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

Paulette Mhawech, MD,[1] Margaret Berczy, MT,[1] Mounia Assaly, MD,[1] Francois Herrmann, MD,[4] Hanifa Bouzourene, MD,[5] Abdelkarim S. Allal, MD,[3] Pavel Dulguerov, MD,[2] and Jürg Schwaller, MD[1]

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

DOI: 10.1309/QD0K9Q1JBH6B5GQQ

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.*

A real-time polymerase chain reaction assay was developed, permitting the comparative determination of hASH1 *mRNA levels in triplicate in a double-blind pilot study including 24 frozen cases of esthesioneuroblastoma and poorly differentiated tumors.*

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.[7,8] 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.[9,10] *mASH1* is critical for olfactory neuronal differentiation; *mASH1*-deficient mice fail to develop mature olfactory neurons.[11] *hASH1,* or *ASCL1*, is the human achaete-scute homologue, which shares 95% identity with *mASH1*. It is located at the 12q22-q23 locus.[12] *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.[9] 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.[3] 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.[3]

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 1**.

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[13] 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.

DOI: 10.1309/QD0K9Q1JBH6B5GQQ

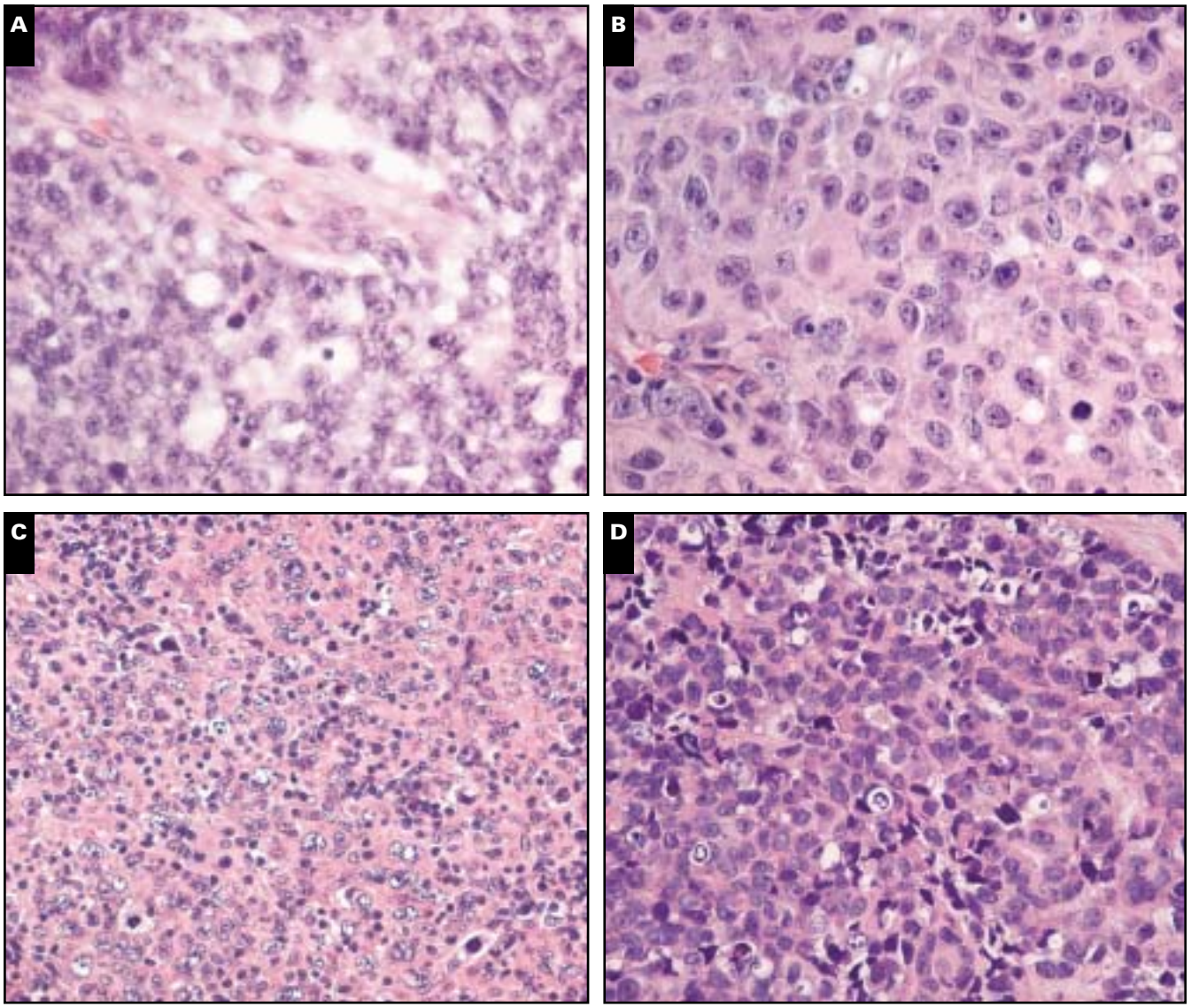

Image 1 Representative cases of esthesioneuroblastoma (**A**, H&E, ×400), malignant melanoma (**B**, H&E, ×400), diffuse large B-cell lymphoma (**C**, H&E, ×200), and nasopharyngeal carcinoma (**D**, H&E, ×400). In all cases, the tumor morphologic features are similar, with diffuse proliferation of large cells and numerous mitotic figures.

