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APPENDIX I

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REVIEW

Involvement of liver-intestine cadherin in cancer progression

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Abstract Cadherins constitute a superfamily of Ca^{2+}-dependent cell adhesion molecules that play critical roles in the maintenance of tissue structure and morphogenesis. Their dysregulation is commonly observed in a variety of cancers. Liver-intestine cadherin (LI-cadherin), which was so named in view of its sole expression in the liver and intestine of the rat, is a structurally unique member of the cadherin superfamily, possessing seven cadherin repeats within the extracellular cadherin domain and only 25 amino acids in the cytoplasmic domain. Its adhesive property does not require any interaction with cytoplasmic components such as catenins, and it responds to small changes in extracellular Ca^{2+} below the physiological plasma concentration. In humans, the distribution of LI-cadherin is limited to the duodenum, jejunum, ileum, colon, and part of the pancreatic duct. Data accumulated from studies of the biological characteristics of LI-cadherin have shown that it plays an important role in the pathophysiology of human cancers. Here, we review recent information about LI-cadherin and its implications for cancer progression.

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Keywords Liver-intestine cadherin · Cadherin-17 · Cancer progression · Biomarker · Therapeutic target

Introduction

Cadherins constitute a superfamily of Ca^{2+}-dependent hemophilic cell–cell adhesion molecules that are involved in the maintenance of tissue structure and morphogenesis [1, 2]. A large number of cadherin superfamily members have been identified to date, and these are classified into six subfamilies (classical/type I cadherins, atypical/type II cadherins, desmocollins, desmogleins, protocadherins, and Flamingo cadherins) besides several solitary members [3]. These molecules are expressed in a variety of different tissues in multicellular organisms [4]. Many previous studies have demonstrated that cadherin-mediated cell adhesion plays a critical role in cancer cell behavior [5, 6].

Liver-intestine cadherin (LI-cadherin), also known as cadherin-17, represents a novel type of cadherin within the cadherin superfamily. The primary structures of rat LI-cadherin and its human homolog (human peptide transporter-1, HPT-1) were reported in 1994 [7, 8]. Thereafter, B lineage-, intestine-, liver-, and leukocyte-cadherin (BILL-cadherin), a mouse homolog of LI-cadherin, was identified in 2000 as a nonclassical cadherin expressed during B-cell development [9]. LI-cadherin has been mapped to rat chromosome 5q13, human chromosome 8q22.1, and mouse chromosome 4 [10, 11]. In contrast to other cadherins, LI-cadherin is expressed in an organ-specific manner, its distribution in the normal rat being confined to the basolateral membrane of hepatocytes and intestinal epithelial cells, whereas in the normal human it is expressed in intestinal epithelial cells and a fraction of pancreatic ductal epithelial cells, but

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not in hepatocytes [7, 8]. The pattern of LI-cadherin expression differs among human cancers of different types. There is increasing evidence that altered expression of LI-cadherin is implicated in cancer progression. In this review, we summarize recent advances in our understanding of LI-cadherin and its implications for cancer progression.

Structural and functional properties of LI-cadherin in noncancerous cells

All members of the cadherin superfamily share common structural features, with some exceptions, having an amino-terminal extracellular region composed of tandemly repeated extracellular cadherin (EC) domains, a single transmembrane domain, and a cytoplasmic domain at the carboxyl terminus [12]. E-cadherin is the best characterized member of the cadherin superfamily. The extracellular domain is composed of five EC domains connected by Ca^{2+}-binding linkers. The most membrane-distal EC (EC1) domain of E-cadherin contains conserved Trp at amino acid residue 2 and the His-Ala-Val cell adhesion recognition sequence at amino acid residues 79–81, which regulate hemophilic binding (Fig. 1). It is now understood that the EC1 domain is vital for cadherin-mediated cell adhesion. The cytoplasmic domain of E-cadherin, about 150 amino acids long, contains two highly conserved regions: a membrane-proximal region and a catenin-binding region. The former binds to p120 and δ-catenin, whereas the latter binds to β-catenin and plakoglobin (γ-catenin). These binding partners form a complex with E-cadherin and play a critical role in actin cytoskeleton interaction and the full adhesive properties of E-cadherin [13].

Liver-intestine cadherin possesses several unique features distinct from E-cadherin (Fig. 1). First, LI-cadherin possesses seven EC domains in its extracellular domain, and in the EC1 domain Trp and the His-Ala-Val sequence are replaced by Gly and the Ala-Ala-Leu sequence, respectively.

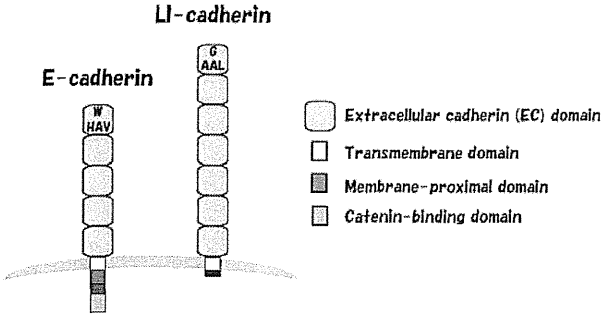

Fig. 1 Domain structures of E-cadherin and liver-intestine (LI)-cadherin

The EC1 sequence of LI-cadherin shows only 44 % protein sequence similarity with that of E-cadherin [3]. Second, LI-cadherin has a short (about 25 amino acids) cytoplasmic domain, which shares no homology with the corresponding region of E-cadherin. In vitro functional studies using mouse L cells or *Drosophila* S2 cells have demonstrated that LI-cadherin is able to mediate Ca^{2+}-dependent cell–cell adhesion without any interaction with catenins or the actin cytoskeleton [14]. The effect of Ca^{2+} on LI-cadherin binding is known to differ markedly from that on E-cadherin [15, 16]. Electron microscopy observations have revealed that more than 60 % of pentamerized recombinant E-cadherin EC domains are engaged in homotypic interactions at an extracellular Ca^{2+} concentration of 0.5 mM. These interactions reached about 80 % in the presence of 1.0 mM extracellular Ca^{2+}. On the other hand, atomic force microscopy and laser tweezer experiments have revealed that LI-cadherin homotypic interactions respond to small changes of extracellular Ca^{2+} within the range 0.6–0.8 mM with a high degree of cooperativity, although the interactions remain negligible at extracellular Ca^{2+} concentrations below 0.5 mM. Third, LI-cadherin participates in intestinal peptide transport [8]. Given that most transporters generally contain multiple membrane-spanning domains distinct from those of LI-cadherin, it cannot be assumed that the protein itself acts as a transporter. For instance, CD98 possesses a single membrane-spanning domain and is covalently linked to an amino acid transporter with which it forms a heterodimer. The CD98 complex fulfills the crucial role of regulating cell adhesion, polarity, and amino acid transport in intestinal epithelia [17]. LI-cadherin may also form a complex with an unidentified transporter, thereby acquiring transport activity. Fourth, it is known that LI-cadherin and E-cadherin are located in different membrane compartments: the former is localized to the basolateral membrane whereas the latter is localized to adherens junctions [7, 18]. A recent in vitro study has revealed that LI-cadherin is localized almost exclusively in cholesterol-rich fractions, whereas E-cadherin is absent from these fractions. Moreover, depletion of cholesterol using mevinolin results in reduced homotypic LI-cadherin adhesion [19]. Further studies are required to determine the physiological significance of these observations.

LI-cadherin protein expression in human cancer cell lines and primary tumors

There is accumulating evidence for a link between LI-cadherin and various cancers. Table 1 summarizes the expression of LI-cadherin protein in human cancer cell lines derived from various organs. LI-cadherin is expressed in cell lines derived from colon, stomach, pancreas, and liver, but not cell lines derived from brain and blood.

