hASH1 expression is closely correlated with endocrine phenotype and differentiation extent in pulmonary neuroendocrine tumors

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The human homolog 1 of the *Drosophila* neurogenic *achaete–scute* genes, *hASH1*, is specifically expressed in fetal pulmonary neuroendocrine cells and in some neuroendocrine tumor cell lines. However, no data have been gathered regarding its *in vivo* expression in tumors. *hASH1* mRNA expression was investigated by *in situ* hybridization in 238 surgically resected lung carcinomas, and the correlations between *hASH1* expression status and immunostaining results of neuroendocrine markers chromogranin A, neural cell adhesion molecule, gastrin-releasing peptide and calcitonin, and clinical outcome were analyzed. *hASH1* expression was detected in 2/20 (10%) adenocarcinomas, 4/30 (13.3%) typical carcinoids, 11/13 (84.6%) atypical carcinoids, 38/67 (56.7%) large-cell neuroendocrine carcinomas and 56/78 (71.8%) small-cell carcinomas, respectively, but not in any squamous cell carcinoma (0/21) or large-cell carcinoma (0/9). The 2 *hASH1*$^+$ adenocarcinomas also expressed multiple neuroendocrine markers. Thus, *hASH1* expression was restricted to lung cancers with neuroendocrine phenotypes. However, not all neuroendocrine tumors expressed *hASH1*. Within the entities of large-cell neuroendocrine carcinoma and small-cell carcinoma, *hASH1* expression correlated very closely with chromogranin A, gastrin-releasing peptide and calcitonin expression ($P < 0.0001$, $r = 0.852$), but was not related to neural cell adhesion molecule expression ($P = 0.8892$), suggesting that *hASH1* expression, at least in lung cancer, is associated with endocrine phenotype expression other than 'neuroendocrine differentiation' in a broad sense. The fact that *hASH1* was virtually absent in almost fully differentiated typical carcinoids, but was expressed in most, if not all, less differentiated atypical carcinoids as well as large-cell neuroendocrine carcinomas and small-cell carcinomas, suggests that *hASH1* expression in lung cancer imitates its early and transient expression in fetal development, and that *hASH1* is instrumental in the establishment, but not in the maintenance, of a cellular endocrine phenotype. Finally, *hASH1* expression correlated with a significantly shortened survival in small-cell carcinoma patients ($P = 0.041$).
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Based on the absence or presence of cellular neuroendocrine differentiation, lung cancer can be grouped into non-neuroendocrine or neuroendocrine tumors. The former is also called non-small-cell lung cancer and largely comprises squamous cell carcinoma, adenocarcinoma and large-cell carcinoma. The latter is also a broad entity ranging from low-grade typical carcinoid, intermediate-grade atypical carcinoid to the most lethal large-cell neuroendocrine carcinoma and small-cell carcinoma.[1–3] The neuroendocrine phenotypes may contribute to the virulence of large-cell neuroendocrine carcinoma and small-cell carcinoma via autocrine loops to promote cell growth and protect tumor cells from apoptosis.[4,5] However, the molecular mechanisms involved in the determination, regulation and maintenance of the neuroendocrine

phenotypes of lung cancer remain to be further elucidated.

Basic helix–loop–helix (bHLH) proteins form a large family of transcriptional regulatory factors, which are essential in cell fate determination and tissue-specific phenotypic differentiation.[6–8] Cells of the diffuse neuroendocrine system and neuronal cells share many common biological and morphological features, and they may utilize similar transcriptional mechanisms to determine cell fate and phenotypic differentiation. In *Drosophila*, the genes of the *achaete–scute* complex are neurogenic determinants and play a critical role in neuronal commitment and differentiation.[9,10] Mammalian and human *achaete–scute* homologs 1, *MASH1* and *hASH1*, were cloned from a rat sympathetic neuronal precursor cell line and a human medullary thyroid carcinoma cell line, respectively.[11,12] Subsequent studies have documented that *MASH1*, like its *Drosophila* homolog, is specifically and transiently expressed in embryonic neuronal precursors and is instrumental in the development of a subset of neurons.[8,13] *MASH1* is also indispensable for the development of pulmonary neuroendocrine cells, thyroid C cells and adrenal chromaffin cells in fetal mice, and homozygous *MASH1* null mice lack pulmonary neuroendocrine cells, while mice deficient in *HES1,* a specific repressor gene of *hASH1,* show a marked increase in pulmonary neuroendocrine cells.[14–17] In vitro studies also documented restrictive *hASH1* expression in tumor cell lines with neuroendocrine or neuronal differentiation such as pulmonary small-cell carcinoma, medullary thyroid carcinoma and neuroblastoma,[12,14,18–23] suggesting a key role for *hASH1* in dictating cellular neuroendocrine differentiation in lung cancer. However, few data about *in vivo hASH1* expression in lung cancer, as well as in other neuroendocrine tumors, are currently available.

