD24 expression might have potential clinical implications. Ninety-five percent of midgut NETs expressed CD24, whereas only 5% of pancreatic and 15% of duodenal NETs had CD24 expression. Importantly, metastatic midgut NETs retained subnuclear CD24 expression. Thus, CD24 proves to be a useful biomarker in determining the origin of metastatic NETs.

**Conclusion**

The incidence of pancreatic and small intestinal NETs has consistently risen over the past decade. Currently, in North America and Europe, in terms of gastrointestinal malignancies, it is second in prevalence only to colorectal carcinoma. In summary, CD24 may be used as a novel marker for identifying midgut NETs as well as a subset of pancreatic NETs with a unique histomorphologic pattern of disease. This distinction is important, since therapeutic...
options depend on the location of the primary tumor. In addition, CD24-reactive NETs could be prospective candidates for anticancer stem cell therapeutic regimens.

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