Table 1 Liver-intestine (LI)-cadherin protein expression in various human cancer cell lines LI-cadherin protein

Organ	Positive	Negative	References
Colon			
Colorectal cancer	Caco-2	HCT116	[8, 20, 28, 31, 33]
	DLD1	RKO	
	LS174T	SW48	
	SW1116	SW480	
	SW837	WiDr	
	SW1463	COLO201	
	COLO320	HCT15	
	LoVo*	LoVo*	[28, 33]
	HT29*	HT29*	[8, 28, 31]
Stomach			
Gastric cancer	HSC39	HGC27	[24, 27]
	SGC7901		
	BGC823		
	MKN28		
Pancreas			
Pancreatic cancer	AsPC-1	PANC-1	[35]
Liver			
HCC	Hep3B	HepG2	[39, 40, 45, 46]
	HuH-7		
	PLC/PRF/5		
	HCCLM3		
	MHCC97H		
	MHCC97L		
ICC	IHGGK	HuCCT1	[49]
		HuH-28	
Brain			
Neuroblastoma		SK-N-MC	[8]
Glioblastoma		U-373	
Blood			
ALL		CCRF-CEM	[8]
MM		IM-9	

HCC hepatocellular carcinoma, *ICC* intrahepatic cholangiocarcinoma, *ALL* acute lymphoblastic leukemia, *MM* multiple myeloma

* Conflicting results

Gastric cancer

Liver-intestine cadherin expression in gastric cancer has been the best studied to date. However, conflicting data have been published, making it difficult to understand the role of LI-cadherin in this malignancy. An initial study suggested that LI-cadherin protein was a useful marker for early detection of gastric intestinal metaplasia (IM) and well-differentiated gastric cancers [20]. A clinicopathological study of 208 gastric cancer cases revealed that LI-cadherin protein was highly expressed in well-differentiated gastric cancers and earlier TNM stages, whereas reduced expression of LI-cadherin was closely associated with lymph node metastasis [21]. Using microarray analysis, Lee et al. have recently screened for genes that are expressed differentially between normal chief cells and two types of metaplastic cells: IM cells and spasmolytic polypeptide-expressing metaplastic (SPEM) cells. They found that eight proteins, including LI-cadherin, were expressed not only in IM or SPEM but also in gastric cancer, and that LI-cadherin protein expression was an independent and prognostically favorable biomarker in stage I or node-negative gastric cancer [22].

In contrast, Oue and colleagues [23] identified LI-cadherin as one of the most upregulated genes in advanced gastric cancer by comparison of the gene expression profile with early gastric cancer. A high level of LI-cadherin mRNA expression was associated with advanced T grade, suggesting a candidate marker gene for tumor progression. In a further study using immunohistochemical analysis, they confirmed that LI-cadherin protein expression was significantly more frequent in patients with advanced gastric cancer than in those with early gastric cancer, and that the prognosis of patients showing positive LI-cadherin protein expression was significantly poorer than that of patients showing negative expression [24]. Other studies have shown that LI-cadherin overexpression is an independent factor associated with lymph node metastasis in gastric cancer [25, 26].

Recently, Liu et al. [27] analyzed the effects of stable LI-cadherin silencing with recombinant lentivirus-mediated microRNA (miRNA) both in vitro and in vivo. They found that LI-cadherin silencing decreased the activities of matrix metalloproteinase (MMP)-2 and MMP-9 markedly, and induced G_1 cell-cycle arrest, thereby inhibiting invasion and proliferation of a gastric cancer cell line in vitro. Moreover, LI-cadherin silencing inhibited tumorigenicity in vivo. Although further analyses using other gastric cancer cell lines will be required to confirm these results, the data suggest that LI-cadherin may participate in the malignant cell behavior of gastric cancer.

Colon cancer

The role of LI-cadherin in normal, metaplastic, and cancerous gastrointestinal (GI) cells had remained elusive until it was identified as a CDX2-regulated gene [28]. CDX2, an intestine-specific transcription factor belonging to the caudal-related homeobox gene family, is reported to have crucial functions in intestinal development, differentiation, and maintenance of the intestinal phenotype [29, 30]. The 5′-flanking region of the LI-cadherin gene contains two CDX2-responsive elements, and CDX2 activates LI-cadherin transcription by directly binding to these elements. As the expression of CDX2 and that of LI-cadherin are tightly correlated in colon cancer cell lines as well as in

normal, metaplastic, and cancerous GI cells, LI-cadherin may be a key factor mediating CDX2 function in intestinal cell fate determination.

To evaluate the role of LI-cadherin in colorectal cancer, the associations between LI-cadherin and clinicopathological parameters were analyzed using immunohistochemistry. We have reported that reduced LI-cadherin expression is associated with a high tumor grade, lymphatic invasion, lymph node metastasis, and an advanced pTNM stage [31]. In a large series of patients with colorectal cancer, it was shown that reduced LI-cadherin expression was associated with poor overall survival and was an independent prognostic parameter [32]. Yu et al. [33] have recently provided supportive evidence that RNA interference (RNAi)-mediated knockdown of LI-cadherin promotes malignant behavior of colon cancer cells through activation of MMP-2 and MMP-9. These findings suggest that reduced LI-cadherin expression may contribute to the progression of colon cancer.

Su et al. [34] have demonstrated that a combination of LI-cadherin and cytokeratin 7 immunoprofiling can differentiate adenocarcinomas of the upper and lower GI tract with high specificity. In their series, 37 of 38 (97.3 %) LI-cadherin (+)/cytokeratin 7 (−) tumors were colorectal adenocarcinomas, whereas 49 of 56 (86 %) LI-cadherin (+)/cytokeratin 7 (+) tumors were gastric, pancreatic, or biliary adenocarcinomas. Thus, LI-cadherin has the potential to be a useful diagnostic marker of GI tract adenocarcinoma.

Pancreatic ductal adenocarcinoma (PDA)

The expression of LI-cadherin was investigated immunohistochemically in a panel of 102 resected PDAs. LI-cadherin expression was focal and weak in normal pancreatic ducts. Well-differentiated PDAs showed strong expression of LI-cadherin, whereas less differentiated areas and poorly differentiated cases showed reduced or no expression. High expression of LI-cadherin was associated with good overall survival and was inversely correlated with tumor dedifferentiation and an advanced tumor stage [35]. The authors also identified galectin-3 as a binding protein for LI-cadherin, and found that the expression pattern of galectin-3 was similar to that of LI-cadherin. Galectins are a family of β-galactoside-binding lectins [36]. To date, 15 mammalian members of the galectin family have been identified. Galectin-3 has been well characterized, and is known to be involved in a variety of biological processes, including cell growth, cell adhesion, differentiation, angiogenesis, and metastasis [37]. A previous study of PDAs has demonstrated that high galectin-3 expression was inversely correlated with tumor dedifferentiation and an advanced tumor stage [38]. Thus, interaction between LI-cadherin and galectin-3 may be of biological significance in PDA, for example, playing a role in cancer progression.

Hepatocellular carcinoma (HCC)

More recently, evidence has accumulated to suggest that LI-cadherin alterations may be of clinical significance in HCC. Wong et al. [39] have reported for the first time that LI-cadherin is overexpressed in HCC cell lines and HCC tissues. Interestingly, the localization of LI-cadherin appears to be unique in HCC, in comparison with other reported malignancies. Although LI-cadherin is primarily localized at the plasma membrane, there is a marked predilection for cytoplasmic versus membrane localization in HCC [39, 40]. This unusual expression pattern is associated with tumor aggressiveness and poor prognosis in patients with hepatitis B virus-positive HCC [40]. It would be interesting to clarify whether these characteristics are also applicable to HCCs with etiologies other than HBV.

Alternative splicing is a critical step for generating protein diversity, and its misregulation is often observed in cancer [41]. Overexpression of splice variants of several genes influences hepatocarcinogenesis or the progression of HCC (see review by Berasain et al. [42]). In HCC tissues, Wang et al. have identified an alternative mRNA splicing variant of the LI-cadherin gene that lacks exon 7. Aberrant expression of this isoform was found to be related to two LI-cadherin gene single-nucleotide polymorphisms (651T and IVS6+35G) and was associated with poor prognosis and a high incidence of tumor recurrence [43]. Furthermore, this specific haplotype (651C>T and IVS6+35A>G) has been shown to be one of the genetic factors involved in the development of HCC in a Chinese population [44].

Liu et al. have recently demonstrated that subcutaneous implantation of LI-cadherin-overexpressing immortalized liver progenitor cells gives rise to tumors in mice, and that overexpression of LI-cadherin mRNA is associated with advanced tumor stages and tumor venous invasion in patients with HCC. Using the RNAi strategy, they further demonstrated that knockdown of LI-cadherin ameliorates the tumorigenic and metastatic phenotypes of highly malignant HCC cell lines and inactivates Wnt/β-catenin signaling (relocalization of β-catenin to the cytoplasm with a concomitant reduction of cyclin D1 and an increase in retinoblastoma), thereby inhibiting tumor growth and enhancing cell death. These findings indicate that LI-cadherin plays a potentially oncogenic role in HCC and is a promising therapeutic target for innovative treatment strategies [45].

