Aberrant Expression of Human Achaete-Scute Homologue Gene 1 in the Gastrointestinal Neuroendocrine Carcinomas

Takashi Shida,1,2 Mitsuko Furuya,1 Takashi Nikaido,4 Takashi Kishimoto,1 Keiji Koda,2 Kenji Oda,2 Yukio Nakatani,3 Masaru Miyazaki,2 and Hiroshi Ishikura1

Departments of 1Molecular Pathology, 2General Surgery, and 3Clinical Pathology, Chiba University Graduate School of Medicine, Chiba, Japan and 4Department of Pathology, Jikei University School of Medicine, Tokyo, Japan

ABSTRACT

Purpose: Gastrointestinal neuroendocrine carcinoma (NEC) is extremely aggressive, but its pathophysiologic features remain poorly understood. There have been no biologically specific markers for this disease. In this study, distinctive up-regulation of human achaete-scute homologue 1 (hASH1) in gastrointestinal NECs was clarified.

Experimental Design: Expression of hASH1 in NECs (n = 10), carcinoid tumors (n = 10), other tumors (10 adenocarcinomas, 2 squamous cell carcinomas and 1 malignant lymphoma), and the corresponding normal mucosa were investigated by in situ hybridization, reverse transcription-PCR (RT-PCR), real-time RT-PCR, and immunohistochemistry.

Results: By in situ hybridization, mild to intense signals of hASH1 mRNA were detected in 9 of 10 NECs, but not in other tumors or normal mucosa, except for focally weak signals in one carcinoid tumor. RT-PCR showed strong expression of hASH1 in a small cell NEC, followed by a moderately differentiated NEC, and a carcinoid tumor, whereas it is undetectable in adenocarcinomas or normal mucosa. By real-time RT-PCR, the amounts of hASH1 mRNA in a small cell NEC were 16,600 times higher than those in adenocarcinomas and 110 times higher than those in a carcinoid tumor. Immunohistochemically, mammalian homologue of hASH1 was positive in 7 of 10 NECs but was negative in the other tumors. Pan-endocrine markers chromogranin A and synaptophysin were positive in almost all carcinoid tumors, in 4 and 7 of the 10 NECs, respectively.

Conclusions: These findings revealed that hASH1 is distinctly up-regulated in gastrointestinal NECs. hASH1 may be used as a more sensitive and specific marker than conventional pan-endocrine markers for clinical diagnosis of gastrointestinal NECs.

Received 7/13/04; revised 9/6/04; accepted 10/13/04. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Mitsuko Furuya, Department of Molecular Pathology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo, Chiba 260-8670, Japan. Phone: 81-43-226-2061; Fax: 81-43-226-2063; E-mail: furuya@faculty.chiba-u.jp.

©2005 American Association for Cancer Research.

INTRODUCTION

Gastrointestinal tumors with neuroendocrine differentiation are common diseases and are divided into some distinct pathologic entities such as typical carcinoid tumors, neuroendocrine carcinomas (NEC), and adenocarcinomas with a component exhibiting endocrine differentiation (1, 2). Typical carcinoid tumors are potentially malignant, but generally grow slowly and show less aggressive features than adenocarcinomas (1–3). On the other hand, NECs are known to be extremely aggressive (1, 2, 4). Although gastrointestinal NECs are less frequent than adenocarcinomas (5), the prognosis of this entity is very poor. The pathophysiologic feature of gastrointestinal NEC is poorly understood, and the effective therapeutic strategies have not yet been established. Based on WHO classification, gastrointestinal NECs are defined as small cell carcinoma, being morphologically similar to the small cell carcinoma of the lung (6, 7). In addition to small cell carcinomas, pathologic studies have shown that the moderately differentiated (also named large cell or intermediate variant; refs. 4, 8) NECs should be classified as high-grade malignant (9) because of their distinct neuroendocrine lineage and biological aggressiveness (4, 8, 10). Both small cell and moderately differentiated NECs need careful differential diagnosis from other malignancies such as poorly differentiated adenocarcinomas, undifferentiated carcinomas, and malignant lymphomas. However, immunohistochemical stainings for pan-endocrine markers such as synaptophysin, neuron-specific enolase and chromogranin A are neither specific nor sensitive to NECs. Carcinoid tumor cells, normal gastrointestinal endocrine cells, and some poorly differentiated adenocarcinoma cells are positive for these pan-endocrine markers, whereas NECs are often negative or contain only a low percentage of positive cells (8, 11). There has been no biologically specific marker for gastrointestinal NECs, and it is very likely that NECs are sometimes misdiagnosed as poorly differentiated adenocarcinomas or undifferentiated carcinomas.

