Liver-Intestine Cadherin Expression Is Associated with Intestinal Differentiation and Carcinogenesis in Intraductal Papillary Mucinous Neoplasm

Katsuya Morimatsu^a Shinichi Aishima^a Tadashi Kayashima^b Akifumi Hayashi^a
Kohei Nakata^a Yasunori Oda^a Tomoaki Taguchi^c Masazumi Tsuneyoshi^a
Masao Tanaka^b Yoshinao Oda^a

Departments of ^aAnatomic Pathology, ^bSurgery and Oncology, and ^cPediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Key Words

Liver-intestine cadherin • Intraductal papillary mucinous neoplasm • Pancreas • Intestinal-type intraductal papillary mucinous neoplasm • Carcinogenesis

Abstract

Objectives: Intraductal papillary mucinous neoplasms (IPMN) are classified into four phenotypes according to the WHO classification. Recently, intestinal-type IPMN has been suggested to grow with a distinct carcinogenetic pathway. Like mucin 2, oligomeric mucus/gel forming (MUC2) and caudal-related homeobox 2 (CDX2), liver-intestine cadherin (LI cadherin) is an intestine-specific marker. We aimed to investigate the roles of LI cadherin expression in IPMN. **Methods:** We examined LI cadherin expression in 135 cases of IPMN by immunohistochemical staining and the quantitative real-time reverse-transcription polymerase chain reaction. **Results:** LI cadherin protein and mRNA levels were significantly higher in intestinal-type IPMN than in nonintestinal-type IPMN (protein level, $p < 0.001$; mRNA level, $p = 0.0312$). A positive correlation was found between protein and mRNA of LI cadherin ($p = 0.0037$). The positivity rates of LI cadherin expression were significantly higher in CDX2-positive cases than in CDX2-negative cases ($p < 0.001$). In 41

intestinal-type IPMNs, LI cadherin-positive rates tended to increase gradually, from IPMN with low-grade dysplasia (IPMN-L) to IPMN with an associated invasive carcinoma (IPMN-IC) [IPMN-L vs. IPMN with high-grade dysplasia (IPMN-H); $p = 0.0357$, IPMN-L vs. IPMN-IC; $p = 0.0230$] and positively correlated with the Ki-67 labeling index ($p = 0.0408$), whereas this tendency was not recognized in nonintestinal-type IPMNs. **Conclusions:** LI cadherin is associated with an intestinal phenotype and an 'intestinal pathway' of carcinogenesis in IPMN.

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Introduction

Intraductal papillary mucinous neoplasms (IPMNs) are characterized by the intraductal proliferation of neoplastic mucinous cells arranged in papillary structures and by the cystic dilation of pancreatic ducts detectable macroscopically; IPMNs are becoming increasingly recognized through worldwide consensus on both the entity and its diagnosis [1–3]. They have a wide spectrum of atypical grades, ranging from low-grade dysplasia to invasive carcinoma [3, 4], and are classified into the following groups according to the new WHO classification sys-

Fig. 1. MUC1, MUC2, and MUC5AC expression in intestinal-type IPMN. Intestinal-type IPMNs are negative for MUC1 (a) but positive for MUC2 (b) and MUC5AC (c).

tem [1]: IPMN with low-grade dysplasia (IPMN-L), IPMN with intermediate-grade dysplasia (IPMN-I), IPMN with high-grade dysplasia (IPMN-H) and IPMN with an associated invasive carcinoma (IPMN-IC).

IPMNs are also subdivided into four groups – gastric (G type), intestinal (I type), pancreatobiliary (PB type) and oncocytic type (O type) – based on morphological features and immunohistochemical findings [1, 5]. Adsay et al. [6, 7] have suggested that intestinal-type IPMN grows to colloid carcinoma with a distinct ‘intestinal pathway’ of carcinogenesis associated with intestinal-related genes, i.e. caudal-type homeobox 2 (CDX2) and mucin 2, oligomeric mucus/gel forming (MUC2). We recently reported that the regenerating islet-derived gene family, member 4 (REG4), was associated with the ‘intestinal pathway’ of carcinogenesis [8].