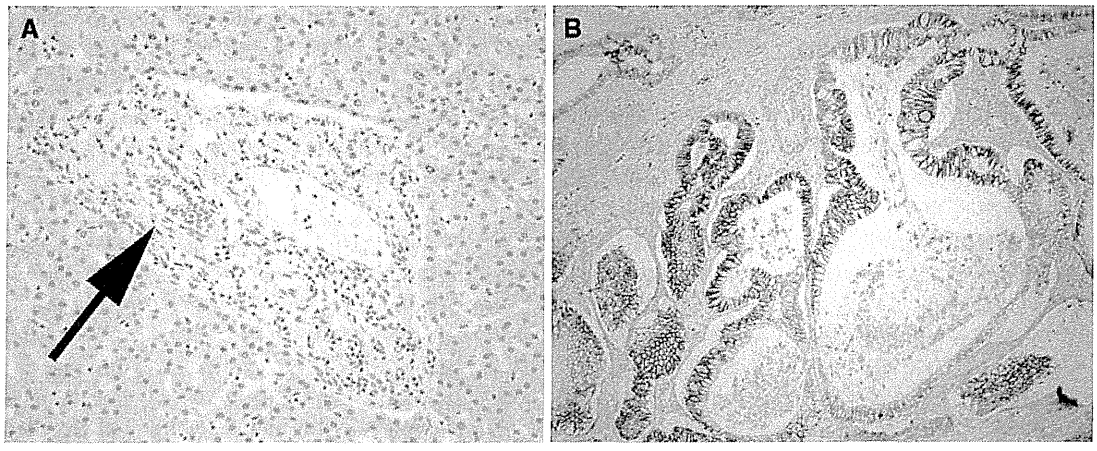

Fig. 2 Expression of LI-cadherin in nonneoplastic biliary epithelium and intrahepatic cholangiocarcinoma. **a** LI-cadherin was absent in nonneoplastic biliary epithelium (*arrow*). **b** LI-cadherin was expressed strongly in well-differentiated carcinoma nests. **a** ×200; **b** ×100

As noted previously, there is evidence that CDX2 regulates LI-cadherin gene expression. Zhu et al. [46] have recently found that hepatocyte nuclear factor 1α (HNF1α), but not HNF1β, binds to the LI-cadherin gene promoter and regulates its expression together with CDX2 in HCC. HNF1 is one of the liver-enriched transcription factors and is composed of HNF1α or HNF1β homo- or heterodimers [47]. An immunohistochemical study has shown that HNF-1α is expressed at a higher level in well-differentiated HCC than in surrounding noncancerous liver tissues, whereas its expression is lower in moderately and poorly differentiated HCCs [48]. HNF1α is thought to play a central role in modulating the expression of LI-cadherin during hepatocarcinogenesis.

Intrahepatic cholangiocarcinoma (ICC)

The potential role of LI-cadherin in ICC has been investigated [49]. In a panel of 34 resected ICCs, LI-cadherin was shown to be absent in nonneoplastic biliary epithelium, whereas it was expressed strongly in well-differentiated carcinoma nests (Fig. 2), but less strongly in dedifferentiated carcinoma nests. LI-cadherin negativity was associated with tumor dedifferentiation and vascular invasion, and thus poor prognosis. Using microarray analysis, the authors screened for genes that were expressed differentially between IHGGK cells transfected with LI-cadherin siRNA and those transfected with scrambled siRNA; they found that metal-responsive transcription factor-1 (MTF-1) was upregulated in LI-cadherin siRNA-transfected IHGGK cells. MTF-1 is a highly conserved zinc finger protein that regulates the expression of metallothioneins and various other genes involved in apoptosis resistance, invasion, and angiogenesis (see Lichtlen and Schaffner [50] for review). Among MTF-1 target genes, placental growth factor (PlGF), a member of the VEGF family, was also upregulated in IHGGK cells transfected with LI-cadherin siRNA. Furthermore, in LI-cadherin-negative ICCs, PlGF expression was increased and the microvessel density was significantly higher than in LI-cadherin-positive ICCs. These findings provide evidence that loss of LI-cadherin results in upregulation of MTF-1, and subsequently PlGF, thereby regulating angiogenesis in ICC.

Biliary intraepithelial neoplasia (BilIN) is an entity that was initially classified into three grades (BilIN-1, -2, and -3) based on the features of intraepithelial atypical/proliferative lesions of the intrahepatic bile ducts in patients with hepatolithiasis [51]. Thereafter, the classification of BilIN was refined and revised as a result of a large interobserver agreement study based on resected liver specimens from patients with primary sclerosing cholangitis, choledochal cysts, and hepatolithiasis [52]. BilIN is now believed to be one of the precursor lesions of ICC associated with hepatolithiasis [53]. Recently, Inoue et al. examined the expression of LI-cadherin in BilIN immunohistochemically in 16 cases of ICC without hepatolithiasis. It was found that the expression of LI-cadherin became gradually upregulated with the histological progression of BilIN, and that high-grade BilIN (BilIN-2/-3) was an independently significant factor associated with LI-cadherin expression, suggesting that overexpression of LI-cadherin might be involved in the development of BilIN [54].

Conclusion

Since the identification and cloning of LI-cadherin, evidence for its expression and role in cancers has been accumulating. LI-cadherin has been shown to play an

important role in the pathophysiology of human cancers, especially those of the gastrointestinal tract and hepatobiliary system. Among other cadherins, the serum level of soluble E-cadherin is reported to be a valuable pretherapeutic prognostic indicator in patients with gastric cancer [55]. Moreover, a pentapeptide drug (ADH-1) that disrupts the N-cadherin adhesion complex has been developed and is currently being evaluated in a clinical trial for treatment of melanoma [56]. Further understanding of the role of LI-cadherin in cancers will facilitate its future application as a novel biomarker and therapeutic target.

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INTERNAL MEDICINE

□ CASE REPORT □

Immunoglobulin G4-Related Disease with Several Inflammatory Foci

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Abstract

We herein report the case of a 62-year-old Japanese man who presented with jaundice, dry eyes and abdominal discomfort. Imaging studies revealed swelling of the periorbital tissue, parotid and submandibular glands, pulmonary hilar lymph nodes, pancreas, bile ducts, gall bladder walls, bilateral kidneys, arterial walls and prostate. A significant increase in the serum level IgG4 was seen, and the patient was diagnosed with IgG4-related disease after undergoing a biopsy of the pancreas and prostate. We herein report a case of IgG4-related disease with multiple ten organ involvement at the onset of the disease that was successfully treated with prednisolone (PSL) therapy.

Key words: IgG4-related disease, autoimmune pancreatitis, corticosteroids, multiple organ, IgG4

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Introduction

Immunoglobulin (Ig) G4-related disease is a new disease entity that was first reported in 2001 (1) and officially named in 2011 (2-5). Inflammation may occur in various organs, thereby causing systemic symptoms. The serum IgG4 levels are related to disease activity and recurrence (2, 6-8). We herein report a case of IgG4-related disease with inflammation in the periorbital tissue, parotid and submandibular glands, pulmonary hilar lymph nodes, pancreas, bile ducts, gall bladder, bilateral kidneys, prostate and arterial walls from the ascending aorta to the left subclavian and bilateral iliac arteries, forming a retroperitoneal lesion. In addition, the patient's serum IgG4 level was significantly high (>1,900 mg/dL). As per our knowledge, this case of multiple organ involvement at the onset of disease is the first if its kind to be reported. Oral corticosteroid administration improved the inflammation and normalized the serum IgG4 level. A discussion of the case presented here is intended to promote wider recognition and understanding of this disease.

Case Report

A 62-year-old Japanese man presented at our hospital with jaundice, dry eyes, abdominal discomfort and pretibial edema in July 2011. A physical examination revealed swelling of the bilateral submaxillary glands and mild tenderness in the epigastric area in addition to the jaundice and pretibial edema. Laboratory test results revealed increased levels of direct bilirubin and the hepatobiliary enzymes aspartate aminotransferase and alanine aminotransferase at 4.4 mg/dL, 121 IU/L and 145 IU/L, respectively. A serum protein analysis revealed an increased IgG level (3,877 mg/dL) that was caused by a significantly high level of an IgG4 subclass (1,920 mg/dL) known as a specific marker of IgG4-related disease. An increase in the serum creatinine level to 1.13 mg/dL, a decrease in creatinine clearance to 60 mL/min and a high level of urinary protein (2.06 g/day) were also de-

