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Lethal Giant Larvae2 Expression Is Reduced or Localized at Cytoplasm in Colon Adenomas and Adenocarcinomas

Tae Jung Jang

Department of Pathology, Dongguk University College of Medicine, Gyeongju, Korea

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Corresponding Author
Tae Jung Jang, M.D.
Department of Pathology, Dongguk University College of Medicine, 707 Seokjang-dong, Gyeongju 780-714, Korea
Tel: +82-54-770-2410
Fax: +82-54-770-2447
E-mail: taejung@mail.dongguk.ac.kr

Background : The Scribble, Par and Crumbs polarity modules are essential for establishing and maintaining apicobasal cell polarity in epithelial cells. The aim of the present study was to investigate the expression pattern of Lethal giant larvae2 (Lgl2) in normal colonic epithelium and epithelial tumors and to examine the relationship between Lgl2 expression and clinicopathological parameters. **Methods :** We examined Lgl2 expression in 66 primary colon cancers and 20 adenomas by immunohistochemistry. **Results :** In normal colonic epithelium, Lgl2 was strongly expressed at the basolateral membrane of cells in the luminal surface but was not expressed at the base of crypts. The expression pattern of E-cadherin in normal epithelium was similar to that of Lgl2. In contrast, tumors did not express Lgl2 or showed diffuse cytoplasmic staining. The Lgl2 positive rate in tumors was significantly lower than in normal epithelium, and its negative rate in tumors was higher in tumors with abnormal E-cadherin expression than in tumors with positive membranous staining. Lgl2 staining intensity was significantly lower in tumor budding sites than in tumor centers. No significant differences were observed between Lgl2 and clinicopathological parameters. **Conclusions :** Lgl2 expression was reduced or localized at the cytoplasm in colon epithelial tumors, suggesting that a perturbation of Lgl2 expression frequently occurs in colon epithelial tumors.

Key Words : Lethal giant larvae2; Colonic neoplasms

Loss of cell polarity has long been recognized as one of the earliest hallmarks of cancer.[1] Cell polarity is the ultimate reflection of complex mechanisms that establish and maintain functionally specialized domains in the plasma membrane and cytoplasm.[2] Normal glandular epithelial cells show a defined organization, displaying asymmetric distribution of proteins along the internal apical-basal axis. The Scribble, Par and Crumbs polarity modules are essential for establishing and maintaining of apicobasal cell polarity in epithelial cells.[3] The Scribble polarity module is composed of three proteins, Scribble (Scrib), Disc large (Dlg) and Lethal giant larvae (Lgl).[4] *Lgl*, first discovered in *Drosophila*, encodes a protein that associates with the submembranous actin cytoskeleton of the basolateral cell domain.[5,6] *Lgl* is known as a tumor suppressor gene because in *Lgl* mutant flies, the imaginal disks and brain fail to differentiate and overproliferate to form disorganized tumor-like lesions.[6,7] There are two homologs of *Drosophila Lgl* in vertebrates, *Lgl1* and *Lgl2*.[8] *Lgl1* knockout mice reveal brain lesions resembling human primitive neuroectodermal tumors.[9] The loss of *Lgl1* in humans is observed in a variety of cancers, including melanoma, prostate, breast and colon and is associated with tumor differentiation and metastasis.[10-13] Furthermore, the localization of polarity proteins is important for precise function. The Lgl1 protein is released into the cytoplasm from the basolateral membrane during human ovarian carcinogenesis.[14] Lgl2 plays a role inhibiting cancer cell invasion at the tumor margins of breast and colon cancers.[15,16] Recently, the loss of Lgl2 protein or its cytoplasmic localization has been observed in gastric dysplasia and gastric adenocarcinoma.[17] The aim of this study was to investigate the Lgl2 expression pattern in normal colonic epithelium, adenoma, and carcinoma and examine the relationship between it and clinicopathological parameters.

MATERIALS AND METHODS

Patients

We selected patients whose paraffin embedded tissues were relatively well preserved and whose medical records were com-

plete. The present study included 66 patients with primary colon cancer patients (age, 31 to 92 years; 30 males and 36 females) who underwent curative surgery and 20 adenoma patients (age, 35 to 65 years; 11 males and nine females) who underwent an endoscopic polypectomy. All of the adenomatous polyps were of the tubular type with low grade dysplasia.

Microscopic examination and immunohistochemistry

Differentiation and depth of tumor as well as lymph node metastasis status were assessed after reviewing each tumor slide. The stage was defined according to the tumor, node and metastasis staging system of the American Joint Committee on Cancer.