Human achaete-scute homologue 1 (hASH1) is a member of the basic helix-loop-helix (bHLH) family of transcription factors, and is known to play a crucial role in neuronal/endocrine determination and differentiation in normal development of nervous system and endodermal endocrine cells (12, 13). With regard to human diseases of neuronal/endocrine lineage, hASH1 is known to be expressed in small cell carcinomas of the lung (14) and of the prostate (15), neuroblastomas (16), and medullary carcinomas of the thyroid (14). On the other hand, in the other neuronal/endocrine carcinomas such as Merkel cell carcinomas (17), medulloblastomas (16), and pheochromocytomas (14), hASH1 was shown to be negative and the other bHLH members, HTH1 and NeuroD, were suggested to play key roles. In the gastrointestinal NECs, it remained unknown whether hASH1 or other important transcriptional regulator(s) contribute to their neuroendocrine lineage.

In this study, we investigated hASH1 expression in human gastrointestinal NECs. Using in situ hybridization (ISH) and quantitative reverse transcription-PCR (RT-PCR) we showed that
hASH1 mRNA was markedly up-regulated in NECs, whereas it was weak in carcinoid tumors, and was scarcely expressed in adenocarcinomas and normal mucosa. Immunohistochemical staining for mammalian homologue of hASH1 (mASH1) supported the findings that this molecule was much more abundant in NECs than in the other types of tumors.

**MATERIALS AND METHODS**

**Samples.** Ten cases of NECs (three in the rectum, three in the colon, three in the stomach, and one in the esophagus), 10 cases of typical carcinoid tumors (seven in the rectum, two in the duodenum, and one in the stomach), and 10 cases of adenocarcinomas (seven in the colon and three in the stomach), two squamous cell carcinomas in the esophagus, one malignant lymphoma (diffuse large B cell type) in the duodenum, and the corresponding nontumor parts of all these cases were examined from surgical specimens of Japanese patients who took biopsy examinations or operations at the Department of General Surgery, Chiba University Hospital and affiliated hospitals between 1999 and 2004. None of the patients had received adjuvant therapy before pathologic examinations. The possibility of metastasis from lung or other primary sites was ruled out based on clinical information. Written informed consent was obtained from each patient prior to biopsy. Among these 33 cases, eight biopsy specimens (one small cell NEC, one moderately differentiated NEC, one typical carcinoid tumor, three adenocarcinomas, and two normal mucosa) were immediately frozen with liquid nitrogen and were kept at −80°C until analysis. Remaining tissues were fixed with 10% formalin and embedded in paraffin. H&E staining was done in all the cases (Table 1). In 3 of 10 NECs (case nos. 2, 3, and 10), reductive surgery was not done because of extensive tumor spread, and biopsy specimens were used for the study.

Pathologic diagnosis of NECs were done based on the criteria generally used in the previous studies on gastrointestinal NECs (8, 10). NECs were divided into two subtypes in the study, i.e., small cell carcinoma similar to the counterpart of the lung, and moderately differentiated NEC composed of polygonal and fusion cells two to three times larger than those of small cell NECs with abundant cytoplasm and prominent nucleoli (10). Each NEC was evaluated independently by three of the authors and classified as either small cell or moderately differentiated NEC. The consensus of the subtypes was reached by simultaneous review defined according to the predominant morphologic feature. Atypical carcinoid tumors