Materials and methods

Tissue Samples

Formalin-fixed, paraffin-embedded tissue samples of 238 surgically resected primary lung cancers from the Kitasato University Hospital, Sagamihara; the National Cancer Center Hospital, Tokyo, and the National Cancer Center East Hospital, Kashiwa, Japan, were used. The cases comprised 50 non-small-cell lung cancers and 188 neuroendocrine tumors. The former included 21 squamous cell carcinomas, 20 adenocarcinomas and nine large-cell carcinomas, and the latter 30 typical carcinoids, 13 atypical carcinoids, 67 large-cell neuroendocrine carcinomas and 78 small-cell carcinomas. The diagnoses were based on the criteria of the 1999 WHO Histological Typing of Lung and Pleural Tumors.[24]

Nonradioactive mRNA Detection by *In Situ* Hybridization

Total RNAs were extracted from LCN1, a large-cell neuroendocrine carcinoma cell line established in our laboratory. A 499-bp *hASH1* fragment corresponding to base pairs 1016–1514 of the *hASH1* cDNA sequence[12] was subcloned into pCR™II (Invitrogen, CA, USA). The digoxigenin-labelled sense and antisense *hASH1* cRNA probes were generated by T7 or SP6 RNA polymerase (Roche Mol. Biochem., Mannheim, Germany), respectively. A modified highly sensitive RNA *in situ* hybridization method with catalyzed signal amplification based on biotinylated tyramines was employed, which allows messengers to be detected readily with routine formalin-fixed and paraffin-embedded samples.[25] In brief, after hybridization with sense or antisense cRNA *hASH1* probe at 50°C for $2\frac{1}{2}$h, the sections were treated with ribonuclease and washed under highly stringent conditions followed by endogenous biotin blocking with a Biotin Blocking System (DAKO, CA, USA). Then, the sections were sequentially incubated with horseradish peroxidase conjugated anti-digoxigenin antibody (DAKO, Denmark), biotinylated tyramide and horseradish peroxidase-labelled streptavidin of the GenPoint kit (DAKO, CA, USA). Hybridization results were visualized with 3,3′-diaminobenzidine and the slides were counterstained with hematoxylin.

A nude mouse xenograft of the LCN1 in which constitutive *hASH1* expression had been confirmed by RT-PCR was used as a positive sample control. Hybridization of a 340-bp antisense beta-2-microglobulin cRNA probe, when necessary, was also used as an internal control to confirm the quality of the messengers. The negative controls included usage of a sense probe and treatment of the antisense probe or sections with ribonuclease. Furthermore, a squamous cell carcinoma without detectable *hASH1* expression by RT-PCR was also used as a negative sample control.

The results were scored as negative, or focally, patchily or diffusely positive when definite hybridization signals were detected in none, less than 10, 10–50% or more than 50% of the tumor cells in individual tumors, respectively. The positive hybridization signals were also scored as mild, moderate or intense, according to intensity.

Immunohistochemical Staining

Immunohistochemical staining of general neuroendocrine markers, chromogranin A (1000 × dilution, Dako, Denmark) and neural cell adhesion molecule (200 × dilution, Nippon Kayaku, Tokyo, Japan), and peptide hormones, gastrin-releasing peptide (2000 × dilution, Dako, Japan) and calcitonin (200 × dilution, Incstar, Stillwater, MN, USA), were performed with the standard labelled streptavidin–biotin method, as described previously.[3,26]