For immunohistochemical staining, we selected a paraffin block containing both tumor tissue with the deepest invasive lesion and adjacent normal mucosa. Formalin-fixed paraffin embedded tissue sections of 4 μm thickness were made and spread on poly-L-lysine coated slides. The sections were deparaffinized and hydrated in a graded series of alcohol. Antigen retrieval was routinely performed by immersing the sections in 0.01 M citrate buffer (pH 6.0) in a pressure cooker and autoclaving for 15 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes and slides were then incubated with primary antibody for 2 hours at room temperature. The primary antibodies used were: anti-Lgl2 (1 : 500, Abcam, Cambridge, UK) and anti-E-cadherin (1 : 1,000, Transduction Laboratories, Lexington, KY, USA). Staining was done with an EnVision kit labeled with peroxidase (Dako, Santa Barbara, CA, USA) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Zymed Laboratories, South San Franciso, CA, USA) as the chromogen. Sections were counterstained for 3 minutes with Mayer's hematoxylin and then mounted. As a negative control, rabbit and mouse IgG isotypes were used instead of the primary antibody. Human salivary glands were used as an Lgl2 positive control.

Membranous E-cadherin expression was graded according to the proportion of positive cells and classified into a positive group (> 90%) and an abnormal group (0-90%). Lgl2 expression was evaluated by assessing the proportion of positive cells and graded as absence to mild (0-25%), moderate (25-50%), or extensive (> 51%). The three immunohistochemical staining grades were divided into positive (moderate and extensive) and negative (absent to mild) for statistical analysis. In adenocarcinomas except for mucinous carcinoma, the Lgl2 expression staining intensity was analysed separately for the tumor center and a tumor budding site showing extensive tumor budding. Tumor budding sites were defined as an isolated single cancer cell and a cluster composed of fewer than five cancer cells.[18] Staining intensity was graded as absent (0), mild (1), moderate (2), or severe (3).

Statistical analysis

The chi-square, Fisher exact test and t-test were used. Statistical significance was considered for a $p < 0.05$. Data are expressed as mean ± standard deviation.

RESULTS

Lgl2 is expressed at the basolateral membrane of epithelial cells in luminal surface in normal mucosa

Normal salivary glands well revealed Lgl2 immunoreactivity at the basolateral membrane of acini (data not shown). The Lgl2 and E-cadherin expression patten in normal colonic epithelial cells is well shown in Fig. 1A and B, respectively. Lgl2 strongly expressed at the basolateral membrane domain of epithelial cells on the luminal surface but was not expressed at the base of the intestinal crypts. Similar to that of Lgl2, E-cadherin expression was also marked at the epithelial cell membrane on the luminal surface and the upper part of the intestinal crypts.

Lgl2 expression was reduced or localized at the cytoplasm in colon epithelial tumors compared with normal mucosa

The Lgl2 expression pattern in colon tumors is well shown in Fig. 1C, D, and E. In contrast to normal colonic epithelium, tumors did not express Lgl2 or showed diffuse cytoplasmic staining without membranous staining. The positive rate of Lgl2 expression was 70% (14/20) in adenomas and 85% (56/66) in adenocarcinomas. This positive rate in adenomas and adenocarcinomas was significantly lower than in adjacent normal colon with a 100% positive rate, respectively ($p < 0.001$). No significant difference in positive rate was observed between adenomas and adenocarcinomas.

Close association between Lgl2 and E-cadherin expression in tumors

Our previous study reported that the positive rate of membranous E-cadherin expression was 22% (2/9) in adenomas and 23% (15/66) in adenocarcinomas.[19] The positive rate of Lgl2