**Table 1** Expression of hASH1 (ISH), pan-endocrine markers (IH), and mASH1 (IH)

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Histology</th>
<th>Organ</th>
<th>Stage</th>
<th>Prognosis (months)</th>
<th>hASH1 (ISH)</th>
<th>CgA</th>
<th>Syn</th>
<th>mASH1 (IH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58/F</td>
<td>NEC (moderate)</td>
<td>rectum</td>
<td>III</td>
<td>NED (11)</td>
<td>2+, moderate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>56/M</td>
<td>NEC (small cell)</td>
<td>rectum</td>
<td>IV</td>
<td>DOD (1)</td>
<td>3+, intense</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>69/M</td>
<td>NEC (small cell)</td>
<td>rectum</td>
<td>III</td>
<td>DOD (3)</td>
<td>3+, intense</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>78/M</td>
<td>NEC (small cell)</td>
<td>colon</td>
<td>IV</td>
<td>DOD (1)</td>
<td>2+, intense</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>45/F</td>
<td>NEC (moderate)</td>
<td>colon</td>
<td>II</td>
<td>NED (12)</td>
<td>3+, moderate</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>59/F</td>
<td>NEC (moderate)</td>
<td>colon</td>
<td>IV</td>
<td>DOD (14)</td>
<td>1+, mild</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>81/F</td>
<td>NEC (small cell)</td>
<td>stomach</td>
<td>IV</td>
<td>DOD (7)</td>
<td>2+, intense</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>83/F</td>
<td>NEC (small cell)</td>
<td>stomach</td>
<td>IIIA</td>
<td>DOD (5)</td>
<td>1+, moderate</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>70/M</td>
<td>NEC (moderate)</td>
<td>stomach</td>
<td>I</td>
<td>NED (24)</td>
<td>++</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>56/M</td>
<td>NEC (small cell)</td>
<td>esophagus</td>
<td>IV</td>
<td>DOD (4)</td>
<td>3+, intense</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>55/F</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (14)</td>
<td></td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>76/M</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (7)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>13</td>
<td>72/M</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (11)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>14</td>
<td>60/F</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (14)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>15</td>
<td>44/M</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (15)</td>
<td>1+, mild</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>16</td>
<td>74/M</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (25)</td>
<td></td>
<td>++</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>17</td>
<td>59/F</td>
<td>typical carcinoid</td>
<td>rectum (T1)</td>
<td>NED (14)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>18</td>
<td>48/M</td>
<td>typical carcinoid</td>
<td>duodenum (T1)</td>
<td>NED (16)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>19</td>
<td>42/M</td>
<td>typical carcinoid</td>
<td>duodenum (T1)</td>
<td>NED (9)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>20</td>
<td>40/M</td>
<td>typical carcinoid</td>
<td>stomach (T1)</td>
<td>NED (63)</td>
<td></td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>21</td>
<td>72/F</td>
<td>Adenoca (Por)</td>
<td>colon</td>
<td>III</td>
<td>NED (11)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>22</td>
<td>67/F</td>
<td>Adenoca (Well)</td>
<td>colon</td>
<td>I</td>
<td>NED (10)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>23</td>
<td>67/F</td>
<td>Adenoca (Mod)</td>
<td>colon</td>
<td>II</td>
<td>NED (8)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>24</td>
<td>48/F</td>
<td>Adenoca (Por)</td>
<td>colon</td>
<td>II</td>
<td>NED (41)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>25</td>
<td>69/M</td>
<td>Adenoca (Mod)</td>
<td>colon</td>
<td>III</td>
<td>NED (39)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>26</td>
<td>38/F</td>
<td>Adenoca (Por)</td>
<td>colon</td>
<td>III</td>
<td>AWD (4)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>27</td>
<td>66/F</td>
<td>Adenoca (Well)</td>
<td>colon</td>
<td>I</td>
<td>NED (10)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>28</td>
<td>55/M</td>
<td>Adenoca (Mod)</td>
<td>stomach</td>
<td>IA</td>
<td>NED (37)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>29</td>
<td>68/F</td>
<td>Adenoca (Mod)</td>
<td>stomach</td>
<td>IB</td>
<td>NED (36)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>30</td>
<td>63/F</td>
<td>Adenoca (Mod)</td>
<td>stomach</td>
<td>II</td>
<td>NED (19)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>31</td>
<td>82/F</td>
<td>squamous cell</td>
<td>esophagus</td>
<td>I</td>
<td>NED (37)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>32</td>
<td>76/M</td>
<td>squamous cell</td>
<td>esophagus</td>
<td>III</td>
<td>NED (24)</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>33</td>
<td>48/M</td>
<td>ML</td>
<td>duodenum (T1)</td>
<td>NED (11)</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: IH, immunohistochemistry; CgA, chromogranin A; Syn, synaptophysin; F, female; M, male; Adenoca, adenocarcinoma; Well, well differentiated; Mod, moderately differentiated; Por, poorly differentiated; squamous cell, squamous cell carcinoma; ML, malignant lymphoma; (T1), tumor was limited to submucosa; NED, no evidence of the disease; AWD, alive with the disease; DOD, dead of the disease; ND, not done.
and mixed carcinoid-adenocarcinomas were not included in the study to avoid confusion with NECs. In the carcinoid tumors used in the study, none showed metastasis or lympho-vascular space invasion, and all the patients were alive with no evidence of disease after either endoscopic mucosal resection (eight cases) or operation (two cases). In the adenocarcinomas, squamous cell carcinomas and a malignant lymphoma, no cases showed heterogenous components. The stages were judged based on the classification of International Union Against Cancer. Since both typical carcinoid tumors and a malignant lymphoma were limited to the submucosa, they were described as T1 (Table 1).

