

# Human ASH1 expression in prostate cancer with neuroendocrine differentiation

Ida Rapa<sup>1</sup>, Paolo Ceppi<sup>1</sup>, Enrico Bollito<sup>1</sup>, Rosj Rosas<sup>1</sup>, Susanna Cappia<sup>1</sup>, Elisa Bacillo<sup>1</sup>, Francesco Porpiglia<sup>2</sup>, Alfredo Berruti<sup>3</sup>, Mauro Papotti<sup>1</sup> and Marco Volante<sup>1</sup>

<sup>1</sup>Division of Pathology, Department of Clinical and Biological Sciences, San Luigi Hospital, University of Turin, Turin, Italy; <sup>2</sup>Division of Urology, Department of Clinical and Biological Sciences, San Luigi Hospital, University of Turin, Turin, Italy and <sup>3</sup>Division of Oncology, Department of Clinical and Biological Sciences, San Luigi Hospital, University of Turin, Turin, Italy

**Neuroendocrine differentiation in prostate cancer correlates with overall prognosis and disease progression after androgen-deprivation therapy, although its specific mechanisms are currently poorly understood. A role of Notch pathway has been reported in determining neuroendocrine phenotype of normal and neoplastic tissues. The aim of this study was to analyze whether this pathway might affect neuroendocrine differentiation in prostate cancer. Human achaete-scute homolog 1 (hASH1), a pivotal member of the Notch pathway, was investigated in 80 prostate cancers selected and grouped according to chromogranin A immunohistochemistry, as follows: prostate cancers without neuroendocrine differentiation, untreated (25 cases); prostate cancers with neuroendocrine differentiation, untreated (40 cases); prostate cancers with previous androgen-deprivation therapy, all having neuroendocrine differentiation (15 cases). Human ASH1 protein was analyzed by immunohistochemistry, whereas the presence of hASH1 mRNA transcripts was investigated on paraffin material by real-time PCR. By immunohistochemistry, hASH1 was colocalized with chromogranin A in neuroendocrine cells of normal prostatic gland. It was absent in all but one prostate cancers without neuroendocrine differentiation, whereas it was positive in 25% of prostate cancers with neuroendocrine differentiation/untreated, with a significant correlation with the extent of neuroendocrine features ( $P=0.02$ ). Moreover, comparing untreated and treated prostate cancers with neuroendocrine differentiation, a positive association with androgen-deprivation therapy was observed ( $P=0.01$ ). In prostate cancers with neuroendocrine differentiation, RNA analysis confirmed the association of higher transcript levels in androgen deprivation-treated compared with untreated patients ( $P=0.01$ ). In addition, hASH1 mRNA analysis in microdissected chromogranin A-positive and chromogranin A-negative areas within the same tumor demonstrated a two- to sevenfold increase of hASH1 mRNA expression in chromogranin A-positive tumor cell populations.**

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Prostate cancer is the leading malignancy in the aging male population. Prostate cancer biology is notoriously heterogeneous, some tumors showing a very aggressive course leading to patient's death in few years, others depicting an indolent behavior and remaining silent during the whole life.<sup>1</sup> Accurate prediction of prognosis is important to tailor the optimal therapeutic approach for individual patients. To date, tumor stage, grade and serum PSA (prostate-specific antigen) levels are the only validated prognostic parameters.

Several prostate cancers display focal neuroendocrine differentiation at diagnosis, usually revealed by immunohistochemistry as scattered individual cells or nests of cells in the context of otherwise conventional adenocarcinomas.<sup>2</sup> The biological significance of this phenomenon is incompletely elucidated. It has been suggested that the coexistence of a neuroendocrine phenotype in predominantly exocrine tumors correlates with overall prognosis<sup>3,4</sup> and seems of special importance in facilitating prostate cancer progression after androgen-deprivation therapy. Several mechanisms have been identified in this respect: neuroendocrine cells are androgen receptor negative, therefore they survive androgen deprivation; as an alternative, neuroendocrine cells produce peptides, hormones and growth factors that could stimulate the proliferation of exocrine prostate cancer cells and

Correspondence: Dr M Volante, MD, PhD, Department of Clinical and Biological Sciences, San Luigi Hospital, University of Turin, Regione Gonzole 10, Orbassano, Turin I-10043, Italy.