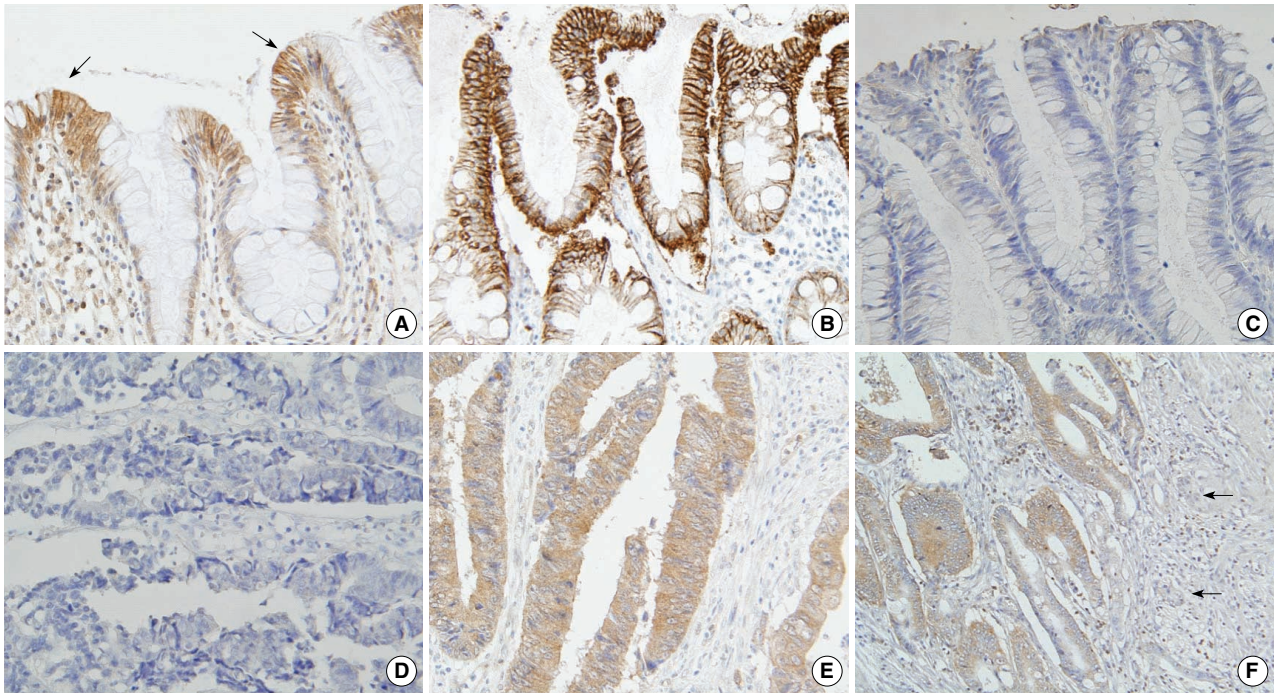

Fig. 1. Immunohistochemical staining of Lethal giant larvae2 (Lgl2) (A, C-F) and E-cadherin (B) in normal colon (A, B) and adenoma (C) as well as adenocarcinoma (D-F). Lgl2 is strongly expressed at the basolateral membrane domain of epithelial cells in the luminal surface (arrows) but is not expressed at the base of the intestinal crypts (A). The E-cadherin expression pattern is similar to that of Lgl2 in normal mucosa (B). Tumors do not express Lgl2 (C, D) or show diffuse cytoplasmic staining without membranous staining (E). Lgl2 immunoreactivity decreased significantly in tumor budding sites compared to the tumor center (arrows) (F).

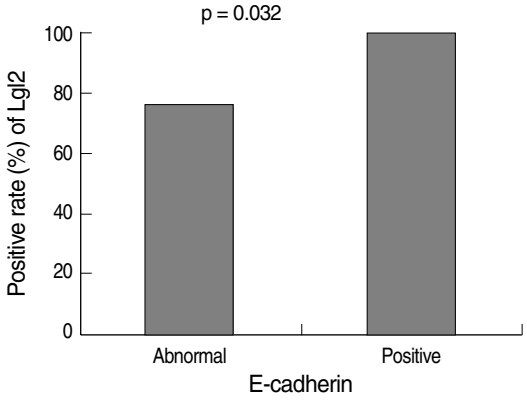

Fig. 2. Relationship between Lethal giant larvae 2 (Lgl2) and E-cadherin expression in colonic epithelial tumors. A significant association is observed between Lgl2 and E-cadherin expression (p = 0.032).

expression in tumors was 76% (45/58) in tumors with abnormal E-cadherin expression and 100% (17/17) in tumors with positive membranous E-cadherin expression. A significant association was observed between Lgl2 and E-cadherin expression in tumors (p = 0.032) (Fig. 2). The relationship between Lgl2 expression and clinicopathological parameters is summarized in Table 1. No significant differences were observed between them.

Table 1. Relationship between Lethal giant larvae2 (Lgl2) expression and clinicopatholgical parameters in adenocarcinomas

Parameter		n	Lgl2 positive rate (%)	p-value
				> 0.05
Differentiation	Well	32	84	
	Moderate & poor	26	85	
	Mucinous	8	88	
Depth	Tis	5	60	
	T1	4	100	
	T2	6	83	
	T3 & T4	51	86	
Nodal metastasis	Absence	41	83	
	Presence	25	88	
Stage	0	5	60	
	I	9	89	
	II	25	84	
	III	25	88	
	IV	2	100	

A reduction of Lgl2 immunoreactivity in the tumor budding sites

We compared Lgl2 immunoreactivity between the tumor center and tumor budding sites in adenocarcinomas to examine

the role of Lgl2 in cancer invasion. Its staining intensity was 2.23 ± 0.70 in tumor center and 1.14 ± 0.65 in tumor budding sites. Lgl2 immunoreactivity was significantly decreased in tumor budding sites than in tumor center as shown in Fig. 1F ($p = 0.000$). Our previous study has reported that membranous E-cadherin immunoreactivity was 2.56 ± 0.11 in the tumor center and 1.24 ± 0.13 in tumor budding sites.[18] It was also significantly lower than at tumor budding sites than in the tumor center ($p = 0.000$).