**RNA Isolation.** Total RNAs from the eight frozen tissues were obtained using RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions.

RNA from human small cell lung carcinoma cell line, NCI-H1688, was used as a positive control because small cell lung carcinomas are known to highly express hASH1 mRNA (18). The expression of hASH1 mRNA in NCI-H1688 was confirmed by subcloning and sequencing described below.

**RT-PCR and real time RT-PCR.** Expression of hASH1 was examined by RT-PCR and real-time RT-PCR with the following primers: 1018F (TCCTCCCAAAGGCGCAAGTACTC) and 1250R (CCCTCCCAACGCCACTG) for hASH1 (18), 447F (GGCGGACATTCTGGAAATGA) and 547R (GGTACTTCTCCCAGCACACTTG) for HES1, 900F (GACCAGCTGCAGAATGTATCT) and 1000R (TCGGA-CAAGCCTTGATGTA) for HATH1, 244F (AAGCTGGA-CAACGAC) and 1250R (CCCTCCCAACGCCACTG) for GAPDH. cDNA synthesis was done using 0.1 nmol of random primer (Takara, Ohtsu, Japan). RT-PCR amplification was done using AmpliTaq Gold PCR Master Mix (PE Applied Biosystems, Foster City, CA) and PCR thermocycler (PE 9700, PE Applied Biosystems). The conditions for PCR of Notch1 were followed as in a previous report (19). Conditions for PCR of the other molecules were as follows: at 95°C for 15 seconds, 60°C for 1 minutes, with an extension step of 72°C for 1 minutes, 35 cycles at 95°C for 1 minutes, 60°C for 1 minutes, 40 cycles of 95°C for 30 seconds and 60°C for 2 minutes. GAPDH was used as an internal control gene. The standard curve for GAPDH and hASH1 were determined using the cell line NCI-H1688. hASH1 mRNA levels were determined as the absolute number of copies normalized against GAPDH mRNA.

**In situ hybridization.** For nonradioactive ISH, digoxigenin-labeled sense/antisense RNA probes were generated by PCR amplification of 431 bp hASH1 products and incorporation of a T3/T7 promoter into the sense/antisense primer. In vitro transcription was done with digoxigenin RNA labeling reagent and T3/T7 polymerase (Roche, Penzberg, Germany). Formalin-fixed tumors and normal tissue were sectioned at 4 µm. All sections were permeabilized with pepsin, and incubated with RNA probe (200 ng/mL) overnight at 55°C. After washing with 50% formamide and 2× SSC, sections were incubated at 37°C with RNase mixture (Ambion, Austin, TX) diluted 1:20 in TNE. Slides were stringently washed with 0.1% SSC and 0.01% SSC at 60°C (GenPoint Kit, DAKO Cytomation, Carpinteria, CA). Before immunodetection, tissues were treated with peroxidase blocking reagent (DAKO Cytomation) and blocked with 1% BSA in TBS. Monoclonal antibody against mASH1 (BD PharMingen, San Diego, CA, clone 24B72D11.1) was used in the detection. Monoclonal antibody against synaptophysin (DAKO Cytomation) was used to catalyze the deposition of biotin-tyramide. Signal was detected with 3,3′-diaminobenzidine.

The staining pattern was scored as negative (0-5%), 1+ (5-10%), 2+ (10-50%), or 3+ (>50%). The intensity of the signal was also scored as mild, moderate, or intense.