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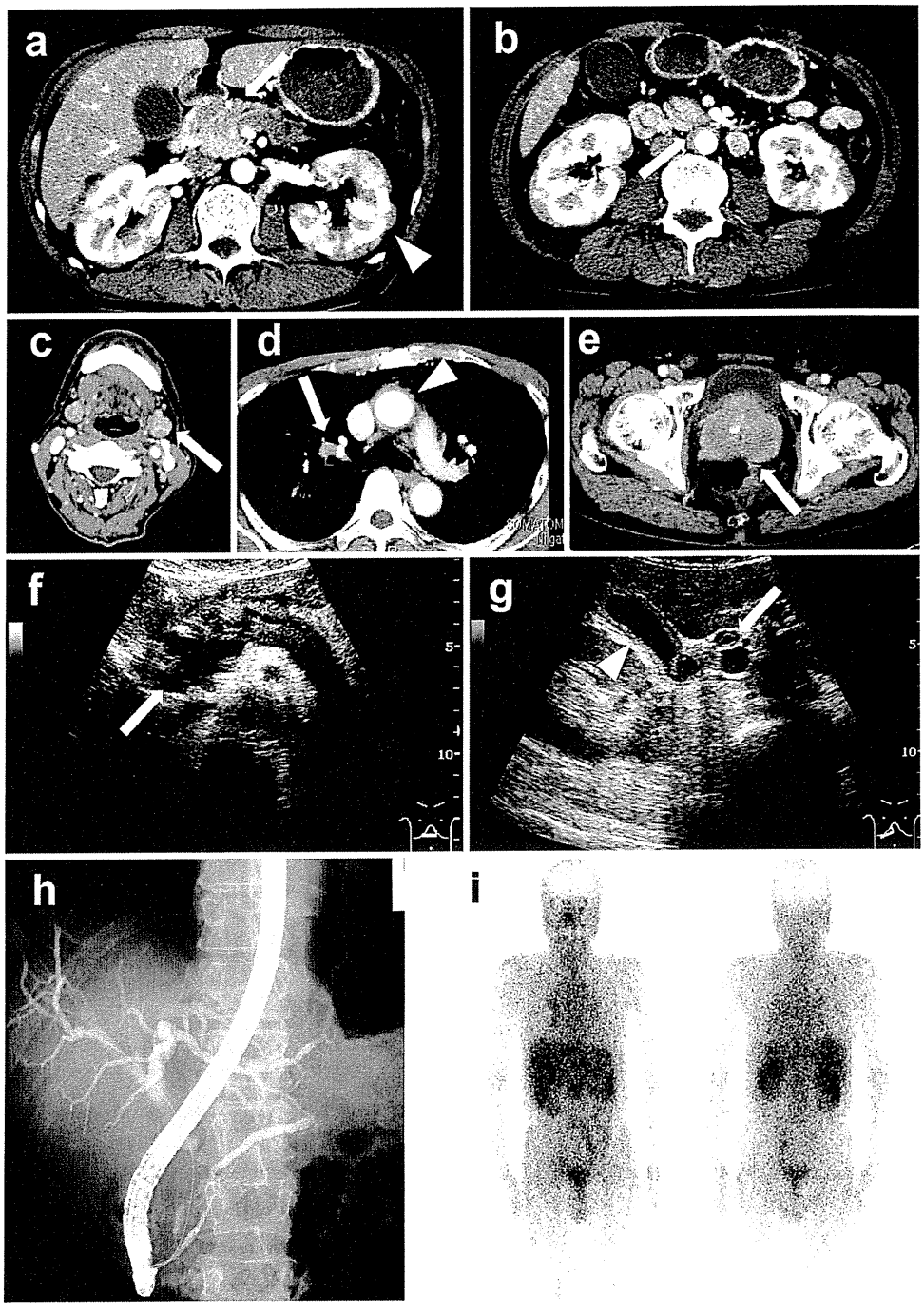

Figure 1. The multiple inflammatory lesions of IgG4-related disease found in our case. Contrast-enhanced computed tomography (CT) showed diffuse enlargement of the pancreas with tumorous changes in the head (a, white arrow), multiple, small, low-attenuation lesions with mild swelling in the bilateral kidneys (a, white arrowhead) and homogeneous thickness in the arterial walls of the abdominal aorta (b, white arrow). Swelling of the submandibular glands (c, white arrows), swelling of the pulmonary hilar lymph nodes (d, white arrow), wall thickness in the ascending aorta (d, white arrowhead) and swelling of the prostate (e, white arrow) were marked. Ultrasonography (US) revealed tumorous swelling in the pancreatic head (f, white arrow), mild dilatation of the pancreatic duct on the tail side and diffuse thickening of the bile duct (g, white arrow) and gall bladder walls (g, white arrowhead). Endoscopic retrograde cholangiopancreatography (ERCP) revealed an irregular surface pattern in the pancreatic and common bile duct walls. A stricture was observed on the pancreatic head side, and mild dilatation was observed in the pancreatic tail and peripheral bile duct (h). Gallium-scintigraphy revealed accumulation in the periorbital tissues, salivary glands, pulmonary hilar lymph nodes, kidneys, prostate and retroperitoneum (i).

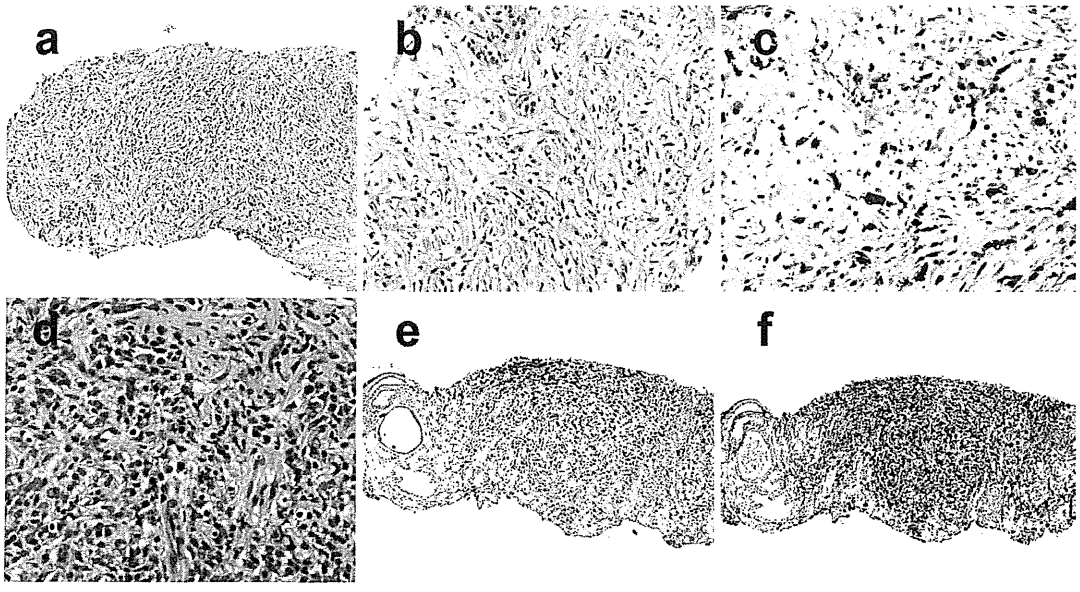

Figure 2. Histopathological findings. Endoscopic US-guided fine needle aspiration of the pancreatic lesion was performed (a-c), and a needle biopsy specimen was obtained from the prostate (d-f). Both tissues revealed storiform fibrosis, lymphoplasmacytic infiltration (a, b, d) and an increased number of IgG4-positive plasma cells (c, e). The ratio of IgG4-positive plasma cells (e) to IgG-positive plasma cells (f) exceeded 50% in the prostate. a, b, d. Hematoxylin and Eosin staining. c, e. IgG4 staining. f. IgG staining, a, e, f, ×40; b, c, ×200; d, ×400.

tected. There were no increases in tumor markers, including carcinoembryonic antigen, carbohydrate antigen 19-9, alpha-fetoprotein and prostate-specific antigen. Furthermore, no SS-A or SS-B autoantibodies were detected.

Imaging studies

Contrast-enhanced computed tomography (CT) revealed diffuse enlargement of the pancreas with homogeneous density. At the pancreatic head, a 50-mm tumorous lesion with a weak and heterogeneous enhancement pattern was observed. The pancreatic duct was diffusely narrowed (Fig. 1a). The bilateral kidneys exhibited multiple, small, low-attenuation lesions with mild swelling (Fig. 1a, b). Homogeneous thickness was observed in the arterial walls from the ascending aorta to the left subclavian and bilateral iliac arteries, thus forming a retroperitoneal lesion (Fig. 1b). Swelling of the parotid glands, submandibular glands, pulmonary hilar lymph nodes and prostate was marked (Fig. 1c-e). Magnetic resonance imaging (MRI) revealed swelling of the periorbital tissue. Ultrasonography (US) revealed tumorous swelling at the pancreatic head and mild dilatation of the main pancreatic duct on the tail side caused by a stricture of the duct on the head side (Fig. 1f). Diffuse thickening of the bile duct and gall bladder walls was clearly observed (Fig. 1g). Structural changes in the common bile duct resulted in dilatation of the intrahepatic bile duct. Endoscopic retrograde cholangiopancreatography (ERCP) revealed an irregular surface pattern in the pancreatic and common bile duct walls. The stricture was observed especially on the pancreatic head side, most likely due to the unevenness of the pancreatic swelling. In addition, mild dilatation was observed in the pancreatic tail and peripheral bile duct (Fig. 1h). Gallium scintigraphy revealed inflammatory foci in the following organs: pancreas, periorbital tissues, salivary glands, pulmonary hilar lymph nodes, kidneys, prostate and retroperitoneum (Fig. 1i).

