Lung Cancer 74 (2011) 405–410

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

The balance between the expressions of hASH1 and HES1 differs between large cell neuroendocrine carcinoma and small cell carcinoma of the lung

Ryo Nasgashio[a,b], Yuichi Sato[b,d,*], Toshihide Matsumoto[c], Taihei Kageyama[c], Manabu Hattori[c,d], Akira Iyoda[e], Yukitoshi Satoh[e], Shinichiro Ryuge[f], Noriyuki Masuda[f], Shi-Xu Jiang[g], Makoto Saegusa[g]

[a] Pathology Division, National Cancer Center Research Institute, Tokyo, Japan
[b] Department of Molecular Diagnostics, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan
[c] Department of Clinical Cytology, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan
[d] Department of Applied Tumor Pathology, Graduate School of Medical Sciences, Kitasato University, Kanagawa, Japan
[e] Department of Thoracic and Cardiovascular Surgery, School of Medicine, Kitasato University, Kanagawa, Japan
[f] Department of Respiratory Medicine, School of Medicine, Kitasato University, Kanagawa, Japan
[g] Department of Pathology, School of Medicine, Kitasato University, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 27 November 2010
Received in revised form 19 February 2011
Accepted 20 April 2011

Keywords:
Achaete-scute complex homolog 1
Hairy/enhancer of split 1
In situ hybridization
Lung cancer
Large cell neuroendocrine carcinoma
Small cell lung carcinoma

ABSTRACT

To clarify the biological differences between small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC), we investigated the expression of two bHLH type transcription factors, human achaete-scute homolog 1 (hASH1) and hairy/enhancer of split 1 (HES1), which positively and negatively regulate the neuroendocrine differentiation of respiratory epithelial cells, respectively. Eighty-eight formalin-fixed and paraffin-embedded pulmonary carcinomas (32 SCLC, 32 LCNEC, 14 adenocarcinomas, and 10 squamous cell carcinomas) and 14 SCLC and 1 LCNEC derived cell lines were used. hASH1 and HES1 mRNA were detected using a highly sensitive *in situ* hybridization method with digoxigenin-labeled cRNA probes and biotinylated tyramide. The staining results were scored from 0 to 12 by multiplying the staining intensity by the percentage of positive tumor cells. The mean staining score of hASH1 mRNA was significantly higher in SCLC than in LCNEC ($p < 0.01$); conversely, that of HES1 mRNA was lower in SCLC than in LCNEC ($p < 0.01$). These findings reveal that SCLC more strongly expresses the neuroendocrine phenotype, while LCNEC shows characteristics more similar to the ciliated epithelium phenotype, suggesting that the biological characteristics of these two tumors are different.

1. Introduction

It is now widely recognized that neuroendocrine tumors of the lung range from low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) [1,2]. Generally, neuroendocrine tumors share some common morphological characteristics such as organoid structures, a palisading basal cell arrangement, and rosette formation. In 1991, Travis et al. introduced the term LCNEC to describe a distinct entity of high-grade neuroendocrine tumors with light microscopic characteristics that differ from those of high-grade SCLC [1]. Although LCNEC is considered to be a disease entity that is distinct from SCLC, these two types of lung cancer share a great number of features, such as similar gene expression profiles and clinical characteristics [2–4]. Pro-gastrin-releasing peptide (pro-GRP) is a specific serodiagnostic marker of SCLC [5]; however, its positivity ratio is low in the early stages of SCLC, and no specific markers have been developed for LCNEC. Many studies have reported that no significant differences in outcome between LCNEC and SCLC patients were observed [3,6,4], and it is difficult to differentiate LCNEC from SCLC, and no definitive discrimination points except for its morphological characteristics and the details of its biological behavior (including tumor aggressiveness and the degree of differentiation) have been established.

Transcriptional factors play an important role in the neuroendocrine differentiation of immature respiratory epithelial cells, and two basic-helix–loop–helix transcription factors, achaete-scute complex homolog 1 (ASCL1, termed hASH1 in humans, MASH1 in rodents) and hairy/enhancer of split 1 (HES1), regulate neuronal differentiation positively and negatively, respectively [7,8]. hASH1 plays a crucial role in neural commitment and differentiation [9] and is selectively expressed in normal fetal pulmonary neuroendocrine cells and lung cancers with neuroendocrine features [7,10].

* Corresponding author at: Department of Molecular Diagnostics, School of Allied Health Sciences, Kitasato University, Kitasato 1-15-1, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan. Tel.: +81 42 778 8013; fax: +81 42 778 9854.
E-mail address: yuichi@med.kitasato-u.ac.jp (Y. Sato).

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doi:10.1016/j.lungcan.2011.04.012

Disruption of the MASH1 gene affects neuroendocrine differentiation during fetal development, resulting in the loss of pulmonary neuroendocrine cells [11].

HES1, a key effector of the Notch signaling pathway, is expressed broadly in non-neuroendocrine cells in the airway epithelium [12]. HES1 directly represses hASH1 expression by binding to a class C site in the hASH1 promoter [13]. In the developing lung, Notch1 and HES1 are strongly expressed in non-neuroendocrine airway epithelial cells, whereas MASH1 is restricted to clustered pulmonary neuroendocrine cells [11,12]. In the mice deficient in HES1, the number of pulmonary neuroendocrine cells increased markedly, and MASH1 was overexpressed [12]. Conversely, MASH1 gene knockout disrupted neuroendocrine differentiation and resulted in the loss of pulmonary neuroendocrine cells [11]. Consistent with its well-established role in inhibiting commitment and differentiation in neuronal precursors, Notch signaling appears to play a critical role in restricting neuroendocrine cell development within the airway epithelium. In human tumors, it is reported that SCLC cells express hASH1 but lack HES1 expression. On the contrary, non-neuroendocrine carcinoma cells do not express hASH1, but show high HES1 expression [13]. However, no studies have been carried out to examine the expression levels and the balance between the expression levels of hASH1 and HES1 in LCNEC and SCLC. In this study, we compared the expression levels of hASH1 and HES1 mRNA in both tumors using a highly sensitive *in situ* hybridization (ISH) method employing biotinylated tyramide.

