Abstract

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Human *achaete-scute* Homologue (hASH1) mRNA Level as a Diagnostic Marker to Distinguish Esthesioneuroblastoma From Poorly Differentiated Tumors Arising in the Sinonasal Tract

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**Key Words:** Esthesioneuroblastoma; Olfactory neuroblastoma; Poorly differentiated tumors; Human achaete-scute homologue (hASH1) expression

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**Abstract**

Distinction of high-grade esthesioneuroblastomas from other poorly differentiated tumors arising in the nasal cavity is an important diagnostic challenge because it determines patient management and prognosis. The human *achaete-scute* homologue (hASH1) gene is critical in olfactory neuronal differentiation and is expressed in immature olfactory cells; therefore, it could have potential use as a diagnostic marker. The aim of the present study was to determine the value of hASH1 messenger RNA (mRNA) levels in differentiating esthesioneuroblastoma from other poorly differentiated tumors.

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All 4 positive cases were esthesioneuroblastomas, and all 19 poorly differentiated tumors were negative. In addition, there was an inverse association between the grade of esthesioneuroblastomas and hASH1 mRNA levels. The hASH1 mRNA level might represent a useful tool for distinguishing esthesioneuroblastoma from poorly differentiated tumors of the sinonasal region.

Esthesioneuroblastoma (ENB), also known as olfactory neuroblastoma, is a rare malignant neoplasm arising in the nasal cavity and the paranasal sinuses. It accounts for 1% of all malignant neoplasms of this region and occurs in a broad age range with high frequency in adults.1,2 The tumor is thought to originate from the olfactory mucosa,3 which is distributed over the superior turbinate, cribriform plate, and superior third of the nasal septum.4 ENB often is a locally aggressive malignant neoplasm, but it also can metastasize via lymphatic and hematogenous routes. The prognosis seemed to depend on the degree of tumor differentiation and lymph node metastasis.5

The differentiation of ENB was subdivided into 4 groups by Hyams.6 When the tumor is well differentiated (groups I and II) and consists of a proliferation of small round cells with fibrillary eosinophilic background material, the diagnosis is relatively easy. However, in high-grade and poorly differentiated lesions (groups III and IV), which are characterized by marked nuclear pleomorphism, frequent mitosis, areas of necrosis, and absence of fibrillary background, the diagnosis is challenging. In those circumstances, the differential diagnosis includes other poorly differentiated tumors (PDTs) arising in the nasal cavity such as malignant melanoma, lymphoma, nasopharyngeal undifferentiated carcinoma, and sinonasal undifferentiated carcinoma (SNUC). Although nasopharyngeal undifferentiated carcinoma does not arise from the nasal cavity, it can involve this region when the lesion manifests at an advanced stage, as often is the case.

In addition to basic morphologic examination, the other diagnostic tool available to distinguish ENB from other PDTs essentially is immunohistochemical analysis, and in second
place, electron microscopy. With the wide acceptance and widespread use of immunohistochemical analysis as an adjunct in the diagnosis of problematic cases, the inference has been that the role of electron microscopy has declined. Nowadays, the role of electron microscopy has been limited to a few problematic cases, and it is used widely in kidney diseases such as glomerulopathy. While immunohistochemical analysis has made a significant contribution to routine histopathologic diagnosis, it still has some limitations. Thus, an additional, more accurate, and objective method would be of great value.

The mammalian *achaete-scute* homologue gene of *Drosophila* (*mASH1*) is expressed in neural crest cells and in neural crest–derived progenitors of sympathoadrenal lineage. *mASH1* is critical for olfactory neuronal differentiation; *mASH1*-deficient mice fail to develop mature olfactory neurons. *hASH1*, or *ASCL1*, is the human achaete-scute homologue, which shares 95% identity with *mASH1*. It is located at the 12q22-q23 locus. *mASH1* and *hASH1* belong to the family of basic helix-loop-helix transcription factors, which regulate the capacity of the ectodermal cells to become developing neuroblasts in the peripheral sensory and central nervous systems. To our knowledge, the only study in the literature evaluating the expression of the *hASH1* gene in ENB was reported by Carney et al in 1995. By using reverse transcriptase–polymerase chain reaction (RT-PCR) on formalin-fixed material in 7 ENB specimens, the authors were able to prove the olfactory epithelial origin of the ENB tumors.