Histopathology

A diagnosis of systemic IgG4-related disease was suspected on the basis of the information obtained from the laboratory data and imaging studies. To confirm the diagnosis, endoscopic US-guided fine needle aspiration of the pancreatic lesion was performed (Fig. 2a-c) and a needle biopsy specimen was obtained from the prostate to rule out prostate cancer (Fig. 2d-f). The analysis of the pancreatic tissue revealed lymphoplasmacytic infiltration and storiform fibrosis with disruption and atrophy of the acinar component (Fig. 2a, b). The analysis of the prostatic tissue showed fibrosis with lymphoplasmacytic infiltration, lymphoid follicles, scattered eosinophilic infiltration and a decrease in the number of glands (Fig. 2d). A high number of IgG4-positive plasma cells per high-power field was observed in both tissue specimens (Fig. 2c, e), and the ratio of IgG4-positive plasma cells to IgG-positive plasma cells (Fig. 2f) exceeded 50%. No malignant cells were evidenced in either tissue.

Clinical course

On the basis of the above mentioned findings, a diagnosis of IgG4-related disease with pancreatitis, sialadenitis, dacryoadenitis, pulmonary hilar lymphadenopathy, sclerosing

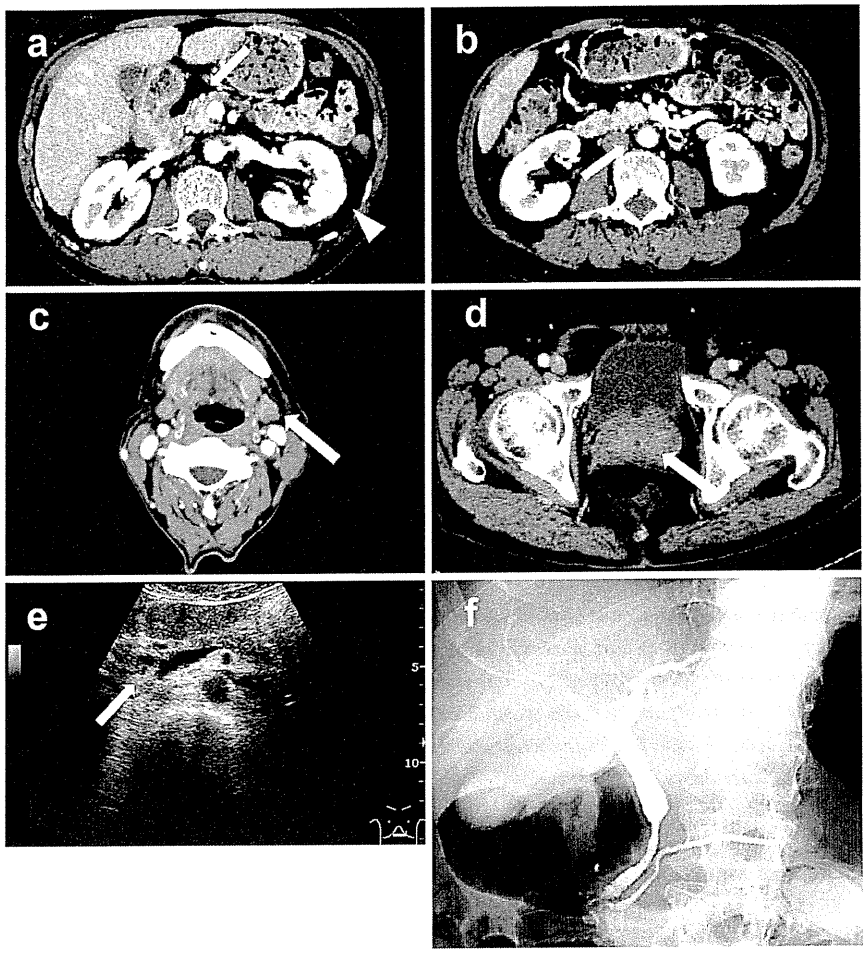

Figure 3. Imaging studies performed four weeks after the administration of prednisolone. CT showed marked decreases in pancreatic swelling (a, white arrow), the kidneys (a, white arrowhead), the retroperitoneal lesion (b, white arrow), the submandibular glands (c, white arrows) and the prostate (d, white arrow). The decrease in pancreatic swelling was marked on US (e, white arrow). ERCP revealed a marked decrease in the irregular surface pattern and narrowing of the pancreatic duct. The stricture of the common bile duct was not fully recovered four weeks after the administration of prednisolone (f).

cholecystitis and cholangitis, tubulointerstitial nephritis, prostatitis and retroperitoneal fibrosis was made. Since September 2011, corticosteroid therapy with 40 mg/day of oral prednisolone (PSL) was initiated. The dose per day was tapered to 35 mg after four weeks of 40 mg administration, then to 30 mg for the next four weeks and tapered by 2.5 mg every four weeks thereafter until July 2012. Since then, the patient has been consuming 10 mg/day for 12 weeks. The symptoms of jaundice, epigastric discomfort, dry eyes, edema and enlarged submandibular glands decreased within four weeks of treatment initiation. Imaging studies showed a marked decrease in pancreatic swelling (Fig. 3a) and the low-attenuation lesions in the kidneys (Fig. 3b). Swelling also diminished in the retroperitoneal lesion (Fig. 3b), submandibular glands (Fig. 3c) and pulmonary hilar lymph nodes. Swelling of the parotid glands and prostate (Fig. 3d) recovered slowly in two months after the administration of PSL. There was a marked decrease in pancreatic swelling, the stricture of the pancreatic duct, the dilatation on the tail side and the thickness of the bile duct and gall bladder walls on US (Fig. 3e). ERCP revealed a marked decrease in the irregular surface pattern on the pancreatic duct and common bile duct wall along with narrowing of the pancreatic duct (Fig. 3f); however, the stricture in the common bile duct did not fully recover after four weeks of PSL treatment and improved six months after the administration of PSL. The serum IgG4 concentrations gradually decreased to 612 mg/dL within four weeks, after which they continuously decreased to reach 150 mg/dL within three months. Normal levels were maintained thereafter. No relapse of disease activity has been observed as of September 2012.

These data suggest that the disease activity and response to PSL in the various affected organs were significantly related to the serum IgG4 level in this case.

Discussion

IgG4-related disease is a relatively new disease entity. In 2001, Hamano et al. reported an increased frequency of high serum IgG4 levels in patients with autoimmune pancreatitis (1). The term IgG4-related disease was chosen for this condition at an international symposium in 2011. Six characteristic features have been identified to date: systemic involvement; solitary or multiple lesions showing diffuse or localized swelling, masses, nodules and/or wall thickening on imaging; a high serum IgG4 level (>135 mg/dL); abundant infiltration of lymphoplasmacytes and IgG4-bearing plasma cells; a positive response to corticosteroid therapy; and complications similar to those seen in other IgG4-related diseases (2-5). Kamisawa et al. recognized the condition as being a systemic disease exhibiting sclerosing changes in various extrapancreatic organs, including the periorbital tissues, salivary glands, meninges, lymph nodes, thyroid glands, lungs, aorta, retroperitoneum, kidneys, pancreas, biliary tree, gall bladder, liver and prostate (9). Fujinaga et al. summarized 90 cases of autoimmune pancreatitis and analyzed the imaging features of extrapancreatic lesions for the differential diagnosis from lesions of corresponding organs. They found extrapancreatic lesions in 92.2% of the cases, including lachrymal and salivary gland lesions (47.5%), lung hilar lymphadenopathy (78.3%), bile duct wall thickness (77.8%), peri- and para-aortic lymphadenopathy (56.0%) and so on (10). Zen et al. reported the organ-specific pathological features of IgG4-related disease. They found head and neck, thoracic, hepatic and pancreatobiliary, retroperitoneal and systemic lesions in 20.1%, 14%, 23.7%, 11.4% and 30.7% of cases, respectively (11). Since the symptoms are characterized by the organs affected by inflammation, e.g., involvement of the periorbital tissues and salivary glands resembles the symptoms of Mikulicz's syndrome, this information is useful for the differential diagnosis of other diseases and might be related to the response to the administration of PSL. Indeed, our patient showed a slower response in the parotid glands, prostate and common bile duct, most likely due to the organ-specific features of this disease entity. Histologically, IgG4-related disease is characterized by dense lymphoplasmacytic infiltrates rich in IgG4-positive cells that show fibroinflammatory changes. The laboratory data of the case reported here revealed elevated serum IgG and IgG4 levels. The activity of systemic lesions is known to be related to the IgG level (2). Hamano et al., in a review of 64 cases, reported that involvement of one, two, three and four extrapancreatic organs was identified in 31.3%, 26.6%, 21.9% and 9.4% of cases, respectively, and showed that patients with three extrapancreatic lesions appear to have significantly higher IgG4 levels than those without lesions. This result suggests that patients with multiple extrapancreatic lesions have an active disease. Interestingly, no cases showed involvement of more than five organs, and in 10.9% of the cases, no extrapancreatic lesions were found (6). A similar result was reported by Ohara et al., suggesting that a high level of IgG4 may indicate the existence of extrapancreatic lesions (12). Therefore, conducting systemic screening and initiating the administration of PSL is necessary if a high level of IgG4 is found. The case described here is a rare and unique case showing involvement of as much as 10 organs at the onset of disease with a high level of serum IgG4.