**Immunohistochemistry.** Immunohistochemistry was done on formalin-fixed paraffin-embedded sections, using labeled streptavidin-biotin-peroxidase and autoclave antigen retrieval technique. Monoclonal antibody against chromogranin A and polyclonal antibody against synaptophysin (DAKO Cytomation) were used to analyze neuroendocrine differentiation. Monoclonal antibody against mASH1 (BD PharMingen, San Diego, CA, clone 24B72D11.1) was used in the study, because this antibody was shown to react to hASH1 of human small cell carcinoma of the lung and prostate in the paraffin-embedded tissues (15). Working dilutions of mASH1 and chromogranin A were 1:100 and 1:50, respectively, and synaptophysin polyclonal antibody was ready-to-use. Mouse IgG was used as a negative control, with a dilution of 1:100. Appropriate positive controls known to contain the antigens in question were processed simultaneously. The staining pattern was scored as follows: −, no staining or < 5% of the tumor cells positive; +, occasional weak staining; ++, moderate staining; and +++, intense staining.

**Statistical Analysis.** $\chi^2$ test was used for the statistical evaluation of ISH data. Statistical significance was assumed when $P < 0.05$ was obtained.
RESULTS

ISH Revealed That hASH1 Signals Were Diffusely Expressed in the Tumor Cells of NECs, but Sarcely Expressed in the Other Types of Tumors or in the Endocrine Cells of Normal Mucosa. The study on hASH1 mRNA localization by ISH showed moderate to intense signals of hASH1 mRNA in the tumor nests in 8 of 10 NEC cases (Fig. 1B and D), and mild signals in one case. All these cases showed clinically advanced stages (Table 1). In one case which showed negative for hASH1 (case 9), tumor was limited to the slight submucosa, and curative endoscopic mucosal resection was done. Among the 10 NECs, 3 cases accompanied adenocarcinoma components in the peripheral zones (cases 5, 6, and 9), and neither these adenocarcinomas nor the corresponding normal mucosa showed significant signals (data not shown). In the 10 cases of carcinoid tumors, mild signals for hASH1 mRNA were focally detected in 1 case (case 15) and no significant signals were observed in the other 9 cases (Fig. 1F). Compared with the strong stainings of hASH1 in NECs, the signals in this carcinoid tumor were far less intense. None of the 10 adenocarcinomas or endocrine cells of normal mucosa showed detectable signals of hASH1 mRNA in this study (Fig. 1H). The expression patterns of hASH1 were significantly correlated with histologic entities between NECs and carcinoid tumors ($\chi^2$ test; $P = 0.0003$).

RT-PCR Showed That Expression Levels of hASH1 mRNA Were Much Higher in NECs Than Those in Non-NEC Gastrointestinal Tissues. In RT-PCR, the bands of hASH1 were strongly detected in a small cell NEC (case 10), and less strongly detected in a moderately differentiated NEC (case 1). PCR products were also detectable in a carcinoid tumor (case 15), in which hASH1 signals were shown by ISH. On the other hand, hASH1 bands were scarcely observed in the adenocarcinomas (cases 21, 22, and 23) and in the normal mucosa. HATH1, another member of the bHLH family, was clearly detected in all the examined cases except for one moderately differentiated NEC, in which the band was weaker than those of others. Notch1, a primary regulator of the bHLH family of transcription factors involved in neuroendocrine differentiation, was expressed in all the examined cases including normal mucosa. HES1 and HES5, the main suppressors of hASH1 in normal neuronal/endocrine differentiation, were also detectable in all the examined cases (Fig. 2).

Real Time RT-PCR Revealed Much Higher Expression of hASH1 mRNA in NECs Than in Adenocarcinomas. These eight cases of biopsy tissues used in RT-PCR analysis were subjected to the quantification of hASH1 mRNA. Real time RT-PCR showed that the amounts of hASH1 mRNA normalized by GAPDH in the small cell NEC and the moderately differentiated NEC were 99,800 $\times 10^{-6}$ and 2,290 $\times 10^{-6}$, respectively. On the
Fig. 1 continued. E, HE staining of the carcinoid tumor (case 13, ×100). F, signals of hASH1 mRNA were not detected in the carcinoid tumor (serial section of E, ×100). G, HE staining of the adenocarcinoma and the corresponding normal mucosa (case 22, ×100). H, signals of hASH1 mRNA were not detected either in the adenocarcinoma or in the corresponding normal mucosa (serial section of G, ×100).

Fig. 2 Expression of hASH1 mRNA in the NECs, carcinoid tumor, adenocarcinomas, and normal mucosa. Total RNA was prepared from homogenized tissue of surgical specimens. RT-PCR was done and PCR product samples were subjected to 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. Seven representative cases are shown. SNEC, small cell NEC (case 10); MNEC, moderately differentiated NEC (case 1); Carcinoid, typical carcinoid tumor (case 15); Adenoca., adenocarcinoma (case 21, lane 4, and case 22, lane 5); Normal, normal mucosa of the colon. RNA from human small cell lung carcinoma cell-line, NCI-H1688, is used as positive control (lane 8).