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Statistical Analysis

Correlation of categorical variables between different groups was assessed using Fisher's exact test, Mann–Whitney U-test or the χ^2-test. Comparison of survival rates was performed using Kaplan–Meier survival rate curves analyzed with the log-rank test. A probability value (P) less than or equal to 0.05 was considered significant. All analyses were performed with StatView statistical software, version 5.0.
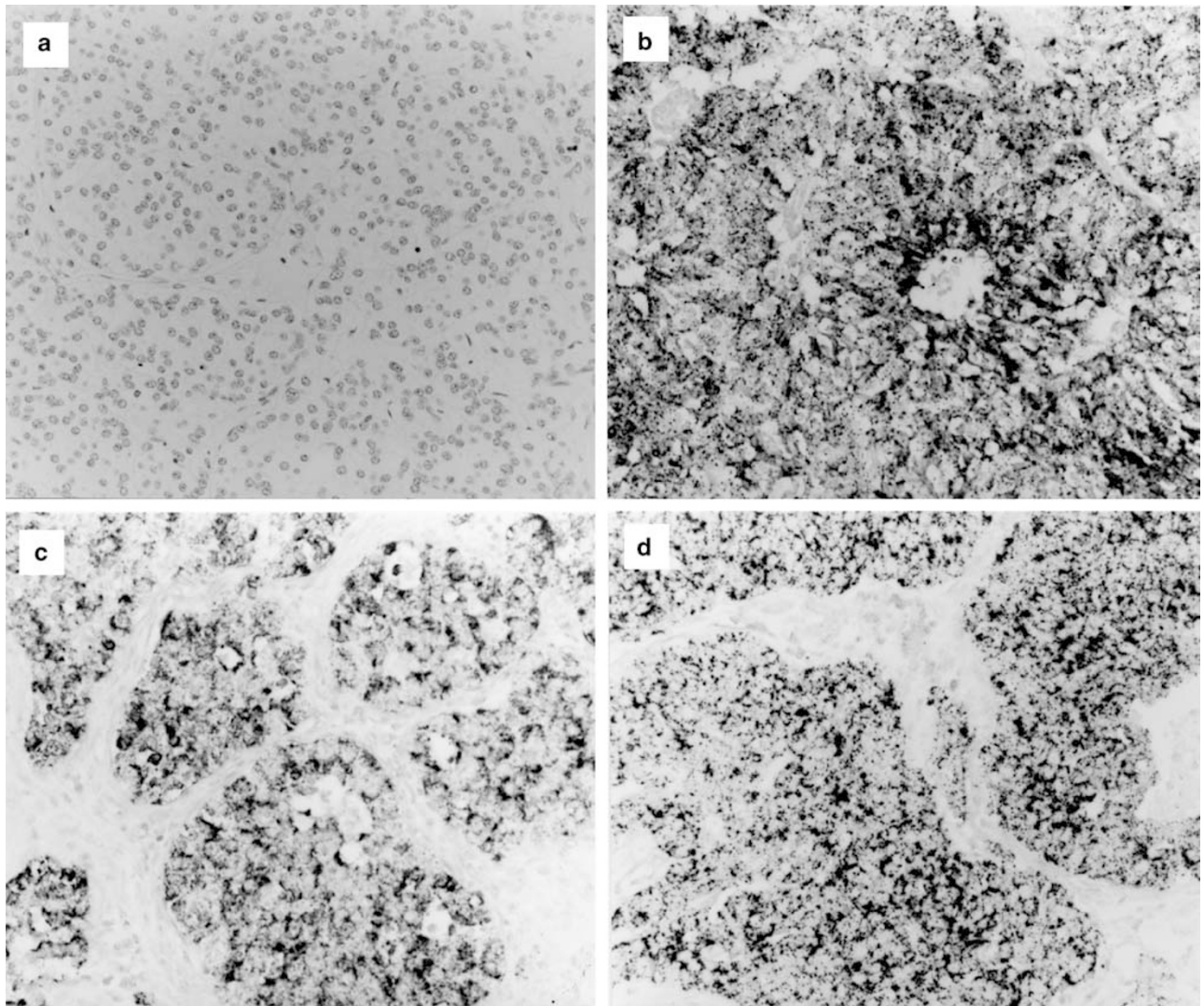
Results

hASH1 Expression in Non-small-cell Lung Cancer

No *hASH1* hybridization signals could be detected in all of the 21 squamous cell carcinomas and nine large-cell carcinomas. Two (10%) of the 20 adeno-carcinomas expressed *hASH1* and both showed patchy and moderate hybridization signals.

hASH1 Expression in Carcinoid Tumors

Of the 30 typical carcinoids, only four (13.3%). cases showed *hASH1* expression, with a diffuse pattern in two and a focal pattern in another two cases (Figure 1a). One was intense, while the other three revealed weak hybridization signals. On the other hand, 11 (84.6%) of the 13 atypical carcinoids were *hASH1*$^+$ and each of them demonstrated hybridization signals in a diffuse pattern (Figure 1b). The hybridization signals were intense in three, moderate in seven and weak in one case, respectively. There was a significant difference in *hASH1* expression incidence between typical carcinoids