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 **Table 1**. 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

DOI: 10.1309/QD0K9Q1JBH6B5GQQ

Table 1
hASH1* Primers and Probe Sequences

Primer	Sequence	Position	Product (base pairs)
hASH1 forward	5' GAG CAG GAG CTT CTC GAC TTC A 3'	1114-1135	—
hASH1 reverse	5' GAT GCA GGT TGT GCG ATC AC 3'	1198-1217	103
Probe	5' AGG CCC TGG TGC GAA TGG ACT TTG3'	1166-1189	—

hASH1, human *achaete-scute* homologue.
* Genbank accession number: NM_004316, *hASH1* mRNA.

test. 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.

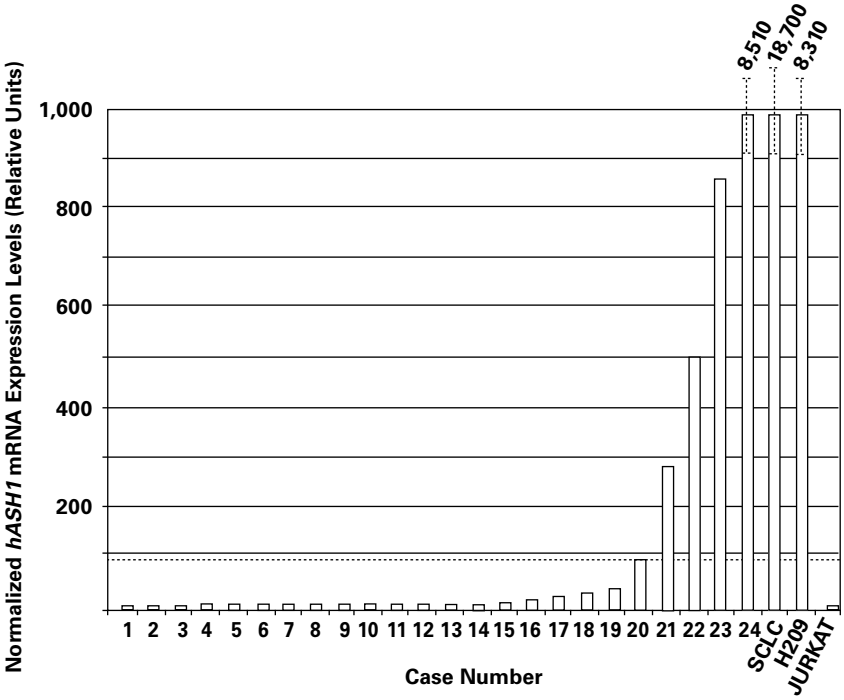

Figure 1 Comparative human *achaete-scute* homologue (*hASH1*) messenger RNA (mRNA) expression analysis in esthesioneuroblastoma (ENB) vs non-ENB tumors (high-grade lymphoma, melanoma, and nasopharyngeal carcinoma) of the head and neck region as determined by real-time polymerase chain reaction (TaqMan, 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.

DOI: 10.1309/QD0K9Q1JBH6B5GQQ

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),[5] and, therefore, the differential diagnosis from other poorly differentiated tumors of the sinonasal tract is a frequent dilemma for surgical pathologists.[14] However, this distinction is critical because the therapeutic management and prognosis vary with the specific histopathologic diagnosis and clinical staging.[15-17]

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.[7,8] 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.[18] 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.[18] 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.[19]

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.[20-22]

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.[23] SNUC and poorly differentiated ENB share numerous characteristics such as clinical manifestations, immunohistochemical profile, poor prognosis and survival, and treatment.[14] 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

DOI: 10.1309/QD0K9Q1JBH6B5GQQ

diagnosis between poorly differentiated ENB and SNUC is not of great importance.[24] 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.

From the Departments of [1]Clinical Pathology, [2]Head and Neck Surgery, and [3]Radiation Oncology and the [4]Department of Rehabilitation and Geriatrics, Geneva University Hospital, Geneva, Switzerland; and the [5]Department of Clinical Pathology, University Hospital of Lausanne, Lausanne, Switzerland.

Address reprint requests to Dr Mhawech: c/o Johnny Depaolo, 4010 Wyne St, Houston, TX 77017.