E-mail: marco.volante@unito.it

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increase their aggressiveness through apoptosis inhibition and neoangiogenesis stimulation.<sup>5–7</sup> Two recent studies in patients with hormone-refractory disease have demonstrated an independent poor prognostic role of elevated levels of circulating chromogranin A, a marker of neuroendocrine differentiation,<sup>8,9</sup> although in patients with hormone naive disease a significant relationship between amount of neuroendocrine cells and disease stage and grade but not with disease-free survival and overall survival has been unequivocally proven.<sup>10–12</sup>

Neuroendocrine differentiation regulatory mechanisms might play a role in the transition from an androgen-dependent to an androgen-resistant phenotype in prostate cancer, and *in vivo* studies, both in animals<sup>13</sup> and humans,<sup>14</sup> have shown that the neuroendocrine prostate cancer compartment increases after androgen deprivation.

Neuroendocrine cell development and differentiation is a complex and still poorly understood mechanism. Many experimental *in vitro* and *in vivo* models show that the evolutionarily conserved Notch signaling pathway negatively regulates a system of transcription factors, the basic helix-loop-helix (bHLH) family, during neuronal and endodermal endocrine mammalian development<sup>15–17</sup> via a system of effectors, including hairy and enhancer of split 1 (Hes1), which repress bHLH-mediated transcription.<sup>18</sup> Members of the bHLH family, like Math1, neurogenin 3 and neurogenic differentiation 1, have been demonstrated to regulate endocrine cell differentiation in the gastrointestinal tract and pancreas of mouse models. Among them, human achaete-scute homolog 1 (hASH1) plays a pivotal role in the development and differentiation of neuronal and endocrine cells, with special reference to foregut and midgut derivatives.<sup>19</sup> With regard to human tumors of neuroendocrine lineage, hASH1 transcripts are known to be expressed in poorly differentiated neuroendocrine—small cell—carcinomas of the lung,<sup>20–22</sup> gastrointestinal tract,<sup>23</sup> as well as in neuroblastomas<sup>24</sup> and medullary thyroid carcinomas,<sup>25</sup> but not in well-differentiated endocrine tumors (carcinoids).<sup>21,23</sup> No data are currently available on the possible role of hASH1 in driving divergent neuroendocrine differentiation in non-neuroendocrine adenocarcinomas, such as prostate carcinoma, or in mixed endocrine—exocrine cancers. Recently, in a mouse model of prostate neuroendocrine carcinoma, exceptionally high expression levels of hASH1, in concert with a wide range of neuroendocrine markers including L-dopa decarboxylase, have been demonstrated.<sup>26</sup> On the basis of such findings, hASH1 likely represents a critical actor to drive neuroendocrine phenotype development in prostate cancer: the present study was designed to analyze expression at both transcriptional and protein levels in a large series of prostatic cancers with various degrees of neuroendocrine differentiation, with and without androgen-deprivation therapy administration.

## Materials and methods

### Case selection

#### *Group A: PC-NE/Untreated*

To analyze hASH1 expression in prostate cancers with neuroendocrine differentiation, a series of 40 cases with neuroendocrine features at diagnosis, proven by chromogranin A immunohistochemistry, and without any previous treatment was collected.

#### *Group B: PC-NonNE*

To compare possible differential expression of hASH1 in non-neuroendocrine prostate cancers, a control group of previously untreated 25 prostate cancers showing no evidence of neuroendocrine differentiation by chromogranin A immunohistochemistry was entered in the study.

#### *Group C: PC-NE/Treated*

In addition, to investigate the possible effects of hormonal treatment in modulating hASH1 expression, a third group of 15 prostate cancers with neuroendocrine differentiation and previous androgen-deprivation therapy was also collected. All these patients were treated by endocrine therapy, consisting of androgen deprivation by LHRH (luteinizing hormone-releasing hormone) analog administration, for a period ranging from 6 to 86 months prior to sampling of the tissue specimen analyzed in this study.

Three additional cases of pure large cell neuroendocrine carcinomas primary of the prostate were also included in the study.

Total tissue material corresponded to 62 surgical samples and 21 needle biopsies or transurethral endoscopic resections. Formalin-fixed paraffin-embedded tissue material was available for histological review and further analysis. In 20 cases, fresh frozen tumor tissue (selected by frozen section examination of the corresponding tissue fragment) as well as parallel normal tissue, were also available. All tissue samples were anonymized by a staff member of the Pathology Department not involved in the study, and the study was approved by the Local Ethic Committee.