Figure 1 Representative *hASH1* mRNA *in situ* hybridization results in pulmonary neuroendocrine tumors. No *hASH1* hybridization signals can be detected in typical carcinoid (**a**), while atypical carcinoid (**b**), large-cell neuroendocrine carcinoma (**c**) and small-cell carcinoma (**d**) are strongly and diffusely positive for *hASH1*.

and atypical carcinoids (Fisher's exact test, $P < 0.0001$).

hASH1 Expression in Large-cell Neuroendocrine Carcinoma and Small-cell Carcinoma

Of the 67 large-cell neuroendocrine carcinomas, 38 (56.7%) cases showed readily detectable *hASH1* hybridization signals. A total of 30 cases showed diffuse staining, while only six and two cases showed patchy and focal staining, respectively. The hybridization signals were intense in 16, moderate in 13 and weak in nine cases (Figure 1c).

hASH1 hybridization signals were observed in 56 (71.8%) of the 78 small-cell carcinomas; diffuse in 49, patchy in five and focal in two cases. The hybridization signals were intense in 21, moderate in 23 and weak in 12 cases, respectively (Figure 1d).

There was no statistical difference in *hASH1* expression incidence between large-cell neuroendocrine carcinoma and small-cell carcinoma, although *hASH1* mRNA was detected in a higher percentage of the latter (Fisher's exact test, $P = 0.0807$). The same was true for the distribution range and intensity of *hASH1* hybridization signals between large-cell neuroendocrine carcinoma and small-cell carcinoma (Mann–Whitney *U*-test, $P = 0.0549$ and 0.1417, respectively).

Of the 67 large-cell neuroendocrine carcinomas and 78 small-cell carcinomas, eight and 11 cases were combined tumors,[24] respectively, in which an element of squamous cell carcinoma or adenocarcinoma coexisted. Of these 19 combined carcinomas, 10 expressed *hASH1,* and the hybridization signals were strictly restricted to the large-cell neuroendocrine carcinoma and small-cell carcinoma regions, while the squamous cell carcinoma and adenocarcinoma elements were completely negative for *hASH1* (Figure 2).