Fig. 3 hASH1 mRNA levels in the NECs, carcinoid tumor, adenocarcinomas, and normal mucosa. The levels of hASH1 mRNA normalized by GAPDH in eight different clinical samples. The amounts of hASH1 mRNA normalized by GAPDH in small cell NEC (SNEC, case 10) is the highest (99,800 × 10⁻⁶), followed by moderately differentiated NEC (MNEC, case 1; 2,290 × 10⁻⁶). The amount of hASH1 mRNA in the carcinoid tumor (Carcinoid, case 15) is 910 × 10⁻⁶. The ratios of hASH1/GAPDH in three adenocarcinomas (Adenoca.) are 6.0 × 10⁻⁶ (case 23, moderately differentiated), 20 × 10⁻⁶ (case 22, well differentiated), and 24 × 10⁻⁶ (case 21, poorly differentiated). The ratios of hASH1/GAPDH in the normal mucosa are 3.4 × 10⁻⁶ and 7 × 10⁻⁶.
other hand, the ratios of hASH1/GAPDH in the normal mucosa were $3.4 \times 10^{-6}$ and $7 \times 10^{-6}$. The ratios of hASH1/GAPDH in the three adenocarcinomas were between $6 \times 10^{-6}$ and $24 \times 10^{-6}$. The ratio of hASH1/GAPDH in a typical carcinoid tumor was $9 \times 10^{-6}$. These results showed that hASH1 mRNA in a small cell NEC was the highest, followed by a moderately differentiated NEC, and the levels of NECs were approximately 100 to 16,600 times higher than those in adenocarcinomas. hASH1 mRNA in a typical carcinoid tumor was 150 to 455 times higher than those in adenocarcinomas, but 2.5 to 110 times lower than those in NECs (Fig. 3).

Immunohistochemistry Supported the Results of ISH That hASH1 was Abundantly Expressed in NECs but Not in the Other Types of Tumors or in Normal Mucosa. Immunohistochemistry for hASH1 was done using anti-mASH1 monoclonal antibody (mASH1 known to cross-react to hASH1 in the human prostate and lung NECs; ref. 15). In this study, nuclear staining was counted as mASH1-positive and cytoplasmic staining was not counted based on the positive staining pattern in small cell lung carcinomas: 7 of 10 NECs were shown to be positive (Fig. 4B). Normal endocrine cells, carcinoid tumors and adenocarcinomas showed no stainings for mASH1 (Fig. 4E and H). With regards to pan-endocrine markers, 7 of 10 NECs showed positive for synaptophysin, and among these 7 cases, 4 cases were also positive for chromogranin A (Fig. 4C). All the carcinoid tumors showed positive for synaptophysin, and 8 of 10 cases were also positive for chromogranin A (Fig. 4F). None of the adenocarcinomas and normal glands showed positive stainings for the pan-endocrine markers except for the normal enterochromaffin-like cells (Fig. 4I, arrows).

Fig. 4 Immunohistochemistry of mASH1 and synaptophysin in the NEC, carcinoid tumor, adenocarcinoma, and normal mucosa. A, HE staining of small cell NEC (case 7, ×400). B, immunohistochemistry of mASH1. The tumor cells show diffuse nuclear staining pattern (×400).
Other tumors (two squamous cell carcinomas and a malignant lymphoma) used in this study did not show positive stainings for hASH1 mRNA, hASH1 protein, or pan-endocrine markers (data not shown).

These results show that hASH1 mRNA can be measured in clinical biopsy samples, and that the expression of hASH1 is distinctly more abundant in gastrointestinal NECs than in the other types of tumors.

**DISCUSSION**

Gastrointestinal NECs are known to be highly aggressive, but their pathophysiologic feature remains largely unknown. It might owe to the infrequency of this disease, and also to the possible difficulty in differential diagnosis, because there has been no specific marker for NECs and the sensitivity of pan-endocrine markers is limited (8). Indeed, 4 of 10 NECs in this study were negative for chromogranin A, and 3 of them were also negative for synaptophysin.