### Immunohistochemistry

Five-micron-thick sections, serial to those used for conventional histopathological examination, were deparaffinized and then treated with graded alcohols and rehydrated in PBS pH 7.5. Endogenous peroxidase activity was blocked by absolute methanol and 0.3% hydrogen peroxide for 15 min. To assess the presence of neuroendocrine phenotype, a primary monoclonal antibody to chromogranin A (LK2H10, diluted 1:800; NeoMarkers, Fremont, CA, USA) was used. Human hASH1 protein was detected by means of a monoclonal antibody (24B72D11.1, diluted 1:150; BD Biosciences, San Jose, CA, USA).

Antigen-retrieval procedure was performed for both chromogranin A and hASH1 by microwave heating (three 5 min passages at 750 W), in citrate buffer (pH 7.5) and EDTA buffer (pH 8.0) solutions, respectively. Immunoreactions were revealed by a dextran-chain (biotin-free) detection system (EnVision; DakoCytomation, Glostrup, Denmark), using 3,3'-diaminobenzidine (DAB; Dako) as a chromogen. A small cell lung carcinoma sample served as positive control for both antibodies. Negative control reactions were obtained by omitting the primary antibodies.

For both hASH1 and chromogranin A, the immune reaction was scored independently by two different researchers of the Pathology Department (IR and MV) as negative or positive using a semiquantitative three-tier system according to the percentage of reactive tumor cells (0: <5%; 1: 5–20%; 2: >20%).

### Double immunohistochemical procedure

On selected normal and neoplastic prostate samples hASH1 and chromogranin A were investigated by double immunohistochemical reactions, using immunoperoxidase procedure for hASH1, followed by immunoalkaline phosphatase method Envision-AP (Dako) with Vector blue alkaline phosphatase substrate kit III (from Vector Laboratories, CA, USA) for chromogranin A, at the same conditions reported above. Moreover, to test the proliferative capability of hASH1-positive cells, double immunofluorescence experiments with hASH1 and Ki-67 antibodies were performed, as follows. After antigen-retrieval procedure by microwave heating in citrate buffer solution (pH 7.5), sections were incubated with rabbit monoclonal anti-Ki-67 (clone SP6; Lab Vision, Fremont, CA, USA) 1:200 overnight at 4°C, and then incubated with FITC-conjugated goat anti-rabbit immunoglobulin (Invitrogen Corporation, Carlsbad, CA, USA) diluted 1:200. Samples were then incubated with mouse anti-hASH1 diluted at 1:150, overnight at 4°C, followed by anti-mouse biotin-conjugated antibody (Dako) diluted 1:200 and detected with streptavidin Texas red-conjugated (Invitrogen). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Vysis Downers Grove, IL, USA) and immunostained slides were examined under a fluorescence microscope Olympus BX41 (Olympus Shinjuku Monolith, Tokyo, Japan) equipped with appropriate filters and analyzed by Soft Imaging System software.

### RNA isolation from frozen tissues and qualitative RT-PCR of hASH1, Notch1 and Hes1

To screen hASH1, Notch1 and Hes1 RNA expression in prostate normal tissues and cancers, total RNA from snap-frozen normal and neoplastic tissues of 20 prostate cancer cases was extracted using QIAzol

lysis Reagent (Qiagen, Tokyo, Japan), and cDNA was transcribed using 500 µg/ml oligodT (Roche Applied Science, Penzberg, Germany) and 500M-MLV RT (200 U/µl) (Invitrogen) according to standard protocols.

Expression of hASH1, Notch1 and Hes1 transcripts, were examined by qualitative real-time (RT)-PCR using primers and PCR conditions previously reported.<sup>23</sup>

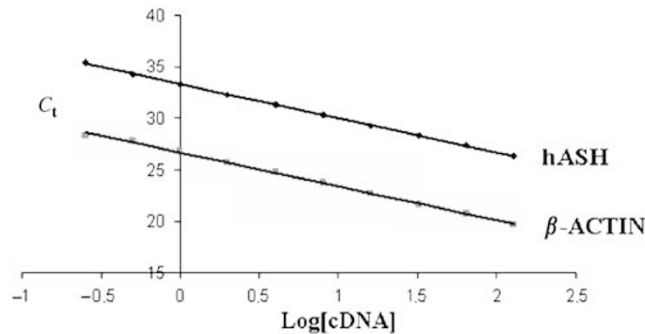
### RNA isolation from paraffin-embedded tissues and hASH1 quantitative real-time PCR