The aims of the present study were to develop an assay for comparative determination of hASH1 mRNA expression levels in small biopsy specimens and to validate this assay in a double-blind pilot study as being a useful tool to distinguish ENBs from PDTs arising in the sinonasal region.

### Materials and Methods

#### Tissue Specimens

Biopsy samples of lesions diagnosed as ENBs (n = 5) and samples from various PDTs from the head and neck region (undifferentiated nasopharyngeal carcinoma, 10; high-grade diffuse large B-cell lymphoma, 5; malignant melanoma, 4) were obtained by the departments of clinical pathology at Geneva and Lausanne university hospitals, immediately frozen after biopsy, and stored at –80°C. H&E-stained sections of each of the 4 entities are shown in Image II.

The diagnoses for these cases were confirmed by using an immunohistochemical panel that included total keratin, synaptophysin, chromogranin, leukocyte common antigen, MHB45, S-100, and melanin A. None of the cases were analyzed using electron microscopy. Of the 5 ENB cases, 3 were high-grade (groups III and IV) and 2 were low-grade (groups I and II) lesions. The morphologic and immunohistochemical profiles of all cases were examined by 1 pathologist (P.M.). Thus, all of those cases have a histologic diagnosis performed by 2 pathologists (first by the pathologist in charge of the case and second by the reviewing pathologist [P.M.]). In addition, lymphoma cases were confirmed by molecular biologic studies for IgG heavy chain and T-cell receptor rearrangements. For total RNA isolation, 20 serial sections of 10 µm were cut and processed immediately. The middle section of each series was stained with H&E to confirm the diagnosis and to estimate the percentage of tumor cells within the biopsy specimen.

#### Cell Lines

The NCI-H209 (ACC 499) small cell lung cancer cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained in 80% RPMI 1640 medium with 20% fetal bovine serum. NCI-H209 cells have been shown to express high levels of hASH1 mRNA and, therefore, were used as positive control samples to calibrate the sensitivity of the quantitative hASH1 cDNA PCR assay. JURKAT cells, a T-cell acute leukemia/lymphoma line obtained from American Type Culture Collection (Rockville, MD) without neuroendocrine features, were kept in RPMI 1640 with 10% fetal bovine serum and used as a negative control sample for hASH1 mRNA expression.

#### RNA Isolation and Complementary DNA Synthesis

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The obtained total RNA was quantified (absorbance, 260-280: 20-150 µg), and 0.5 µg was transcribed directly into first-strand complementary DNA (cDNA) using the first-strand cDNA synthesis kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer’s instructions. The quality of the cDNA was checked further by PCR amplification of a 200-base-pair fragment of the β-actin mRNA (position 2661-2974), according to the National Institutes of Health (Bethesda, MD) Genbank.

#### Quantitative RT-PCR

To compare the hASH1 mRNA expression levels in ENB and other PDTs arising in the sinonasal tract, a real-time RT-PCR assay using TaqMan technology (Applied Biosystems, Rotkreuz, Switzerland) was set up. Relative hASH1 mRNA levels were normalized against expression levels of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene.
Primers and a TaqMan probe covering the exon 1 and exon 2 boundaries of hASH1 were selected by using Primer Express software (PE Biosystems, Rotkreuz, Switzerland) and purchased from Mycrosynth (Balgach, Switzerland). The probe was linked covalently at the 5’ end with the reporter dye FAM and the TAMRA quencher dye at the 3’ end. The real-time reaction was performed in a final volume of 20 µL containing 1× Platinum qPCR Supermix UDG (Invitrogen, Rotkreuz, Switzerland), a 600-nmol/L concentration of primers, a 200-nmol/L concentration of probe, and 1 µL of 10 times diluted cDNA reaction. The real-time PCR cycling conditions were as follows: 2 minutes at 50°C, followed by 10 minutes of denaturation at 95°C and 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute.

hASH1 mRNA levels were normalized to the expression of the GAPDH gene, for which the mRNA level was measured by the aforementioned predeveloped TaqMan assay. For quantitative comparative analysis, standard curves for GAPDH and hASH1 expression were established using serial dilutions of H209 (hASH1-positive) and JURKAT (hASH1-negative) cDNA in duplicate reactions. The molecular pathologist (J.S.) had no knowledge of the histologic diagnosis.