DISCUSSION

Loss of cell polarity and disruption of cell junctions occur in carcinomas. Apical-basal polarity, asymmetric distribution of proteins along the internal axis, plays a role in the organization of normal differentiated epithelial cells. The Scribble, Par and Crumbs polarity modules are essential for establishing and maintaining apical-basal polarity in epithelial cells.[3] Previous studies have demonstrated that, similar to the *Drosophila* Lgl, mammalian Lgl1 and Lgl2, a component of the Scribble polarity module, bind to Par6/atypical protein kinase C (aPKC), a component of the Par complex, and aPKC phosphorylates Lgl.[20] Once phosphorylated, Lgl is localized to the cytoplasm and basolateral membrane from the apical membrane domain.[21] Lgl, in turn, inhibits aPKC function at the basolateral domain.[22] Mutual inhibition of Lgl and aPKC plays an important role in preserving the apical-basal polarity of epithelial cells. Lgl depletion or aPKC ectopic expression causes cells to overproliferate and to suppress the formation of cell polarity.[23] Lgl is relocated to the membrane from the cytoplasm in MDCK cells once polarization is established.[21] A recent ovarian cancer study demonstrated that aPKCζ, spreading from apical sites along the lateral domain, phosphorylates Lgl1 leading to cytoplasmic release in an inactive form.[14] Furthermore, Lgl2 expression disappears or is localized at the cytoplasm in gastric dysplasia and adenocarcinomas.[17] The present study showed that Lgl2 expression was salient at the basolateral membrane domain of epithelial cells in the luminal surface of colonic crypts in normal colonic mucosa, whereas it was lost or localized at the cytoplasm in adenomas and adenocarcinomas. This Lgl2 expression pattern could cause the loss of apical-basal cell polarity in colonic tumor cells, which may begin during the early stage of colonic carcinogenesis. This phenomenon results in accidental cross talk between multiple signaling molecules that normally localize to distinct cellular compartments,[8] which could potentially cause the disorganization of multiple signaling pathways and provoke uncontrolled cell proliferation.

E-cadherin is functionally linked to the generation of epithelial cell polarity.[24-26] E-cadherin colocalizes with Dlg in cultured mammalian epithelial cells.[27] The ectopic expression of E-cadherin in cadherin-null cells causes the relocation of both Dlg and Scribble into cell-to-cell contact areas.[28] E-cadherin is induced and localized to the cell membrane upon ectopic expression of Lgl1 in melanoma cells.[11] E-cadherin expression is reduced after Lgl2 knockdown in Sw480 cells, and its expression is increased at the HEK293 cell membrane in cells ectopically expressing Lgl2.[16] In our study, the E-cadherin expression pattern was similar to that of Lgl2 in normal colonic mucosa, and there was a close association between Lgl2 and E-cadherin expression in tumors. These findings support the observation that E-cadherin plays an important role in epithelial cell polarity by interacting with polarity determinants at the basolateral membrane domain.

Lgl1 expression decreases in 75% of colonic adenocarcinomas and its loss is associated with advanced clinical stage and lymph node metastasis.[13] A recent study has reported that Lgl2 decreases in colorectal carcinomas and this reduction is more severe in poorly differentiated carcinomas.[16] However, the authors did not show any relationship between Lgl2 expression and prognostic factors. In our study, Lgl2 immunoreactivity was also significantly lower in tumors than in adjacent normal colon mucosa. However, we did not find any significant relationship between Lgl2 and clinicopathological parameters. Furthermore, a study examining Lgl2 expression in gastric tumors also failed to show data regarding its biologic behavior.[17] Thus, an additional clinicopathological studies are necessary to determine the biological behavior of Lgl2.

The progression of carcinomas involves the loss of polarized cell architecture, a process of epithelial-to mesenchymal transition in tumor budding sites. Previous studies have demonstrated that E-cadherin expression is downregulated in tumor budding sites of colon carcinomas.[18,29] It was reported recently that Lgl2 expression is reduced by zinc finger E-box-binding protein homeobox 1 in tumor budding sites of colorectal carcinomas.[16] Our study also showed a reduction in Lgl2 immunoreactivity in tumor budding sites.

In conclusion, Lgl2 expression is reduced or localized at the cytoplasm in colon epithelial tumors, suggesting that a perturbation of Lgl2 expression frequently occurs in colon epithelial tumors.

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