To parallel immunohistochemical data with hASH1 mRNA expression, 10 µm section, serial to those immunostained for chromogranin A, were cut in RNase-free conditions and stained with nuclear Fast Red (Sigma-Aldrich, St Luis, MO, USA) to highlight the tissue architecture. Under stereomicroscopic assistance, representative tumor areas positive and negative for chromogranin A in the parallel section, as well as normal peritumoral tissue, were identified, isolated by means of microdissection using a scalpel at a magnification of ×5–10, and analyzed separately. RNA isolation was performed by commercially available RNA extraction kits designed for paraffin material according to the manufacturer's instructions (High Pure RNA Paraffin Kit; Roche Applied Science, Milano, Italy). Relative cDNA quantification for hASH1 and an internal reference gene ( $\beta$ -actin) was performed using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System—Taqman; Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probe used for  $\beta$ -actin have been previously published.<sup>27</sup> Those used for hASH1 are listed in Table 1 and were designed according to the Ref Seq NM\_004316 (<http://www.ncbi.nlm.nih.gov/LocusLink>). The PCR product size generated with these primers was 71 bp long and validated through gel electrophoresis and serial dilutions<sup>28</sup> (Figure 1). The PCR mixture consisted of 1200 nmol/l of each primer, 200 nmol/l of the probe, 200 nmol/l each of dATP, dCTP, dGTP and dTTP, 3.5 mmol/l MgCl<sub>2</sub> and 1 × Taqman Universal PCR Master mix to a final volume of 20 µl (all reagents were from Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. Each measurement was performed in duplicate. To compare hASH1 expression in different tumors, the relative gene expression levels were expressed as ratios (differences between the  $C_t$  values) between two absolute measurements (genes of interest/internal reference gene). To test differences of hASH1 expression of different areas within individual tumors, the  $\Delta\Delta C_t$  values were calculated (subtracting  $\Delta C_t$  values of chromogranin A-positive and chromogranin A-negative tumor areas, analyzed separately), and converted to ratio by the following formula:  $2^{-\Delta\Delta C_t}$ .

**Table 1** Primer and probe sequences for hASH1 real-time quantitative PCR

Primer	Location (position)	Sequence 5' → 3'
hASH1 forward	Exon 1 (1291–1310)	CCTGGTGCGAATGGACTTT
hASH1 reverse	Exon 2 (1361–1383)	GCCACTGACAAGAAAGCACTAA
hASH1 probe	Exon 1/2 junction (1316–1338)	AGGGTGATCGCACAAACCTGCAT

hASH1: human achaete-scute homolog 1.



**Figure 1** Several runs with serial control cDNA dilutions were performed. The slope of the plot  $C_t$  vs log of input cDNA was  $-3.28$  for hASH and  $-3.32$  for  $\beta$ -actin (being  $-3.33$  100% efficiency). The two efficiencies were comparable at a satisfactory level ( $<5\%$ ) with a correlation coefficient ( $R^2$ )  $>0.98$ . Thus, the slope of the  $\Delta C_t$  values vs log of input cDNA was  $<0,1$  (not shown).

### Statistics

$\chi^2$  test was used to analyze the correlation between hASH1 protein expression and the clinicopathological characteristics, including age, Gleason score, tumor stage, PSA levels at the time of diagnosis, extent of neuroendocrine differentiation and therapy (see Table 2). *t*-Test was used to evaluate hASH1 mRNA expression levels in treated vs untreated patients. Statistical significance was set at  $P=0.05$ .