The relationship between the IgG4 levels and relapse was described by Kawa et al., who suggested that the IgG and IgG4 levels may be a marker of disease relapse (8). In the case reported here, no evidence of relapse has been found to date, and the patient's IgG4 level has remained within the normal range for more than 11 months with oral PSL therapy. However, due to the significantly high IgG4 level and the presence of multiple lesions with various associated symptoms, providing careful follow-up, including measuring the serum IgG4 levels and performing imaging studies such as CT, US and MRI, is necessary.

IgG4-related disease is a relatively new entity. In addition to PSL (13), B-cell depletion therapy with rituximab may be effective in some, but not all, cases of recurrent or refractory IgG4-RD (14). Further evaluation of cases of IgG4-related disease is required in order to provide a comprehensive understanding of this disease, which will aid the development of a standard therapy.

The authors state that they have no Conflict of Interest (COI).

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Human T cell leukemia virus type 2 (HTLV-2) Tax2 has a dominant activity over HTLV-1 Tax1 to immortalize human CD4$^+$ T cells

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Abstract While human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia, a close relative, HTLV-2, is not associated with any leukemia. HTLV-1 and HTLV-2 encode the Tax1 and Tax2 proteins, respectively, which are essential for the immortalization of human T cells by the respective viruses, thereby causing persistent infection. In this study, we compared Tax1 and Tax2 with respect to their immortalization activity in human T cells. Lentivirus-mediated transduction of the *tax2* gene into human peripheral blood mononuclear cells stimulated with phytohemagglutinin and interleukin-2 in 96-well plates induced outgrowing T cells in most wells, but the cells infected with the control viruses died within 3 weeks. Surprisingly, the number of outgrowing cells induced by Tax2 was much higher than that induced by Tax1, and the appearance of outgrowing cells by Tax2 was earlier than that induced by Tax1. Nevertheless, both Tax2 and Tax1 preferentially immortalized CD4$^+$ T cells, but not CD8$^+$ T cells. Our study showed that HTLV-2 Tax2 can immortalize human CD4$^+$ T cells, and the activity is much higher than that of Tax1. The distinct T cell immortalization activities of Tax2 and Tax1 might therefore play a role in the different pathogeneses observed for these two viruses.

Keywords HTLV-1 · HTLV-2 · Tax2 · CD4 · ATL

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) [1–3]. HTLV-1 infection is generally asymptomatic throughout life, but 3–5 % of HTLV-1-infected individuals develop ATL, with an average age of onset at 60 years. These findings indicate that multiple host and environmental factors are associated with the development of the disease [4]. HTLV-1 immortalizes primary human T cells in the presence of interleukin(IL)-2 in vitro, and a fraction of cells, as a rare event, progress to acquire IL-2-independent growth properties [5, 6]. These studies suggest that the immortalization of T cells by HTLV-1 is therefore of critical importance to establish a persistent infection in vivo [7].

In addition to the structural genes, HTLV-1 has at least two oncogenic genes, *tax1* and *HBZ* (HTLV-1 bZIP factor). In transgenic mice, Tax1 and HBZ independently induce T cell lymphoma [8–10]. In addition, Tax1, but not HBZ, is essential for the immortalization of human T cells. For instance, the inactivation of the *tax1* gene in recombinant HTLV-1 abrogates the T cell immortalization activity of HTLV-1 [11].

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Moreover, the transduction of the *tax1* gene without other viral genes into primary peripheral blood mononuclear cells (PBMC) establishes immortalized T cells [12, 13].

HTLV-2 is a close relative of HTLV-1, and it also immortalizes human T cells with equivalent efficiency to HTLV-1 [14]. Nevertheless, there is no etiological association of HTLV-2 with malignancies. Therefore, comparative studies between HTLV-1 and HTLV-2 provide insight into the molecular mechanism of HTLV-1 leukemogenesis. To obtain information about the distinct pathogenesis between HTLV-1 and HTLV-2, we investigated whether HTLV-2 Tax2 can immortalize human T cells, and how strong (relative to Tax1) the effects of Tax2 are on immortalizing human T cells. We found that Tax2 can immortalize T cells in vitro, and surprisingly, that the activity was much higher than that of Tax1. These findings will be discussed in the context of the distinct pathogenesis between HTLV-1 and HTLV-2.

Materials and methods

Cells and culture conditions

Jurkat and SLB-1 cells are HTLV-1-negative and HTLV-1-positive human T cell lines, respectively. These T cell lines were cultured in RPMI1640 medium supplemented with 10 % fetal bovine serum (FBS), 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (RPMI/10 %FBS). The 293T cells were derived from a human kidney, and the cells were cultured in Dulbecco's modified Eagle medium supplemented with 10 % FBS, 4 mM glutamine, 0.1 mM MEM non-essential amino acids (aa), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Plasmids

CSII-EF-EGFP, CSII-EF-Tax1, CSII-EF-Tax2B, and CSII-EF-Tax300 are the lentiviral expression vectors encoding EGFP, Tax1, Tax2B, and Tax300, respectively [15–17]. CSII-EF-IG is a lentiviral bicistronic EGFP expression vector and was also used for Tax gene expression [16]. pEFneoTax1, pEFneoTax2B, and pEFneoTax300 were used as the expression vectors encoding Tax1, Tax2B, and Tax300, respectively [16, 17]. The *tax* mutant gene, *tax300*, is a chimeric gene that contains the N-terminal region of Tax2B from aas 1 to 299 and the C-terminal region of Tax1 from aa 300 to 353. *tax300* is the same gene as *tax221* which was described in a previous study [18]. pNFAT-Luc is a luciferase expression plasmid that is regulated by three copies of the NFAT site (−286 to −249 of human IL-2 gene) and the human IL-2 promoter (−64 to −47) [19]. The κB-Luc is a luciferase expression plasmid regulated by the κB element of the IL-2 receptor α-chain gene and the minimal HTLV-1 promoter [20]. pGK/β-gal expresses β-galactosidase under the control of the phosphoglycerate kinase promoter and is used to normalize the transfection efficiency.

Immortalization assay

Human PBMCs were isolated from the blood of a healthy donor. They were stimulated with 10 μg/ml phytohemagglutinin (PHA) (Sigma Aldrich) in RPMI/20 %FBS supplemented with 55 μM 2-mercaptoethanol (2-ME) for 2 days and then further cultured in RPMI/20 %FBS with 0.5 nM IL-2 and 2-ME for 2 days. To generate recombinant lentiviruses, 293T cells were transfected with pCAG-HIVgp, pCMV-VSV-G-RSV-Rev (provided by Dr. H. Miyoshi, RIKEN Tsukuba Institute) and the respective lentiviral vectors encoding Tax1, Tax2B, or Tax300 using FuGENE 6 (Roche). At 72 h after the transfection, the supernatant was collected and used to infect PHA-stimulated PBMC (4×10^5 cells) in a final volume of 1 ml of RPMI/20 %FBS containing 8 μg/ml polybrene, 0.5 nM IL-2, and 2-ME. The virus titer was measured by a Lenti-X qRT-PCR Titration Kit (Clontech), and the viruses containing 1.2×10^8 copies of viral genomic RNA were used for the infection. At 48 h after the infection, the cells were resuspended in RPMI/20 %FBS with IL-2 and 2-ME and cultured in 96-well flat-bottom plates at the density of 1.33×10^3 cells/0.1 ml/well, 4.0×10^3 cells/0.1 ml/well, or 1.2×10^4 cells/0.1 ml/well for 10 weeks. Half of the volume of culture medium was exchanged with new medium once every 4–5 days. The number of wells containing outgrowing cells was counted by light microscopy.

Western blotting analysis

To prepare total cell extracts, the cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2 % SDS, 62.5 mM Tris–HCl pH 6.8, 10 % glycerol, 50 mM dithiothreitol, 0.01 % bromophenol blue) and heated at 95 °C for 5 min. Then they were size-separated by electrophoresis under reducing conditions in 10 % polyacrylamide gel with SDS. The proteins in the gel were electrotransferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was incubated with TBS-T [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1 % Tween 20] in 5 % skimmed milk for 1 h at room temperature to inhibit non-specific binding and was further incubated with either an anti-Tax1 mouse monoclonal antibody (Taxy-7) [21] or rabbit anti-Tax2 polyclonal serum (GP3738) [22]. After being washed with TBS-T, the membranes were incubated with either anti-mouse (for Tax1) or anti-rabbit (for Tax2) immunoglobulin G

conjugated with horseradish peroxidase (Bio-Rad Laboratories). Protein bands in the membrane recognized by the antibodies were visualized using the ECL Western blotting detection system (GE Healthcare).