Statistical Analysis

The distributions of gene-relative expression among the diagnoses were compared by using the Kruskal-Wallis
Because this variable is not normally distributed, standard procedures for normalization were applied successfully, and logistic regressions with sensitivity analysis were performed to find the best threshold value of the natural logarithm of “mASH1 gene-relative expression” for predicting ENB. Statistics were performed using Stata release 8.1 software (Stata, College Station, TX).

**Results**

PCR amplification plots showed threshold cycle values ranging from 18 to 38 cycles when hASH1 mRNA expression was analyzed in H209 cells, 2 ENB cases, and a case of PDT. As shown in **Figure 1**, relative hASH1 mRNA (normalized to GAPDH) expression levels ranged from not measurable (0) to 18,700 relative units in all cases analyzed.

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Product (base pairs)</th>
</tr>
</thead>
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<tr>
<td>hASH1 forward</td>
<td>5′ GAG CAG GAG CTT CTC GAC TTC A 3′</td>
<td>1114-1135</td>
<td>—</td>
</tr>
<tr>
<td>hASH1 reverse</td>
<td>5′ GAT GCA GGT TGT GCG ATC AC 3′</td>
<td>1198-1217</td>
<td>103</td>
</tr>
<tr>
<td>Probe</td>
<td>5′ AGG CCC TGG TGC GAA TGG ACT TTG 3′</td>
<td>1166-1189</td>
<td>—</td>
</tr>
</tbody>
</table>

hASH1, human achaete-scute homolog.

* Genbank accession number: NM_004316, hASH1 mRNA.

**Figure 1** Comparative human achaete-scute homologue (hASH1) messenger RNA (mRNA) expression analysis in esthesioneuroblastoma (ENB) vs non-ENB tumors (high-grade lymphoma, melanoma, and nasopharyngeal carcinoma) of the head and neck region as determined by real-time polymerase chain reaction (TaqMan, Applied Biosystems, Rotkreuz, Switzerland). hASH1 mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels (see the “Materials and Methods” section). All values represent the medians of 4 to 6 values determined in 3 different experimental settings. All analyses were performed without knowing the diagnoses. Cases 1-14 and 16-20 are non-ENB cases, including 5 cases of high-grade lymphoma (diffuse large B-cell lymphoma), 4 cases of malignant melanoma, and 10 cases of poorly differentiated tumors; cases 15 and 21 are low-grade ENBs, and cases 22-24 are high-grade ENBs. A case of small cell lung cancer (SCLC) with neuroendocrine features and the H209 SCLC cell line were used as positive control samples to calibrate the assay; human T-cell leukemia/lymphoma JURKAT cells were used as negative control samples. Dotted line, sensitivity threshold value.
The highest values were observed in a primary small cell lung cancer biopsy specimen and in the H209 small cell lung cancer line. In general, ENB cases showed higher hASH1 mRNA relative expression levels than PDT cases. The distribution of hASH1 mRNA relative expression showed a statistically significant difference ($P = .02$) between positive and negative cases.

The natural logarithm of hASH1 mRNA relative expression explains 61.2% of the variability of having ENB in the logistic regression model, with an odds ratio of 3.34 (95% confidence interval, 1.13-9.91; $P = .030$); 4 of 5 ENB cases and all 19 non-ENB cases were identified correctly. Sensitivity analysis yielded a threshold value of 2.2597 for the log of hASH1 mRNA relative expression, which corresponds to 9.58 on a normal scale, and the area under the corresponding receiver operating characteristic curve reached 95% (not shown). The ENB case with hASH1 mRNA expression levels (1.17 ± 0.5) under the “diagnostic” threshold value (9.58) for being diagnosed as ENB corresponded histologically to a low-grade, well-differentiated lesion.