## Results

### hASH1 protein expression correlates with NE phenotype and antiandrogenic therapy administration

Human ASH1 nuclear protein was detected in scattered cells within normal and hyperplastic prostate glands. Double immunohistochemical procedure identified these cells as chromogranin A-positive neuroendocrine cells (Figure 2a). A strong and diffuse hASH1 expression was found in all three cases of pure primary large cell neuroendocrine prostate carcinomas (all scores 2) (Figure 2b). In adenocarcinomas, a strong correlation between hASH1 protein expression and the presence and extent of neuroendocrine phenotype was observed. In fact, it was absent in all prostate cancers lacking neuroendocrine features (PC-nonNE group) except for one case where nuclear hASH1 staining was observed in isolated tumor cells. By contrast, it was observed to a higher extent in therapy-naive prostate

cancers showing neuroendocrine features (PC-NE/untreated group), with a strong correlation with the extent of chromogranin A-positive immunohistochemistry ( $P=0.02$ ) (Figure 2c). By either double immunohistochemistry or comparison of parallel serial sections, hASH1 and chromogranin A distribution was localized in the same tumor areas. In addition, hASH1-positive immunohistochemistry was stronger (hASH1 score 2 cases) in tumor samples with more prominent chromogranin A reactive cells (score 2 cases), although this latter finding did not reach statistical significance ( $P=0.09$ ). By means of double hASH1 and Ki-67 immunofluorescence, we could demonstrate that hASH1 was coexpressed in a fraction of proliferating tumor cells (Figure 2d). When comparing PC-NE/untreated with PC-NE/treated groups, a higher rate of hASH1-positive cases was observed in the latter group, ( $P=0.01$ ). Human ASH1 protein expression was not correlated with any further clinicopathological parameter considered.

### Constitutive hASH1, Notch1 and HES1 transcript expression in normal and neoplastic frozen prostate tissues

To screen transcript expression of Notch1, hASH1 and Hes1 in neoplastic prostate tissue, we tested by qualitative RT-PCR 20 tumors and the corresponding normal gland. Among them, five cases displayed on the corresponding paraffin tissue neuroendocrine features as represented by chromogranin A-positive immunohistochemistry. All 20 cases displayed detectable mRNA amounts of the three genes in both normal and neoplastic tissue specimens, without any correlation with the presence of the neuroendocrine phenotype. Therefore, we focused our further attention on hASH1 expression—which represent the final effector of the pathway—on paraffin-embedded specimens by means of quantitative RT-PCR, as detailed in the next paragraph.

### High hASH1 mRNA expression levels in antiandrogen therapy-treated prostate cancers with NE features

To validate hASH1 immunohistochemical data and quantify transcript expression in chromogranin A-positive prostate cancers, all radical prostatectomy samples in groups PC-NE/untreated and PC-NE/treated were analyzed by RT-PCR from paraffin

**Table 2** hASH1 protein expression in prostate adenocarcinoma and correlation with clinicopathological features and neuroendocrine differentiation