The LCN1 xenograft, the positive sample control, demonstrated intense and diffuse *hASH1* hybridization signals. No signals could be observed in the squamous cell carcinoma, the negative sample control, or when a sense probe or ribonuclease pretreated antisense probe or sections were used.
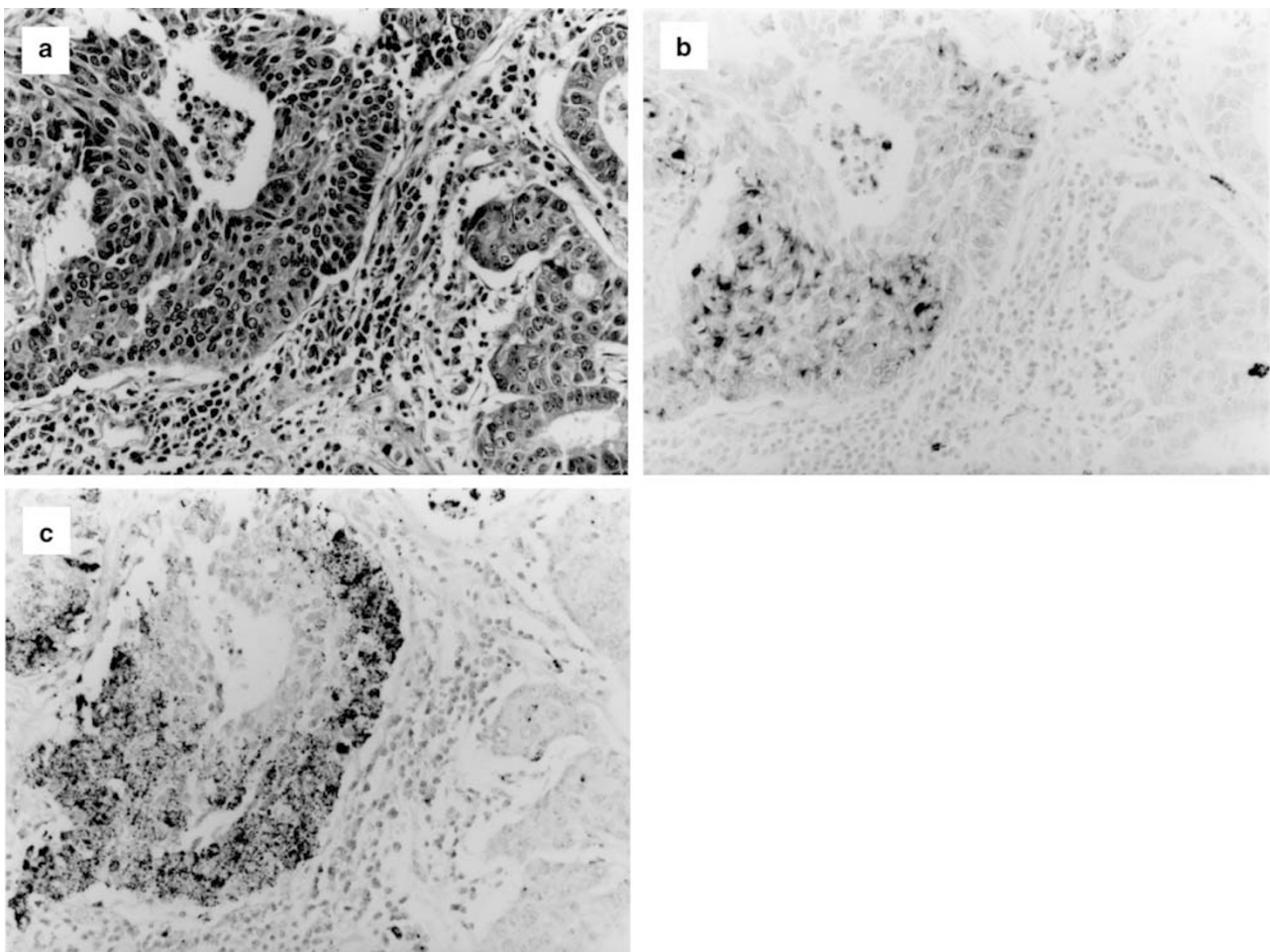
Figure 2 (**a**) A combined large-cell neuroendocrine carcinoma (left) and adenocarcinoma (right). (**b**) Only the large-cell neuroendocrine carcinoma element is positive for chromogranin A (left). (**c**) On a consecutive section, the *hASH1* hybridization signals are also confined to the large-cell neuroendocrine carcinoma element.

All the *hASH1*[-] tumors showed beta-2-microglobulin hybridization signals, indicating that the lack of *hASH1* signals in these specimens was not due to degraded tumor mRNA.

Immunohistochemical Staining Results

As mentioned above, two adenocarcinomas showed patchily moderate *hASH1* hybridization signals. Also, in the same areas of these two tumors, positive staining for chromogranin A and calcitonin was observed on consecutive sections, and one of them was also positive for gastrin-releasing peptide. The remaining 18 *hASH1*[-] adenocarcinomas, and all the 21 squamous cell carcinomas and nine large-cell carcinomas, were completely negative for the general neuroendocrine markers or peptide hormones.

Of the 43 typical carcinoids and atypical carcinoids, all and 38 cases were positive for chromogranin A and neural cell adhesion molecule, respectively, and 32 and 17 cases were positive for gastrin-releasing peptide and calcitonin, respectively, with all 17 calcitonin[+] cases being also positive for gastrin-releasing peptide.

Of the 145 large-cell neuroendocrine carcinomas and small-cell carcinomas, 88 and 133 were positive for chromogranin A and neural cell adhesion molecule, respectively, with all cases expressing at least one marker and 80 cases being positive for both the markers, confirming their neuroendocrine differentiation. In all, 95 and 51 tumors also expressed gastrin-releasing peptide and calcitonin, respectively, with 45 cases expressing both the peptide hormones. When compared with *hASH1* expression status, an obvious tendency for *hASH1* expression in those tumors expressing chromogranin A was found. 79/94 *hASH1*[+] large-cell neuroendocrine carcinomas and small-cell carcinomas were positive, while the majority (42/51) of *hASH1*[-] tumors were negative for chromogranin A; and there was a close positive correlation between *hASH1* and chromogranin A expressions (χ^2-test, $P < 0.0001$, $r = 0.649$). On the other hand, *hASH1* expression showed no relationship to neural cell adhesion molecule expression status (Table 1, χ^2-test, $P = 0.8892$).