Liver-intestine cadherin (LI cadherin), also called cadherin-17 (CDH-17) or human transporter-1 (HPT-1), is distinguished from other cadherins by its unique structural and functional features [9, 10]. In classical cadherins, such as E-, N-, and P-cadherins, the cytoplasmic domain consists of 150–160 amino acids. In contrast, LI cadherin has a short cytoplasmic domain consisting of only 20 amino acids. Because of its short cytoplasmic domain, LI cadherin does not interact with the catenin network or the actin cytoskeleton [11]. In mice and humans, LI cadherin is expressed only in the epithelial cells of the small and large intestine [12, 13]. LI cadherin expression has been investigated in various malignant tumors, such as gastric [14–16], hepatocellular [17], colorectal [18], pancreatic [19] and intrahepatic cholangiocellular [20] carcinoma. In gastric and hepatocellular carcinoma, LI cadherin is overexpressed and associated with lymph node metastasis and poor prognosis [14, 15, 17], but lower ex-

pression of LI cadherin is associated with lymph node metastasis, dedifferentiation and poor prognosis in colorectal, pancreatic and intrahepatic cholangiocellular carcinoma [18–20]. In gastric carcinoma, LI cadherin is associated with intestinal phenotype [16]. However, LI cadherin expression in pancreatic IPMN has not yet been investigated.

In this study, we aimed to understand the roles of LI cadherin expression and investigated the relationships between LI cadherin expression and the intestinal phenotype or atypical grade in IPMN.

Materials and Methods

We obtained 135 formalin-fixed tissue samples of IPMNs that had been diagnosed at the Department of Anatomic Pathology (Pathological Science, Graduate School of Medical Science, Kyushu University, Japan) between July 1986 and May 2010. Our study protocol was accepted by the Institutional Review Board of Kyushu University (22-81) and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. For strict privacy protection, identifying information was removed from all samples before analysis and classified into four groups: IPMN-L (n = 54), IPMN-I (n = 30), IPMN-H (n = 24), and IPMN-IC (n = 27), according to the WHO classification system [1]. We evaluated the lesions showing the highest degrees of architectural and cytological atypia because IPMNs showed heterogeneous atypia in the same samples. If the lesion showed two different phenotypes and the same degree of dysplasia in the different phenotypes (e.g. intestinal-type IPMN with intermediate-grade dysplasia and gastric-type IPMN with intermediate-grade dysplasia), we evaluated the dominant component of the lesion and classified the case into the dominant phenotype. All samples were also classified into four groups: gastric (n = 70), intestinal (n = 41), pancreatobiliary (n = 9), and oncocytic types (n = 5), based on morphological features and immunohistochemical findings of MUC1, MUC2 (fig. 1), MUC5AC and CDX2. IPMNs that could not be catego-

rized specifically into any of these subtypes were placed into an unclassified type (n = 10).

Immunohistochemical Staining and Evaluation

Immunohistochemical staining was performed by the streptavidin-biotin-peroxidase complex method. Briefly, 4-μm-thick tissue sections were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H₂O₂ for 30 min. Antigen retrieval was achieved by microwave heating in 10 mM citrate buffer (pH 6.0) for 10 min (LI cadherin and Ki-67) or 20 min (CDX2). After nonspecific binding of the antibodies was blocked by 10-min incubation in 10% rabbit serum, the slides were incubated with primary antibodies at 4°C overnight. We used the following primary antibodies: goat polyclonal anti-LI cadherin (sc-6978; 1:200 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-CDX2 (EA10; 1:100 dilution; BioGenex) and mouse monoclonal anti-Ki-67 (MIB-1; 1:50 dilution; R&D Systems). The specificity of the anti-LI cadherin antibody we used was confirmed in a previous report [21]. The labeled antigens were detected with a Histofine SAB-POkit (Nichirei Pharmaceutical) and visualized by 3,3'-diaminobenzidine tetrahydrochloride as a chromogen and completed by counterstaining with hematoxylin. We used colonic mucosa as positive control and colonic mucosa without the primary antibody as negative control.

To evaluate the expression of LI cadherin and CDX2, we used the following scoring scale: negative = 0; <33% = 1+; <66% = 2+; >67% = 3+, and a score >2+ was considered positive for LI cadherin and CDX2. The Ki-67 labeling index was calculated by counting Ki-67-positive nuclei among 1,000 tumor cells, according to a previous report [22]. In IPMNs-IC, all immunohistochemical expressions were evaluated based on the invasive component.

Microdissection-Based Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

Nineteen frozen tissue samples of 135 cases were cut into 8-μm sections and subclassified into four groups according to the morphological and immunohistochemical examination: intestinal (n = 7), gastric (n = 6), pancreatobiliary (n = 4) and oncocytic (n = 2). The total RNA of tumor cells was collected selectively from frozen tissue samples using a laser microdissection and pressure catapulting system (PALM Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols [23, 24].