Flow cytometric analysis

The cells were incubated with phycoerythrin (PE)-labeled anti-human CD4 and fluorescein isothiocyanate (FITC)-labeled anti-human CD8 monoclonal antibodies or with isotype matched control antibodies for 30 min at 4 °C. After washing with PBS containing 2 % FBS, the cells were analyzed by flow cytometer (FACScan, Becton–Dickinson) using the Cellquest software program (Becton–Dickinson). PE-labeled mouse anti-human CD8 and FITC-labeled anti-CD4 were purchased from eBioscience.

Transient transfection and luciferase assays

Jurkat cells in RPMI/10 %FBS were seeded at 2×10^5 cells/1.0 ml/well in a 12-well plate. The cells then were cotransfected with the Tax expression plasmid and pGK/β-gal, together with either pNFAT-Luc or κB-Luc by using TransFectin (Bio-Rad Technologies) according to the manufacturer's instructions. At 48 h after transfection, the cell lysates were harvested, and the luciferase and β-galactosidase activities in the lysates were determined using a Luciferase assay system (Promega) and Galacto-Light System (Applied Biosystems). The activity of luciferase was normalized to that of β-galactosidase. The assay was carried out three times to confirm the reproducibility.

Measurement of nuclear NFATc2 and NF-κB p65 proteins

NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) were used to prepare cytoplasmic and nuclear fractions from Tax-immortalized cells and Jurkat cells. The cytoplasmic (10 μg) and the nuclear (5 μg) fractions were characterized by a Western blotting analysis using anti-p65 (F6; Santa Cruz Biotechnology), anti-NFATc2 (4G6-G5; Santa Cruz Biotechnology), anti-Sp1 (PEP2; Santa Cruz Biotechnology), anti-Tubulin (DM1A; Calbiochem), and anti-Tax1 and anti-Tax2 antibodies.

Results

Tax2 immortalizes primary human T cells in the presence of IL-2

The tax2B gene used in this study was derived from the HTLV-2b subtype [15]. The tax300 gene is a chimeric gene that combines tax2B and tax1, and has the N-terminal Tax2B aa (1–299) and the C-terminal Tax1 aa (300–353) (Fig. 1a) [16]. To measure the immortalization activity of the genes in human T cells, we generated lentivirus vectors encoding tax2B, tax300, and tax1 by using 293T cells, and the virus titers in the culture supernatant of 293T cells were adjusted by quantification of the viral genome by real time polymerase chain reaction (PCR). The viruses containing 1.2×10^8 copies of viral genomic RNA were then used to infect two types of cells, human PBMCs stimulated with 10 μg PHA and recombinant IL-2 for 2 days, or a human T cell line (Jurkat). The control lentivirus expressed green fluorescent protein (GFP). The anti-Tax1 antibody detected Tax1 and Tax300 proteins, but not Tax2B, in both PBMCs and Jurkat cells infected with the respective viruses, although the expression level in PBMCs was lower than in Jurkat cells (Fig. 1b, c). An anti-Tax2 antibody detected all three Tax proteins in both cells. However, it should be noted that the anti-Tax2 antibody was raised against Tax2 peptides, and detects Tax2 and Tax300 proteins more efficiently than Tax1 protein [16, 22]. On the other hand, anti-Tax1 antibody detects Tax1 and Tax300 proteins more efficiently than Tax2B protein. Collectively, the results of the Western blotting analyses indicated that the amounts of Tax2B, Tax1, and Tax300 proteins in PBMCs infected with the respective viruses were either almost equivalent or the

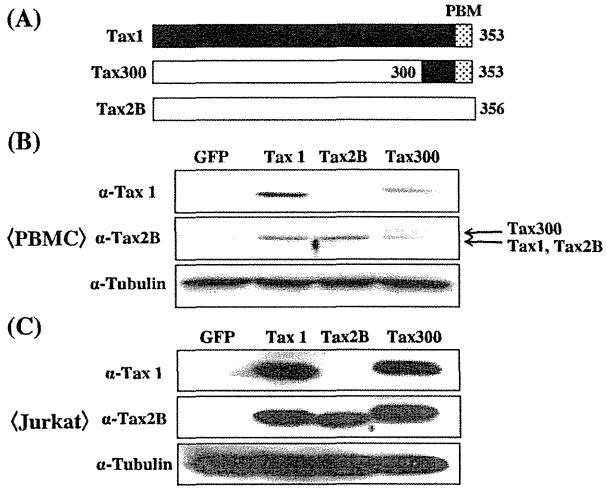

Fig. 1 Lentiviral introduction of *tax1*, *tax2B*, and *tax300* into PBMCs and Jurkat. **a** Tax1 and Tax2B consisted of 353 and 356 aa, respectively. Tax300 has the N-terminal region of Tax2B from aa 1 to 299 and the C-terminal region of Tax1 from aa 300 to 353. PBM indicates the PDZ domain binding motif present only in Tax1 but not in Tax2B, which is essential for the augmented transforming activity of Tax1 in CTLL-2 relative to Tax2 [32, 34]. **b**, **c** The PHA-stimulated PBMCs (**b**) and Jurkat cells (**c**) were infected with the indicated viruses. At 48 h after the infection, the cell lysates were prepared, and the amounts of Tax1, Tax2B, Tax300, and Tubulin in the lysates were measured by a Western blotting analysis using anti-Tax1 (Taxy-7), Tax2, and anti-Tubulin antibodies

amount of Tax1 was more than that of Tax2B. Tax300 was always detected as a higher molecular protein than Tax1 by our Western blotting analysis, although both Tax300 and Tax1 consist of 353 aas. This difference between Tax300 and Tax1 might be caused by their distinct conformation or distinct post-translational modification.

Next, these virus-infected PBMCs were cultured in the presence of IL-2 in a 96-well plate at cell densities of 1.2×10^4, 4×10^3, and 1.33×10^3 cells/well (Fig. 2a, b). At 7- to 10-days post-infection, PBMCs that were infected with Tax2B-viruses showed multiple small clumps of cells in all the wells, and the number of clumps was much higher than that of the cells infected with the control virus. Thereafter, these Tax2B-infected cells continuously proliferated, and 80 out of 96 wells (1.2×10^4 cells/well) contained obvious outgrowing cells at 10-weeks post-infection, whereas all the cells infected with the control virus had died within 3 weeks. At 7- to 10-days post-infection, Tax1 also induced small clumps of cells similar to Tax2B in all the wells, but they then stopped proliferating for 4–5 weeks. Thereafter, some cells started to regrow, and at 10-weeks post-infection, 10 out of 96 wells contained obvious outgrowing cells. Tax300 also induced outgrowing cells from PBMCs, and at 12-weeks post-infection, 36 out of 96 wells contained obvious outgrowing cells, the number of which was lower than that induced by Tax2B. Three independent experiments confirmed the augmented immortalization activity of Tax2B relative to Tax1. We transferred several outgrowing cells to a 24-well plate, and then to a culture flask, and the expression levels of Tax in such cells were characterized by a Western blotting analysis. Three Tax2B-transduced cells express the Tax2B protein, but not Tax1, whereas three Tax1-transduced cells expressed Tax1 protein, but not Tax2B (Fig. 2c). These results indicated that Tax2 is therefore able to immortalize PBMCs, and this activity is stronger than that of Tax1.

Tax1 preferentially immortalizes $CD4^+$ T cells [12, 23]. Therefore, it would be interesting to see whether Tax2 also has