Chromogranin A is a constituent of secretory granules of normal as well as neoplastic neuroendocrine cells, and its expression is closely associated with the cellular endocrine phenotype defined as peptide hormone/biogenic amine production, storage and secretion. Thus, the correlation between *hASH1* expression and gastrin-releasing peptide or calcitonin expression was also analyzed. In all, 86 and 49 of the 94 *hASH1*[+] large-cell neuroendocrine carcinomas and small-cell carcinomas were positive for gastrin-releasing peptide and calcitonin, respectively, while 42 and 49 of the 51 *hASH1*[-] tumors were negative for gastrin-releasing peptide and calcitonin, respectively. Thus, a positive correlation

Table 1 Irrelative *hASH1* and neural cell adhesion molecule expression in 145 large-cell neuroendocrine carcinomas and small-cell carcinomas

|	hASH1 *positive*	hASH1 *negative*
Neural cell adhesion molecule positive	86	47
	$P < 0.8892$, $r = -0.012$	
Neural cell adhesion molecule negative	8	4

Table 2 *hASH1* and chromogranin A/gastrin-releasing peptide/calcitonin (CGA/GRP/CT) expression in 145 large-cell neuroendocrine carcinomas and small-cell carcinomas

	hASH1 *positive*	hASH1 *negative*
Any of CGA/GRP/CT positive	94	10
	$P < 0.0001$, $r = 0.852$	
All of CGA/GRP/CT negative	0	41

between *hASH1* expression and gastrin-releasing peptide or calcitonin expression was also found (χ^2-test, $P < 0.0001$, $r = 0.742$ and $r = 0.482$, respectively). Furthermore, when the immunostaining results were taken together, as shown in Table 2, all the 94 *hASH1*[+] large-cell neuroendocrine carcinomas and small-cell carcinomas expressed chromogranin A and/or gastrin-releasing peptide and/or calcitonin, while the vast majority of *hASH1*[-] tumors were negative for these neuroendocrine markers; and there was a very close positive correlation between *hASH1* expression and chromogranin A/gastrin-releasing peptide/calcitonin expression (χ^2-test, $P < 0.0001$, $r = 0.852$).

Clinical Significance of *hASH1* Expression in Pulmonary Neuroendocrine Tumors

Complete follow-up information was available for all cases except for one atypical carcinoid and four small-cell carcinoma patients. The Kaplan–Meier survival curves of the patients are shown in Figure 3a. There was a significant difference in overall survival for typical carcinoid patients *vs* atypical carcinoid patients and for atypical carcinoid patients *vs* large-cell neuroendocrine carcinoma patients (log-rank test, $P = 0.0259$ and 0.0183, respectively), but not for large-cell neuroendocrine carcinoma patients *vs* small-cell carcinoma patients ($P = 0.4692$). One of the four *hASH1*[+] typical carcinoid patients died of the disease, while all 26 *hASH1*[-] typical carcinoid patients survived. Two of the 10 *hASH1*[+] and one of the two *hASH1*[-] atypical carcinoid patients succumbed to the disease,
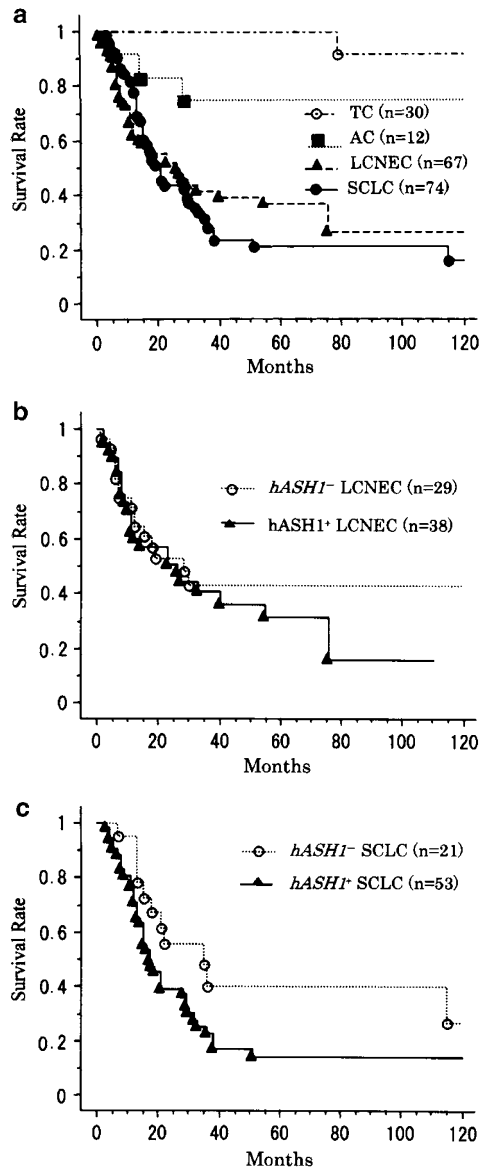
Figure 3 (**a**) Kaplan–Meier survival curves and log-rank test for patients with typical carcinoid (TC), atypical carcinoid (AC), large-cell neuroendocrine carcinoma (LCNEC) and small-cell carcinoma (SCLC). There was a significant difference in overall survival for typical carcinoid *vs* atypical carcinoid and for atypical carcinoid *vs* large-cell neuroendocrine carcinoma ($P = 0.0259$ and $P < 0.0183$, respectively), but not for large-cell neuroendocrine carcinoma *vs* small-cell carcinoma ($P = 0.4692$). (**b**) Kaplan–Meier survival curves and log-rank test for large-cell neuroendocrine carcinoma patients by *hASH1* expression status ($P = 0.4702$). (**c**) Kaplan–Meier survival curves and log-rank test for small-cell carcinoma patients by *hASH1* expression status ($P = 0.041$).