We designed specific primers using primer 3 as follows: LI cadherin, 5'-CCCAATGGCCAGCTTTATTA-3' (forward) and 5'-GCTCCCGTTTGTGTGTTGAT-3' (reverse); 18S ribosomal RNA, 5'-GTAACCCGTTGAACCCATT-3' (forward) and 5'-CCATCCAATCGGTAGTAGCG-3' (reverse) and performed BLASTN searches to conform to the primer specificity.

A quantitative real-time reverse transcription-polymerase chain reaction was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) with a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories) for 40 cycles for 15 s at 95°C and for 1 min at 55°C according to the manufacturer's instructions [25]. The level of mRNA in each sample was calculated from a standard curve constructed with total RNA from the LoVo human colon cancer cell line. The level of LI cadherin mRNA was normalized to that of 18S ribosomal RNA.

Table 1. The relationship between LI cadherin and CDX2 expression

	CDX2 expression		p value
	positive	negative	
LI cadherin expression			<0.001*
positive	24	13	
negative	15	83	
* Statistically significant.			

Statistical Analysis

Statistical analyses were performed by Fisher's exact test and the Mann-Whitney U test. All calculations were carried out using JMP software version 7.0.1 (SAS Institute Inc., Cary, N.C., USA). A p value <0.05 was considered statistically significant.

Results

LI Cadherin Protein Expression and mRNA Level

LI cadherin expression was strongly recognized in the islet cells of Langerhans and focally in the pancreatic normal ducts. In IPMNs, LI cadherin expression was recognized in the basolateral plasma membrane or cytoplasm of the tumor cells. LI cadherin was expressed in 63% (26/41) intestinal-type IPMNs, in 7.1% (5/70) gastric-type IPMNs, in 0% (0/9) pancreatobiliary-type IPMNs, in 20% (1/5) oncocytic-type IPMNs and 30% (3/10) unclassified-type. LI cadherin expression was diffusely recognized in a large number of intestinal-type IPMNs, whereas LI cadherin expression was not recognized or focally recognized in a large number of gastric-type IPMNs (fig. 2). LI cadherin expression was significantly higher in the intestinal type (I-type) than in the nonintestinal type (non I-type) (fig. 3a, p < 0.001). LI cadherin mRNA level was significantly higher in I-type IPMNs than in non-I-type IPMNs (fig. 3b, p = 0.0312). Furthermore, the LI cadherin mRNA level was significantly higher in LI cadherin protein-positive cases than that in negative cases by immunohistochemical staining (fig. 3c, p = 0.0037).

Relationship between LI Cadherin and CDX2 Protein Expression

CDX2 expression was recognized in the nuclei of tumor cells, and LI cadherin was found in the basolateral plasma membrane or cytoplasm (fig. 4a, b). The LI cadherin expression was significantly higher in CDX2-positive cases than in CDX2-negative cases (p < 0.001) (table 1).

Fig. 2. Microphotographs of LI cadherin and MUC2 expression in intestinal- and gastric-type IPMN in the same specimen. LI cadherin is expressed in the basolateral plasma membrane and cytoplasm of intestinal-type IPMN-I (a, right-upper square), but not expressed in gastric-type IPMN-L (a, right-lower square). MUC2 is expressed in the cytoplasm of intestinal-type IPMN-I, but not expressed in gastric-type IPMN-L (b).

Fig. 3. LI cadherin protein expression in each subclass, mRNA level in the intestinal type and the nonintestinal type, and relationship between protein and mRNA of LI cadherin. The protein (a) and mRNA level (b) of LI cadherin are more highly expressed in intestinal-type IPMN than in nonintestinal-type IPMN. The LI cadherin mRNA level is positively correlated with LI cadherin protein expression (c).

Fig. 4. LI cadherin and CDX2 expression in intestinal-type IPMN. LI cadherin is expressed in the basolateral plasma membrane and cytoplasm (a), and CDX2 is expressed in the nuclei of tumor cells (b).

Fig. 5. LI cadherin expression in each atypical grade in intestinal-type IPMN. **a** The positivity rates of LI cadherin show an increasing tendency from IPMN-L to IPMN-IC in intestinal-type IPMNs. **b** This tendency is not recognized and there is no significant difference between any atypical grades in nonintestinal-type IPMNs. **c** 3+ LI cadherin score also shows an increasing tendency from IPMN-L to IPMN-IC in intestinal-type IPMNs.

Fig. 6. Microphotographs of LI cadherin expression in intestinal-type IPMN-H and IPMN-IC. **a** LI cadherin expression in intestinal-type IPMN-IC with colloid invasion. LI cadherin is diffusely expressed both in the non-invasive component (**b**) and the invasive component (**c**).