Acknowledgments: We thank Sophia Taylor, MD, for critical review of the manuscript and Ludovic Metral, Raoul Azoran, Danielle Fontana, and Christelle Cerrato for technical assistance.

References

1. Broich G, Pagliari A, Ottaviani F. Esthesioneuroblastoma: a general review of the cases published since the discovery of the tumor in 1924. *Anticancer Res*. 1997;17:2383-2406.
2. Argiris A, Dutra J, Tseke P, et al. Esthesioneuroblastoma: the Northwestern University experience. *Laryngoscope*. 2003;113:155-160.
3. Carney ME, O'Reilly RC, Sholevar B, et al. Expression of the human achaete-scute 1 gene in olfactory neuroblastoma (esthesioneuroblastoma). *J Neurooncol*. 1995;26:35-43.
4. Dulguerov P, Calcaterra T. Esthesioneuroblastoma: the UCLA experience 1970-1990. *Laryngoscope*. 1992;102:843-849.
5. Dulguerov P, Allal AS, Calacaterra TC. Esthesioneuroblastoma: a meta-analysis and review. *Lancet Oncol*. 2001;2:683-690.
6. Hyams VJ. Olfactory neuroblastoma. In: Hyams VJ, Batsakis JG, Michaels L, eds. *Tumors of the Upper Respiratory Tract and Ear*. Washington, DC: Armed Forces Institute of Pathology; 1988:240-248. *Atlas of Tumor Pathology*; Second series, Fascicle 25.
7. Chan JKC. Advances in immunohistochemistry: impact on surgical practice. *Semin Diagn Pathol*. 2000;17:170-177.
8. Devoe K, Weidner N. Immunohistochemistry of small round-cell tumors. *Semin Diagn Pathol*. 2000;17:216-224.
9. Sommer L, Shah N, Rao M, et al. The cellular function of MASH1 in autonomic neurogenesis. *Neuron*. 1995;15:1245-1258.
10. Johnson JE, Birren SJ, Anderson DJ. Two rat homologues of *Drosophila* achaete-scute specifically expressed in neuronal precursors. *Nature*. 1990;346:858-861.
11. Guillemot F, Lo LC, Johnson JE, et al. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell*. 1993;75:463-476.
12. Ball DW, Azzoli CG, Bylin SB, et al. Identification of a human achaete-scute homolog highly expressed in neuroendocrine tumors. *Proc Natl Acad Sci U S A*. 1993;90:5648-5652.
13. Carney DN, Gazdar AF, Bepler G, et al. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res*. 1985;45:2913-2923.
14. Mills SE. Neuroectodermal neoplasms of the head and neck with emphasis on neuroendocrine carcinomas. *Mod Pathol*. 2002;15:264-278.
15. Oskoulan RJ, Jane JA, Dumont AS, et al. Esthesioneuroblastoma: clinical presentation, radiological, and pathological features, treatment, review of the literature, and the University of Virginia experience. *Neurosurg Focus*. 2002;12:1-9.
16. Thompson LDR, Wieneke JA, Miettinen M. Sinonasal tract and nasopharyngeal melanomas. *Am J Surg Pathol*. 2003;27:594-611.
17. Lund VJ, Howard D, Wei W, et al. Olfactory neuroblastoma: past, present, and future? *Laryngoscope*. 2003;113:502-507.
18. Cau E, Casarosa S, Guillemot F. Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development*. 2002;129:1871-1880.
19. Sriuranpong V, Borges MW, Strock CL, et al. Notch signaling induces rapid degradation of achaete-scute homolog 1. *Mol Cell Biol*. 2002;22:3129-3139.
20. Clegg N, Ferguson C, True LD, et al. Molecular characterization of prostatic small-cell neuroendocrine carcinoma. *Prostate*. 2003;55:55-64.
21. Westerman BA, Neijenhuis S, Poutsma A, et al. Quantitative reverse transcription–polymerase chain reaction measurement of HASH1 (ASCL1), a marker for small cell lung carcinomas with neuroendocrine features. *Clin Cancer Res*. 2002;8:1082-1086.
22. Soderholm H, Ortoft E, Johansson I, et al. Human achaete-scute homologue 1 (HASH-1) is downregulated in differentiating neuroblastoma cells. *Biochem Biophys Res Commun*. 1999;256:557-563.
23. Levine PA, Frierson HF, Stewart FM, et al. Sinonasal undifferentiated carcinoma: a distinctive and highly aggressive neoplasm. *Laryngoscope*. 1987;97:905-908.
24. Miyamoto RC, Gleich LL, Biddinger PW, et al. Esthesioneuroblastoma and sinonasal undifferentiated carcinoma: impact of histological grading and clinical staging on survival and prognosis. *Laryngoscope*. 2000;110:1262-1265.

DOI: 10.1309/QD0K9Q1JBH6B5GQQ