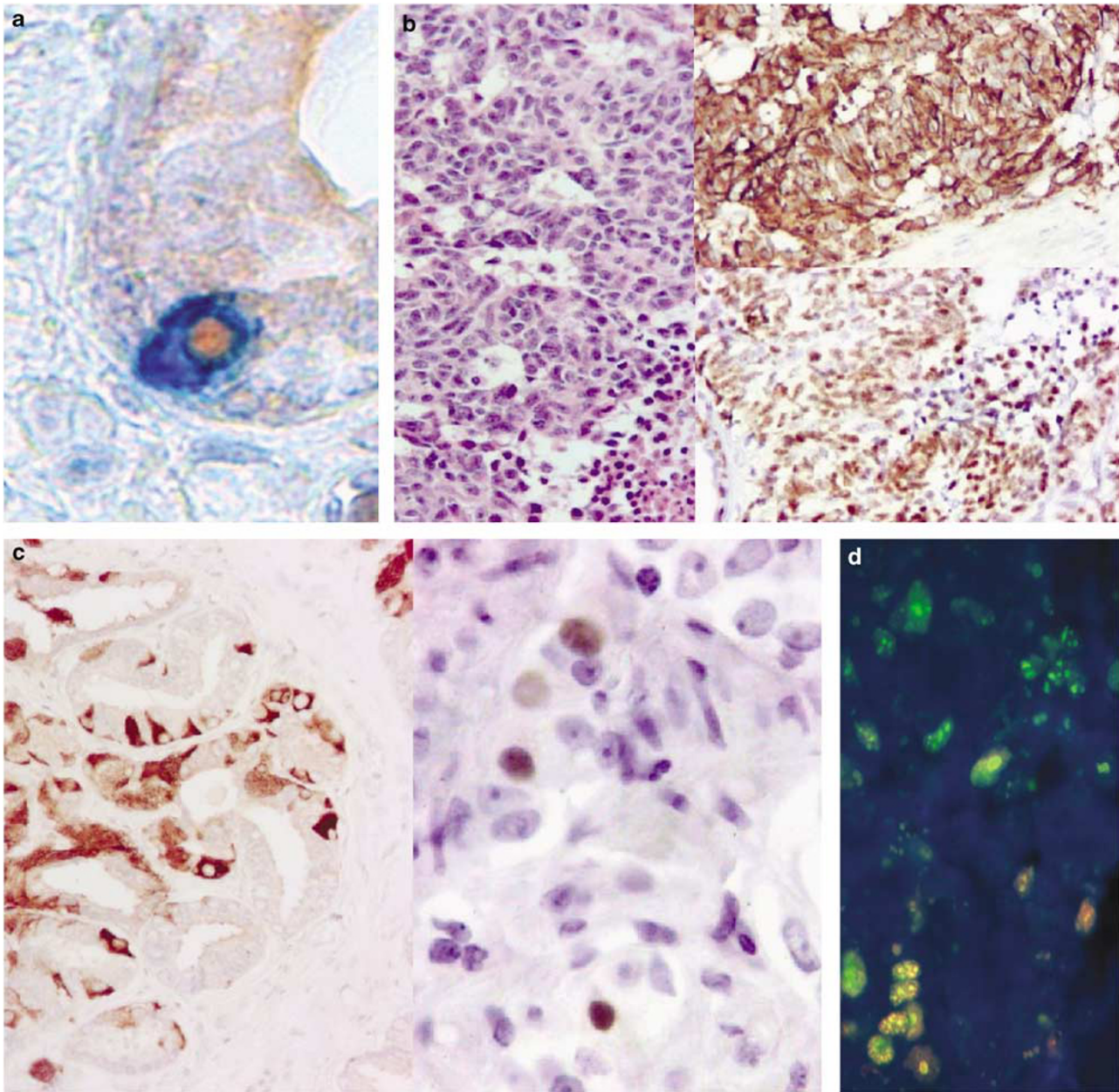
Characteristic	No. of patients	hASH1 IHC		P-value
		Positive	Negative	
Total cases	80	21	59	
Age (mean 67.9 years)				
≤65	28	5	23	P = 0.324
>65	52	16	36	
Gleason score				
≤6	28	4	24	P = 0.873
7–10	37	7	30	
NA*	15	—	—	
Primary tumor stage				
pT2	35	3	32	P = 0.058
pT3	26	8	18	
NA	19	—	—	
Preoperative serum PSA (ng/ml)				
<10	26	4	22	P = 0.219
10–20	20	3	17	
>20	7	3	4	
NA	27	—	—	
Presence of NE differentiation (CgA score)				
PC-nonNE (score 0)	25	1	24	P = 0.02
PC-NE/untreated (score 1)	20	3	17	
PC-NE/untreated (score 2)	20	7	13	
Endocrine therapy administration				
PC-NE/untreated	40	10	30	P = 0.01
PC-NE/treated	15	10	5	

CgA: chromogranin A; hASH1: human achaete-scute homolog 1; IHC: immunohistochemistry; NA: not available; NE: neuroendocrine; PC: prostate cancer; PSA: prostate-specific antigen; \*: not applicable because of previous androgen-deprivation therapy.

material, for a total of 18 cases. In 13 of them, chromogranin A-positive and chromogranin A-negative tumor areas from each individual case were analyzed separately, and the relative ratios compared according to the  $\Delta\Delta C_t$  method. A general upregulation of hASH1 transcripts in chromogranin A-positive as compared with chromogranin A-negative tumor cell populations was observed: 10 out of such 13 samples showed a ratio of hASH1 transcripts >twofold, which was considered significant according to the literature<sup>29</sup> (Figure 3a). To test hASH1 mRNA expression in cases with or without previous androgen ablative therapy, a relative quantification using  $\beta$ -actin as the endogenous control and evaluating expression levels as ratio in a linearized scale was performed in 10 cases from PC-NE/untreated and 8 cases from PC-NE/treated groups. All values ranged from 0.1 to 17.7 (all unitless ratios). A higher hASH1 mRNA expression was observed in PC-NE/treated patients ( $1.6 \pm 6.9$  and  $0.6 \pm 2.8$  in PC-NE/treated and PC-NE/untreated cases, respectively,  $P = 0.01$ ) (Figure 3b). Peritumoral normal prostate tissue showed low hASH1 expression levels ( $0.63 \pm 0.65$ ), without relevant differences among samples from PC-NE/untreated and PC-NE/treated groups.