In general, although the number of ENB cases is small in this pilot study, an inverse association between tumor differentiation and hASH1 mRNA relative expression levels was seen, with median expression values of low-grade ENBs of 14.8 vs 329.1 in high-grade ENBs. Taken together, our assay for comparative quantitative analysis of hASH1 mRNA expression enabled us to distinguish high-grade ENBs from PDTs in all cases in this pilot “double-blind” study. Well-differentiated, low-grade ENB lesions generally showed lower hASH1 mRNA expression levels, but they might be diagnosed by immunohistochemical analysis using a wide panel of neuroendocrine markers.

**Discussion**

About half of the cases of ENB manifest as high-grade lesions (Hyams grades III and IV), and, therefore, the differential diagnosis from other poorly differentiated tumors of the sinonasal tract is a frequent dilemma for surgical pathologists. However, this distinction is critical because the therapeutic management and prognosis vary with the specific histopathologic diagnosis and clinical staging.

Based on morphologic features alone, the differential diagnosis of ENB, especially the high-grade, poorly differentiated lesions, from other poorly differentiated tumors is almost impossible, and the other widely used tool available for pathologists, namely immunohistochemical analysis, has some limitations. Thus, finding a new method to include in the routine setting besides morphologic and immunohistochemical analyses would be of great interest.

Our results clearly show that the hASH1 mRNA level as measured, using RT-PCR, is an objective and accurate method to distinguish ENB from other PDTs arising in the sinonasal tract, with high sensitivity and specificity. All 3 high-grade ENB lesions had hASH1 mRNA levels 2 to 3 log above the median values. One case of low-grade ENB was positive, and the other was negative (ie, under the significance threshold). However, in these low-grade ENBs, traditional morphologic and immunohistochemical analyses usually are sufficient for the correct diagnosis and permit the distinction of ENB from other sinonasal PDTs. In addition, the very high level of hASH1 mRNA levels in all high-grade ENBs and the absolute negativity of all other PDTs highlight the usefulness of this marker in the diagnosis of ENB.

Our study indicated that hASH1 mRNA levels had an inverse association with the degree of tumor differentiation, ie, high-grade, poorly differentiated ENBs showed higher expression of this gene than low-grade, well-differentiated tumors. This result is in accordance with the literature in which the expression of hASH1 seemed to be restricted to immature cells. Consequently, the more the tumor cells are immature or undifferentiated, the higher the expression of this gene, and the more they are mature or differentiated, the lower the expression of this gene. However, it is interesting to note that overexpression of hASH1 in combination with a potent oncogene (SV40 large T) in vivo resulted in poorly differentiated tumors, which raises the question of whether aberrant hASH1 expression might have a more direct role in carcinogenesis.

Determination of the hASH1 mRNA level also can be expected to be of value in the diagnosis of metastatic ENBs in which the differential diagnosis is with other poorly differentiated tumors. However, in those circumstances, we have to keep in mind that this gene is expressed in neuroendocrine malignant neoplasms such as medullary carcinoma of the thyroid and small cell carcinoma of the lung and prostate.

Shortcomings of this study include the small number of ENB specimens, but because of the rarity of this neoplasm, only a few cases are seen in a single institution. Second, our assay requires fresh or frozen tissue samples, which rarely are available. Third, our series of cases did not have cases of SNUC. SNUC is a related tumor that was recognized in 1987 as a distinct entity. SNUC and poorly differentiated ENB share numerous characteristics such as clinical manifestations, immunohistochemical profile, poor prognosis and survival, and treatment. Because of the much worse prognosis of SNUC and of poorly differentiated ENB, their distinction from well-differentiated ENB is vital. Because the hASH1 gene has been shown to be expressed in neuroendocrine malignant neoplasms, we can speculate that this gene also will be present in SNUC cases. However, as far as management and prognosis are concerned, the differential
diagnosis between poorly differentiated ENB and SNUC is not of great importance. Finally, although the small number of specimens prevented us from confirming the regression model with a validation sample, the sensitivity analysis provided interesting results that need to be confirmed by other groups.

To our knowledge, our study is the first to evaluate the role of this gene as a diagnostic marker for the diagnosis of ENB. hASH1 mRNA expression might be used in addition to morphologic and immunohistochemical analyses as a new tool for the diagnosis of ENB.

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