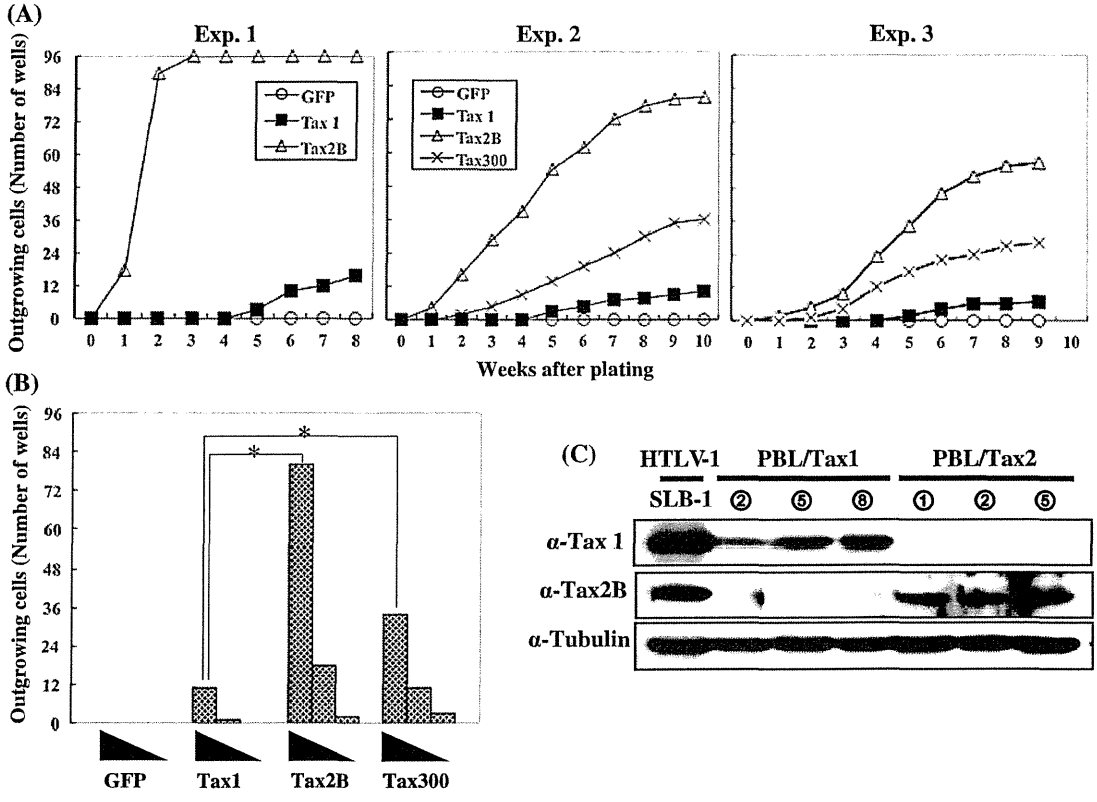

Fig. 2 Tax2B immortalizes human PBMCs. **a**, **b** PHA-stimulated PBMCs infected with lentiviruses encoding *GFP*, *tax1*, *tax2B*, or *tax300* were cultured in RPMI/20 %FBS/IL-2 in 96-well plates at a density of 1.2×10^4, 4.0×10^3, or 1.33×10^3 cells/0.1 ml/well for 10 weeks. The number of wells containing outgrowing cells (seeded at cell density of 1.2×10^4 cells/0.1 ml/well) was counted under a light microscope for 8–10 weeks. The results of three independent experiments are presented in **a**. The number of wells containing outgrowing cells (seeded at cell density of 1.2×10^4, 4.0×10^3, or 1.33×10^3 cells/0.1 ml/well) at 10-weeks post-infection of the experiment 2 in **a** is indicated in **b**. *Asterisk* A significant difference ($p < 0.001$) by the χ^2 test. **c** Three PBL/Tax1 cell lines (PBL/Tax1-2, PBL/Tax1-5, PBL/Tax1-8) and three PBL/Tax2B cell lines (PBL/Tax2B-1, PBL/Tax2B-2, PBL/Tax2B-5) were established from the PBMCs infected with Tax1 and Tax2B lentiviruses as shown above, respectively. The amounts of Tax1 and Tax2B protein in these cells were measured by a Western blotting analysis using anti-Tax1 and Tax2 antibodies

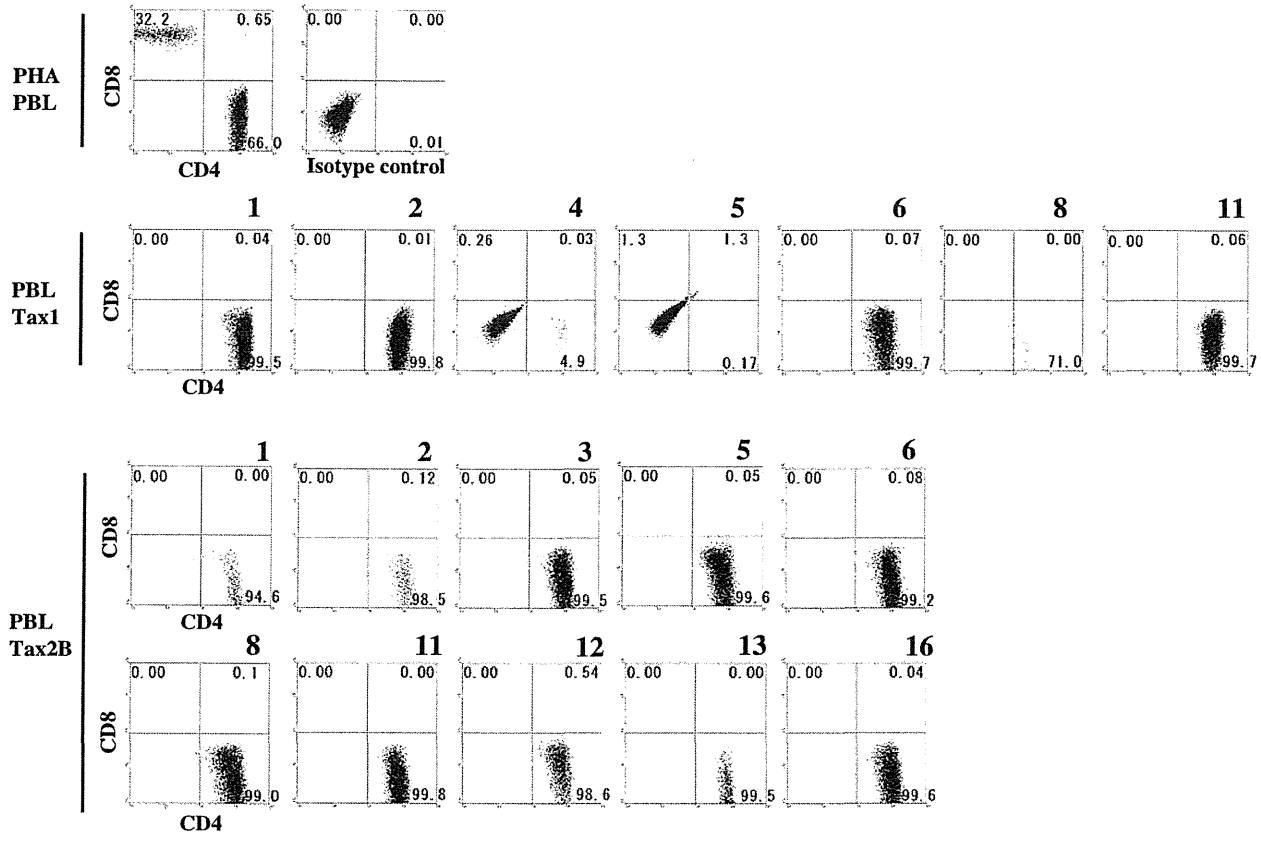

Fig. 3 Tax-immortalized cells express CD4, but not CD8 antigen. The Tax-immortalized T cell lines were characterized for CD4 and CD8 expression using a flow cytometer. Seven PBL/Tax1 cell lines and ten PBL/Tax2B cell lines were established by infection of PBMCs with the Tax1-lentivirus or Tax2B-lentivirus, respectively, and were cultured in the presence of IL-2

an immortalization ability similar to that of Tax1. A flow cytometric analysis showed that all ten Tax2B-immortalized cells were CD4 single positive (Fig. 3). On the other hand, five out of the seven Tax1-immortalized cells were CD4 single positive, one was double negative and the last one (PBL/Tax1-4) was a mixture of double negative cells with a minor population of CD4 single positive cells. These results indicated that Tax2 immortalizes $CD4^+$ T cells, but not $CD8^+$ T cells.

Tax2 activates NFAT-dependent transcription much more efficiently than Tax1

NF-κB and NFAT are transcription factors that control the expression of many genes regulating T cell functions, including cell proliferation and anti-apoptosis, and they are stimulated by both Tax1 and Tax2 [6, 24]. To determine whether the high immortalization activity of Tax2B is related to activation of these transcription factors, we next measured the transcriptional activation by Tax2B, Tax1, and Tax300. The tax expression plasmids and luciferase reporter plasmid under the control of either NF-κB or NFAT were transfected into Jurkat cells using the lipofection method. At 48 h post-transfection, the cell lysates were prepared, and the luciferase activities were measured. Tax2B and Tax300 activated the NF-κB reporter 20- to 25-fold more than the control plasmid, and the activities were half that of Tax1 (Fig. 4). While Tax2B and Tax300 activated the NFAT reporter more than 100-fold, Tax1 only activated NFAT 3- to 10-fold. These results were consistent with those of a previous study [18]. We also examined whether Tax stimulates the nuclear localization of NF-κB p65 and NFATc2, which would indicate the activation of these transcription factors (Fig. 5a). The treatment of Jurkat cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and ionomycin induced an increased nuclear expression of NF-κB p65 and NFATc2 proteins. The transient transduction of Tax1 and Tax2B into Jurkat cells also induced an increased nuclear expression of NF-κB p65 and NFATc2 relative to the control, but the amount of nuclear NFATc2 in Tax1-transduced cells was lower than that of Tax2B-transduced cells, which is consistent with the above described reporter assay.

We then examined the status of NF-κB and NFAT in Tax-immortalized T cells. The amounts of nuclear p65 and NFATc2 in each of three Tax1- and Tax2B-immortalized T cells were more than those of unstimulated Jurkat and they