Fig. 7. Relationship between LI cadherin expression and Ki-67 labeling index. The expression of LI cadherin is positively correlated with the Ki-67 labeling index in intestinal-type IPMN (**a**), whereas the correlation was not recognized in nonintestinal types (**b**).

Correlation of LI Cadherin, Grading of IPMN and Ki-67 Labeling Index

The 41 cases of intestinal-type IPMN included 5 cases of IPMN-L, 16 IPMN-I, 11 IPMN-H, and 9 cases of IPMN-IC. The rate of positive LI cadherin expression tended to increase gradually from IPMN-L to IPMN-IC (IPMN-L vs. IPMN-H; $p = 0.0357$, IPMN-L vs. IPMN-IC; $p = 0.0230$, fig. 5a), whereas the tendency was not recognized in nonintestinal-type IPMNs (fig. 5b). Furthermore, 3+ cases of LI cadherin also tended to increase gradually from IPMN-L to IPMN-IC in intestinal-type IPMNs (IPMN-I vs. IPMN-H; $p = 0.0115$, fig. 5c). LI cadherin was diffusely expressed both in IPMN-H and in IPMN-IC (fig. 6a–c).

In intestinal-type IPMNs, the Ki-67 labeling index was significantly higher in LI cadherin-positive cases than in LI cadherin-negative cases (fig. 7a, $p = 0.0408$), while the correlation was not recognized in nonintestinal-type IPMNs (fig. 7b).

Discussion

It is widely accepted that IPMNs have a wide spectrum of atypical grades, ranging from low-grade dysplasia to invasive carcinoma. Adsay et al. [6, 7] have suggested that intestinal-type IPMNs grow to colloid carcinoma following a distinct pathway of carcinogenesis associated with

intestinal-related genes, i.e. CDX2 and MUC2, which is called the 'intestinal pathway' of carcinogenesis. In the present study, we found that LI cadherin protein and mRNA were highly expressed in intestinal-type IPMN and that the expression of LI cadherin was correlated with that of CDX2. Hinoi et al. [26] have shown that CDX2 upregulates LI cadherin expression in a colorectal cancer cell line. Colocalization of CDX2 and LI cadherin was seen in intestinal metaplasia and adenocarcinoma of the stomach [27]. LI cadherin might be regulated by a complex regulatory mechanism of not only CDX2 but also human hepatocyte nuclear factor-1 α (HNF1 α), metal-responsive transcription factor-1 (MTF-1), and placental growth factor (PIGF) as described in previous reports [20, 28].

We then examined LI cadherin expression in each atypical grade by separating intestinal-type IPMN from nonintestinal-type IPMN. We found that LI cadherin expression tended to increase gradually from IPMN-L to IPMN-IC. In addition, LI cadherin expression was positively correlated with the Ki-67 labeling index in intestinal-type IPMN. Thus, the upregulation of LI cadherin is associated with the 'intestinal pathway' of carcinogenesis. The invasive component of intestinal-type IPMN-IC frequently showed colloid carcinoma with intestinal differentiation. Furthermore, there was a slight upregulation from noninvasive IPMN to invasive IPMN in intestinal-type IPMN (fig. 5a). In contrast, nonintestinal-type IPMN showed a reduced tendency to progress from non-

invasive to invasive IPMN (fig. 5b). Takamura et al. [19] have shown that the low expression level of LI cadherin correlates with tumor dedifferentiation in pancreatic carcinoma. Considering these findings, pancreatic invasive ductal carcinoma may have similar characteristics to nonintestinal-type IPMN with invasion. We reported that programmed cell death 4 [29], the DNA damage checkpoint pathway [30], and REG4 [8] were each associated with carcinogenesis. REG4 was related to the intestinal phenotype as was LI cadherin, and was upregulated in the step of carcinogenesis from IPMN-L to IPMN-I whereas LI cadherin expression was increased from IPMN-L to IPMN-H. Until now, LI cadherin had not been reported to be associated with carcinogenesis in any malignant tumors. Now, our results suggest that LI cadherin is associated with carcinogenesis in intestinal-type IPMN. In gastric and hepatocellular carcinoma, overexpression of LI cadherin is associated with lymph node metastasis and poor prognosis [14, 15, 17], and LI cadherin promotes cell proliferation [30, 31]. Further investigation of the role of LI cadherin in intestinal-type IPMN may reveal LI cadherin to be a useful therapeutic target, as described in gastric and hepatocellular carcinoma [31, 32].

In conclusion, LI cadherin has different roles in intestinal-type IPMN and nonintestinal-type IPMN. Our findings suggest that LI cadherin is associated with the differentiation of intestinal phenotype and the 'intestinal pathway' of carcinogenesis in IPMN.

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