2. Materials and methods

2.1. Cell lines

Eleven classical (Lu24, Lu130, Lu134A, Lu135C, Lu139, Lu140, Lu143, Lu165, N230, N231, and H69) and three variant (H82, N417, and Lu135v) SCLC cell lines and a LCNEC cell line (LCN1) [14] were used in this study. The SCLC cells were donated by Dr. Hirohashi (National Cancer Center, Japan). Each cell line was grown in RPMI1640 (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Auckland, New Zealand). The harvested cells were washed with phosphate-buffered saline without bivalent ions and were fixed in 10% formalin and embedded in paraffin for *in situ* hybridization or were stored at −80 °C until their use in the reverse transcription-polymerase chain reaction (RT-PCR).

2.2. Tumor tissues

Eighty-eight formalin-fixed and paraffin-embedded pulmonary carcinomas, which were surgically resected at Kitasato University Hospital, were used in the present study. They were divided into 32 SCLC, 32 LCNEC, 14 adenocarcinomas (AD), and 10 squamous cell carcinomas (SCC). This study was approved by the Ethical Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave their consent for their samples to be used.

2.3. RT-PCR

Total RNA were extracted from the fifteen cell lines with Isogen (Nippon Gene, Tokyo, Japan) and reverse transcribed with the First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The primers used for the PCR were designed with Oligo Primer Analysis Software, version 6.0 (Takara Bio, Otsu, Japan) according to defined hASH1 mRNA (accession number: NM_004316), HES1 mRNA (accession number: NM_005524), and β-2-microglobulin (β_2M) mRNA sequences (accession number: NM_004048) (Table 1). PCR amplification was performed using Taq DNA polymerase (Roche Diagnostics) and the PCR system 2700 (Applied Biosystems, Warrington, UK). The PCR consisted of denaturing at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C and a final extension step of 7 min at 72 °C.

Table 1
Primer sets used for the reverse transcription-polymerase chain reaction.

hASH1	
Sense primer	5′-TCTCCCCCAACTACTCCAAC-3′
Antisense primer	5′-CGCGTGTGCTGCTCCCTTCT-3′
HES1	
Sense primer	5′-GTCAACACGACACCGGATAAA-3′
Antisense primer	5′-GCGGGTCACCTCGTTCA-3′
β-2-Microglobulin	
Sense primer	5′-TTCTGGCCTGGAGGGCATCC-3′
Antisense primer	5′-ATCTTCAAACCTCCATGATG-3′

hASH1, human achaete-scute homolog 1; HES1, hairy/enhancer of split 1.

2.4. Preparation of the cRNA probes

For non-radioactive ISH, digoxigenin (DIG)-labeled cRNA probes were generated with T7 RNA polymerase promoter region (CTTAATACGACTCACTATAGGG)-tailed PCR, as described in our recent study [15]. The PCR conditions were the same as those used for the RT-PCR, and the core sequences of the primers used were the same as those of the primers used for the RT-PCR mentioned above without the T7 RNA polymerase promoter sequence. The PCR products were transcribed *in vitro* using T7 RNA polymerase and labeled with DIG-dUTP using a DIG *in vitro* transcription kit (Roche Diagnostics) to produce sense and antisense cRNA probes. The specificity of the PCR-generated templates was confirmed by direct sequencing with a cycle sequencing method (Big Dye Terminator cycle sequencing kit, Applied Biosystems).

2.5. Highly sensitive ISH

The ISH was carried out as described previously [15,16] with some modifications. In brief, deparaffinized 3-μm-thick tissue sections and cells were treated with 10 μg/mL proteinase K (Roche Diagnostics) for 20 min at 37 °C and 5 μg/mL proteinase K for 10 min at 37 °C, respectively. The sections were post-fixed in 4% paraformaldehyde and then treated with 0.2 N HCl and 0.25% acetic anhydride in 0.1 mol/L tri-ethanol amine (pH 8.0) for 10 min each. After treatment with 3% hydrogen peroxide for 60 min, the sections were dehydrated and air-dried. Fifty microliters of the hybridization mixture (Hybridization Solution I, Maxim Biotech, Inc., South San Francisco, CA) and 50 ng of the sense or antisense cRNA probe were loaded onto each section and hybridized for 16–18 h at 50 °C. After hybridization, the sections were washed in 50% formamide/2× standard sodium citrate (SSC) for 30 min at 55 °C and then treated with 10 μg/mL RNase A (Roche Diagnostics) for 30 min at 37 °C. The sections were then stringently washed with 2× SSC, 0.2× SSC, and 0.1× SSC for 20 min each at 55 °C. After being placed into 0.01 mol/L Tris–HCl (pH 7.5), 0.3 mol/L NaCl, and 0.1% Tween-20 three times for 5 min each, and then in 0.5% casein/0.01 mol/L Tris–HCl (pH 7.5) and 0.15 mol/L NaCl for 10 min, the sections were reacted with 400 times diluted horseradish peroxidase (HRP)-conjugated rabbit anti-DIG Fab′ fragmented polyclonal antibody (Dako, Glostrup, Denmark), 0.07 μmol/L biotinylated tyramide [17,18], and 500 times diluted HRP-conjugated streptavidin (Dako) for 15 min each. Finally, the sections were visualized with DAB solution (Liquid DAB Substrate Chromogen System) (Dako) and counterstained with Mayer's hematoxylin.