## Discussion

An essential role of the Notch signaling pathway, and specifically of hASH1 transcription factor, has been reported in the development and control of biological activities of both normal and neoplastic neuroendocrine cells. Despite high expression levels of hASH1 have been demonstrated, among others, in poorly differentiated neuroendocrine carcinomas of the lung and gastrointestinal tract,<sup>21,23</sup> no data are currently available on its putative role in determining the occurrence of neuroendocrine differentiation in non-neuroendocrine tumors, a phenomenon that is still controversial from both pathogenetic and clinical sides. With special reference to prostate cancer, the presence of neuroendocrine features was proven to correlate with aggressive clinical behavior and hormone-refractory disease,<sup>8,9,30</sup> and therefore a better knowledge of the mechanisms leading to this phenomenon would have major clinical impact. By qualitative and quantitative PCR and immunohistochemistry, we could identify a pivotal role of hASH1 molecule in the neuroendocrine differentiation process of prostate cancer, as it was associated to the presence of neuroendocrine features, with special regard to

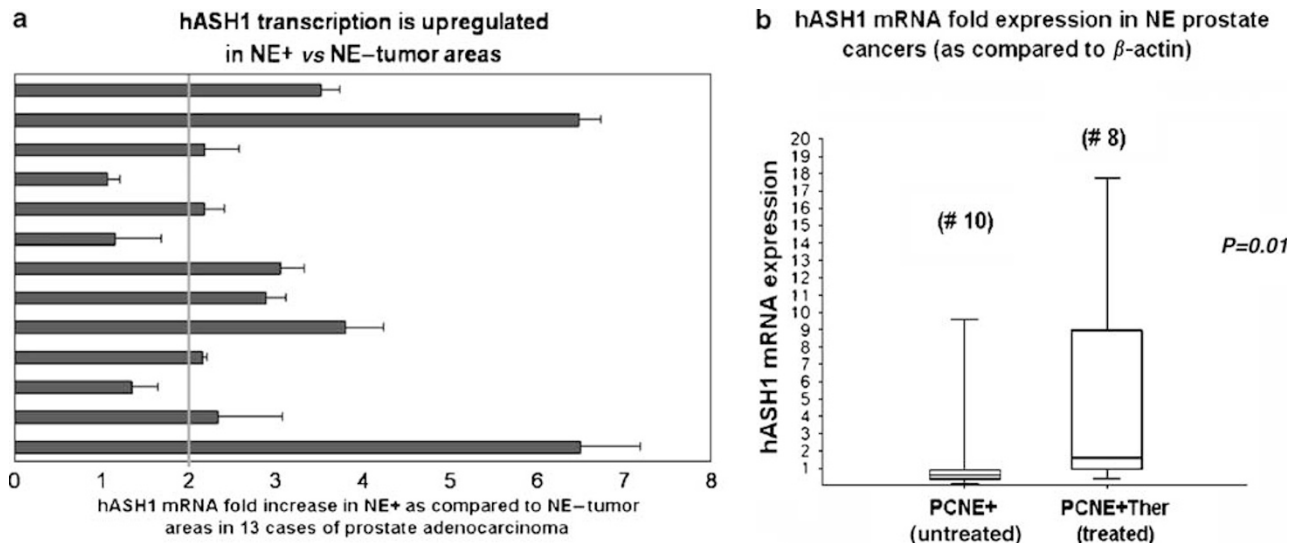


**Figure 2** hASH1 nuclear protein was expressed in normal neuroendocrine cells of the prostate (a; double immunohistochemical procedure with chromogranin A, blue cytoplasmic and hASH1—brown nuclear), pure neuroendocrine carcinomas primary of the prostate (b) and in tumor areas of conventional adenocarcinomas with neuroendocrine differentiation (c), with partial coexpression with Ki-67 (d; hASH1 in red, Ki-67 in green and coexpression in yellow—bottom left) (a; double immunohistochemical procedure, immunoperoxidase and immunoalkaline phosphatase,  $\times 1000$ ; (b) left: H&E  $\times 200$ , right: chromogranin A—top—and hASH1—bottom—immunostaining,  $\times 200$ ; (c) chromogranin A—left—and hASH1—right—immunostaining in the same tumor area, immunoperoxidase,  $\times 200$  and  $400$ ; (d) double immunofluorescence procedure, see text for details).

patients with previous androgen-deprivation hormonal treatment. These data are in agreement with a single study published by other investigators,<sup>26</sup> in which the expression of the mouse homolog of hASH1 was detected in neuroendocrine cancers derived from CR2-Tag transgenic mice prostate model, and in a limited number of human prostate carcinoma samples.