respectively. The small numbers of *hASH1*$^+$ typical carcinoid and *hASH1*$^-$ atypical carcinoid cases impeded statistical analysis for survival difference based on *hASH1* expression status. The 2- and 5-year overall survival rates for the 38 *hASH1*$^+$ and the 29 *hASH1*$^-$ large-cell neuroendocrine carcinoma patients were 51.0 and 31.5%, and 51.9 and 42.1% respectively. Although *hASH1*$^+$ large cell neuro-

endocrine cases had a shorter 5-year survival than *hASH1*$^-$ patients, the difference was not significant (Figure 3b, log-rank test, $P = 0.4702$). However, *hASH1* expression was associated with a worse prognosis in small-cell carcinoma patients. As shown in Figure 3c, the 2- and 5-year survival for patients with *hASH1*$^+$ and *hASH1*$^-$ small-cell carcinoma are 39.0 and 14.6%, and 55.9 and 40.0%, respectively, and there was a significant difference in prognosis (log-rank test, $P = 0.041$).

Discussion

It is important to define, at a molecular level, the mechanisms involved in regulating neuroendocrine differentiation of lung cancers because this may contribute to the virulence of small-cell carcinoma and large-cell neuroendocrine carcinoma.[4,5] In the present study, *hASH1* expression was detected in 4/30 typical carcinoids, 11/13 atypical carcinoids, 38/67 large-cell neuroendocrine carcinomas and 56/78 small-cell carcinomas. Of the 50 non-small-cell lung cancers, only two adenocarcinomas expressed *hASH1* in a patchy pattern. However, both of these two *hASH1*$^+$ adenocarcinomas also expressed neuroendocrine markers. Thus, our data confirmed and extended the findings of previous *in vitro* studies that *hASH1* was specifically expressed in lung cancers with neuroendocrine differentiation,[12,14,18,19] indicating that besides being instrumental in development of a subset of neurons and neuroendocrine cells,[8,13–17] *hASH1* also plays a key role in regulating neuroendocrine differentiation of tumor cells.

The term 'neuroendocrine differentiation' in a broad sense is used to define cells expressing a neural and/or an endocrine phenotype. The markers of the former include cytosolic and cell membrane markers common to neurons and endocrine cells such as neuron-specific enolase, neuron-specific class III beta-tubulin and neural cell adhesion molecule. On the other hand, an endocrine phenotype should be defined as the capacity of a given cell to produce and secrete peptide hormone(s)/biogenic amine(s). Chromogranins, a group of acidic monomeric proteins, are regarded as markers of the endocrine phenotype, as they are constituents of the matrix of secretory granules where peptides/amines are stored.[27–29] It is well known that some pulmonary neuroendocrine tumors express neural marker(s), but are completely negative for either chromogranin A or hormones, indicating these tumors have neural differentiation, but lack an endocrine phenotype. In the present study, most *hASH1*$^+$ large-cell neuroendocrine carcinomas and small-cell carcinomas expressed chromogranin A, while the vast majority of *hASH1*$^-$ large-cell neuroendocrine carcinomas and small-cell carcinomas were completely negative for chromogranin A ($P < 0.0001$). Furthermore, *hASH1* expression was

completely irrelative to neural cell adhesion molecule expression ($P = 0.8892$). Thus, we suspected that a linkage existed between *hASH1* expression and cellular endocrine phenotype other than 'neuro-endocrine differentiation' in a broad sense.