In this study, ISH showed the presence of hASH1 with high sensitivity and specificity. It was shown by ISH that hASH1 mRNA is aberrantly expressed in the gastrointestinal NECs, whereas it is scarcely detectable in adenocarcinomas. Differential expression of hASH1 between neuroendocrine tumors and adenocarcinomas was supported by real time RT-PCR data, although a limited number of cases were examined. Because 1 of the 10 carcinoid tumors (case 15) showed significant hASH1 expression at mRNA level, we cannot exclude the possibility that hASH1 mRNA may be present in some of the carcinoid tumors. However, none of the carcinoid tumors were stained for hASH1 at the protein level. Immunohistochemistry against hASH1 worked as sensitively as synaptophysin and chromogranin A, but showed more specific patterns to NECs than these pan-endocrine markers. In the pathologic study on human lung, hASH1 mRNA was detected in small cell lung carcinomas but almost none were found in typical carcinoid tumors (20). These findings, in addition to our study, strongly suggest that hASH1 may be a highly useful marker in clinical diagnosis of NECs. The expression analysis of hASH1, and its correlation with pathologic features and prognosis in a larger number of gastrointestinal tumors await further investigations.

It is reported that other bHLH family members besides hASH1, for example, HATH1, may play key roles in some neuronal/endocrine diseases (16, 17), but the expression of HATH1 in the alimentary tract remains poorly understood. In the present study, HATH1 did not show significant up-regulation in the gastrointestinal NECs (Fig. 2). In the process of normal embryogenesis, each bHLH member mediates neuronal/endocrine differentiation in a specific period of cell fate (21–23). If hASH1, HATH1, or other bHLH members work in a limited phase of tumor development, the distinctive expression pattern of each bHLH member in different types of neuroendocrine tumors might be determined, at least in part, by the status of cell differentiation.
adenocarcinomas in the absence of hASH1. Because the expression of hASH1 is limited to the early stage of cell specification in normal embryogenesis, the aberrant expression of hASH1 in NECs might reflect the biologically less-differentiated state of tumor cells.

Functional analysis using siRNA in vitro indicates that mASH1/hASH1 may regulate cell cycle and differentiation (15, 26). At present, very limited information is available about the pathophysiologic roles of hASH1 in human neuroendocrine diseases in vivo. In the hASH1 transgenic mice under the control of Clara cell–specific promoter, hyperplasia and metaplasia were observed in the lung, and hASH1-SV40 Tag (SV40 large T antigen) double transgenic mice developed overt lung carcinomas (27). These results suggest that hASH1 may potentially work as a tumorigenic factor in mammalian epithelial cells in vivo.

mASH1/hASH1 is principally regulated by Notch signaling, such that Notch1 activated hairy-encoder of split 1 and 5 (HES1 and HES5), and induce mASH1 degradation (13, 22, 28–30). In the present study, Notch1, HES1, and HES5 were detectable in NECs, but they apparently did not repress hASH1 overexpression in vivo. It might be attributable to the insufficiency of Notch signaling (30). Because Notch is known to play various roles both as oncogene and tumor suppressor gene depending on tumor microenvironments (31–34), possible involvement of Notch-HES pathway in NECs should be further studied.

In this study, we have shown that hASH1 mRNA was detectable from both fresh biopsy samples and paraffin-embedded archival tissues, and that the expression was distinctly upregulated in the gastrointestinal NECs, although it is scarcely expressed in adenocarcinomas. Detection of hASH1 may be very helpful in the diagnosis of NECs, especially in case pan-endocrine markers are negative and differential diagnosis from poorly differentiated or undifferentiated carcinomas is required. Further investigation is necessary to clarify the roles of hASH1 in the gastrointestinal NECs. In addition to studies on the expression of hASH1, studies on regulation of bHLH transcription factors might provide the basis for development of efficient antitumor approaches against this highly malignant tumor.

ACKNOWLEDGMENTS

We thank Drs. S. Yoshida, M. Nishimura, and S. Takano for providing tissue samples, Prof. Dr. M. Takiguchi for helpful discussion and T. Matsui, K. Kawashima, and A. Sasaki for excellent technical assistance.

REFERENCES

Aberrant Expression of Human Achaete-Scute Homologue Gene 1 in the Gastrointestinal Neuroendocrine Carcinomas

Takashi Shida, Mitsuko Furuya, Takashi Nikaido, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/2/450

Cited articles
This article cites 30 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/2/450.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/11/2/450.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.