Both immunohistochemistry and quantitative PCR data topographically ‘localized’ hASH1

molecule in the same areas showing neuroendocrine features. Such observation outlines a patchy expression of hASH1 in prostate cancer, as the result of still unrecognized regulatory mechanisms that act heterogeneously in those prostate cancer cell populations displaying neuroendocrine phenotype, and probably reflect the acquisition of a specific phenotype in prostate cancer cells in which hASH1 expression most likely represents a peculiar functional status. In this respect, the higher prevalence



**Figure 3** By RT-PCR, higher levels of hASH1 transcripts were detected in chromogranin A positive as compared with negative tumor cell populations (**a**; error bars represents s.d., which were calculated according to Applied Biosystems User Bulletin no. 2); higher levels of hASH1 transcripts were also detected in patients subjected to androgen-deprivation therapy (**b**; the line in the middle represents the median values, the boxes represents the range between 25th and 75th percentiles, and the whiskers represents the upper and lower values).

of hASH1 expression at both transcriptional and protein levels in prostate cancer patients previously submitted to androgen ablation therapy suggests that hASH1 expression in prostate cancer (and possibly normal) cells is under hormonal control. Although functional *in vivo* studies are needed to better clarify this aspect, some literature data may help to draw a possible scenario. The Notch pathway is involved in prostate development and differentiation processes, and Notch itself is expressed in progenitor basal cells of the prostate with a positive modulation by androgen deprivation in transgenic mouse model.<sup>31</sup> Since in physiologic conditions, Notch negatively modulates hASH1 expression, this latter finding is apparently in contrast with our data. However, recent reports aimed to define possible mechanisms leading to hormone resistance in prostate cancer identified specific alterations (cytoplasmic vs nuclear localization) of Notch modulators in prostate cancer, namely Hey1, which acts as corepressor of androgen receptor-mediated signaling and represses bHLH transcription factors, such as hASH1.<sup>32</sup> Abnormalities of Notch itself or Notch-positive effectors might be responsible for both the development of androgen-independent prostate cancers as well as of the de-repression of hASH1 transcriptional activities with the development of neuroendocrine-differentiated tumor clones. In this respect, our data on the lack of correlation between Notch1 and Hes1 and the presence of neuroendocrine phenotype in prostate tissues is inconclusive, and more detailed experiments are needed to clarify the activation status of such molecules in prostate cancer with neuroendocrine differentiation.

The functional activity of hASH1 in cell growth control is therefore still largely unknown, possibly varying from transcriptional regulation of cell-cycle-related molecules to auto/paracrine signaling of growth factors. In neuroendocrine prostate cancer cells derived from CT2-TAg transgenic mice, RNA interference studies demonstrated the capability of the mouse hASH1 homolog to negatively regulate cell cycle (ie, promoting cyclin-dependent kinase inhibitors expression) and to increase cAMP signaling pathways as a mechanism to promote neuroendocrine differentiation.<sup>26</sup> By contrast, the same methodological approach in neuroendocrine lung cancer model demonstrated that hASH1 inhibition led to cell-cycle arrest and apoptotic cell death.<sup>22</sup>

In conclusion, our data suggest hASH1 as a critical factor involved in neuroendocrine differentiation in prostate cancer, with special reference to patients treated with androgen-deprivation therapy. These findings are relevant to better characterize the mechanisms that induce and maintain the neuroendocrine phenotype in prostate cancer, which represent an independent poor prognostic indicator and is a major feature responsible for hormone-refractory disease. Future hASH1 functional studies will highlight the potential impact of new therapeutic strategies targeted to the Notch pathway (ie,  $\gamma$ -secretase inhibitors) on growth control and restoration of androgen sensitivity in currently incurable neuroendocrine-differentiated prostate cancers.

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## Disclosure/conflict of interest

All authors declare the absence of any financial and personal relationships between themselves and others that might bias their work and of any potential conflicts of interest.

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