However, 15 *hASH1*[+] large-cell neuroendocrine carcinomas and small-cell carcinomas showed no chromogranin A expression. Although chromo-granin A expression generally parallels hormone expression, it is not uncommon for pulmonary and other neuroendocrine tumors to express hormone(s), but be negative for chromogranin A. One possible explanation for this is that chromogranins B and/or C, but not chromogranin A, are alternatively present in these tumors.[30] According to our previous studies,[3,26] gastrin-releasing peptide and calcitonin are the most frequently expressed hormones in lung cancer, and a combination of immunostainings of these two hormones and chromogranin A can discriminate most, if not all, lung cancers with an endocrine phenotype. In the present study, first, either gastrin-releasing peptide or calcitonin expression was related to *hASH1* expression in large-cell neuroendocrine carcinomas and small-cell carcinomas ($P < 0.0001$, respectively). Second, when taking the staining results together, there was a very close positive correlation between *hASH1* and chromo-granin A/gastrin-releasing peptide/calcitonin expressions ($P < 0.0001$, $r = 0.852$). Thus, the present results based on a substantial number of cases strongly suggested that *hASH1* expression, at least in the context of lung cancer, is associated with a cellular endocrine phenotype. This notion is supported by a recent observation that development of most adrenal medullary cells in *MASH1*-deficient mice were arrested at an early stage, and the medullary cells expressed high levels of neurofilament and resembled immature sympathetic neuro-blast, but failed to acquire an endocrine phenotype.[17]

On the other hand, *hASH1* expression was virtually absent in typical carcinoid, which has the most overt and mature endocrine phenotype. Across the spectrum of pulmonary neuroendocrine tumors, typical carcinoid and small-cell carcinoma represent the two ends of the differentiation extent, with atypical carcinoid and large-cell neuroendocrine carcinoma in between. The *Drosophila* and mam-malian homologs of *hASH1* are only transiently expressed in neuronal progenitor cells and pulmon-ary neuroendocrine cells during early fetal develop-ment,[8,9,11,13] and *in vitro* *hASH1* expression preceded and overlapped induced neuronal differ-entiation of neuroblastoma and P19 cells, but vanished before the overt neuronal phenotype appeared.[22,31] Silencing of *hASH1* expression also paralleled the development of induced mature C cell phenotype of medullary thyroid carcinoma cells.[20] In the present study, *hASH1* expression also depended on differentiation extent, and was de-tected in the vast majority of less differentiated

atypical carcinoids, as well as in large-cell neuro-endocrine carcinomas and small-cell carcinomas, but was virtually absent in almost fully differen-tiated typical carcinoids. These data together strongly suggest that *hASH1* expression in pulmon-ary neuroendocrine tumors imitates its early and transient expression pattern during development, and *hASH1* is instrumental in establishment of a cellular endocrine phenotype, but is not necessary for maintenance of the fully developed endocrine phenotype. Thus, it is not surprising that clinically benign pheochromocytomas showed no *hASH1* expression, while neural crest-derived malignant tumors expressed *hASH1*.[12,20,22,23] Our typical carci-noids and atypical carcinoids were differentially diagnosed based on mitotic count and/or presence or absence of necrosis.[2,24] As one of the four *hASH1*[+] typical carcinoid patients died of the disease while all 26 *hASH1*[−] typical carcinoid patients survived, *hASH1*[+] typical carcinoid diag-nosed with these criteria might be biologically more like an atypical carcinoid.

hASH1 expression was associated with a signifi-cantly shortened overall survival in small-cell carcinoma patients, suggesting the potential prog-nostic significance of *hASH1*. Since peptide hor-mones such as gastrin-releasing peptide can promote cell growth and protect tumor cells from apoptosis through autocrine loops,[4,5] *hASH1* might confer a role on the biological behavior of small-cell carcinoma via its impact on the endocrine pheno-type of the tumor cells. However, *hASH1* expression was not significantly associated with clinical out-come in large-cell neuroendocrine carcinoma patients, although a shorter 5-year survival was observed in patients with *hASH1*[+] tumor. The prognostic significance of *hASH1* expression in pulmonary neuroendocrine tumors remains to be clarified.

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