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Molecular and Phenotypic Analysis of Poorly Differentiated Sinonasal Neoplasms: An Integrated Approach for Early Diagnosis and Classification

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Abstract

Accurate early diagnosis of poorly differentiated tumors of the sinonasal and skull-base sites is critical to modern multimodality management of patients with these tumors. A combined phenotypic and sequential biomarkers approach of a large retrospective cohort of these tumors led to the reclassification of some cases and the confirmation of uncertain diagnoses in others. An integrated algorithm of selected markers and phenotypic features for biopsy-based diagnosis of these tumors is presented and discussed.

Primary poorly differentiated (small round and non-small) sinonasal neoplasms comprise histogenetically and biologically diverse entities with overlapping morphologic features. Because of the limited initial biopsy tissue materials, differential diagnostic difficulties may arise and complicate timely management of some cases. We employed immunohistochemical and molecular marker analyses in a large cohort of these tumors to optimize their early diagnosis and classification. Fifty-two tumors of the skull base and sinonasal regions and, for comparison, 19 poorly differentiated neoplasms of other head and neck sites were analyzed by a panel of immunohistochemical markers including those of epithelial, mesenchymal, melanocytic, and neuroectodermal origin using tissue microarray. RT-PCR analysis of mRNA for EWS-FLI1 and PAX-FKHR fusion transcripts and the *hASH1* gene was performed on 24 of the 52 sinonasal

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tumors and the 19 tumors of other sites for comparison. The immunohistochemical results substantiated the phenotypic assessment and the initial diagnosis in 49 of the 52 tumors. In four instances the integrated markers and phenotypic analyses led to reclassification of three tumors and confirmed the histogenesis of a mesenchymal tumor with aberrant cytokeratin expression. Molecular analysis of the EWS-FLI1 fusion gene transcript revealed four (9.3%) of the 43 tumors to be positive; all were Ewing's sarcomas. The hASH1 gene transcript was identified in 10 (23.8%) of 42 tumors: three of six neuroblastomas, all four neuroendocrine carcinomas, and one each in sinonasal undifferentiated carcinoma, rhabdomyosarcoma, and melanoma. The PAX-FKHR fusion transcript was not detected in any tumors. We conclude that 1) an integrated morphologic and biomarker algorithm may better optimize the early diagnosis of poorly differentiated sinonasal and skull-base tumors; 2) molecular analysis may assist in future biological stratification of certain classes of these tumors; and 3) the hASH1 gene transcript is a nonspecific marker for the diagnosis of neuroblastoma.

Keywords

Sinonasal tumors; Poorly differentiated; Diagnostic profiling; 'small round cell' neoplasms; Molecular classification

Introduction

The skull base and sinonasal regions are host to a variety of poorly differentiated neoplastic entities of small round and non-small cellular composition [1–6]. The close morphologic resemblance and the overlapping cellular features of these tumors, especially on small biopsy materials, may pose a diagnostic challenge. Traditional diagnostic evaluation of these tumors typically includes histopathologic assessment and the use of a broad array of lineage-specific cellular markers by immunohistochemistry (IHC) [3,6]. Because of the importance of accurate diagnosis for early contemporary multimodality management of these patients, efforts to optimize their classification are required. In that context, the formulation of a tiered panel of carefully selected markers representing the spectrum of lineage derivation may allow for efficient and timely classification.

Recent advances in phenotypic and molecular markers in certain solid tumors, especially the identification of fusion genes, have led to the introduction of novel new factors for the diagnosis and biological classification of several tumors, including skull base and sinonasal neoplasms [7–11]. Of the latter category, only rhabdomyosarcoma and Ewing's peripheral neuroectodermal tumors (Ewing's/PNET) have been shown to exhibit specific balanced chromosomal translocations [8,12,13]. These translocations give rise to fusion gene (EWS-FL1 and the PAX-FKHR) transcripts that can be detected in tumor samples [7,8,11–22]. Using fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR) these markers can also be useful in the analysis of archival materials [11,19–22]. It has also been shown that the human achaete-scute homolog-1 (hASH1) gene transcript, a transcription factor in neural development, is highly specific for neuroblastoma [23]. Others, however, contradicted this claim [24–26]. Therefore the status of hASH1 in differentiating tumors of the sinonasal region remains uncertain.

To assess the integration and the complementarity of histogenetic and/or molecular markers in the classification and diagnosis of skull base and sinonasal neoplasms, we analyzed the phenotypic features and the immunohistochemical expression of a panel of markers and the detection of EWS- FLI1 and PAX-FKHR fusion transcripts and the hASH1 gene in a large cohort of these tumors.

Materials and Methods

Tissues

A search of the head and neck tumor bank database identified 52 tumors of skull base and sinonasal sites and 19 tumors for comparison from the head and neck sites from patients seen at The University of Texas M. D. Anderson Cancer Center between 1991 and 2006. The initial diagnoses included rhabdomyosarcoma, olfactory neuroblastoma, Ewing's sarcoma, sinonasal undifferentiated carcinomas (SNUC), neuroendocrine carcinomas, and sinonasal melanomas. Archival, formalin-fixed paraffin-embedded tissues were retrieved and processed for unstained slides for immunohistochemical analysis of various markers. All hematoxylin and eosin-stained slides of these tumors were available and re-evaluated independent of the prior diagnosis by two head and neck pathologists. All fresh specimens were stored at -80°C from the time of procurement. The fresh samples were evaluated for EWS-FLI1 and the PAX-FRHK fusion gene transcripts and the hASH1 gene expression by RT-PCR. The corresponding archival paraffin tissue from tumors with fresh transcript-positive EWS-FLI1, hASH1, and/or PAX-FRHK was also evaluated for assessing the effectiveness of the RT-PCR using paraffin-embedded tissues. Individual archival formalin-fixed paraffin-embedded tissue blocks from all cases were assembled in duplicate in two tissue microarray paraffin blocks for immunohistochemistry (IHC) analysis. The molecular analysis and IHC were performed and interpreted in a blinded fashion without knowledge of the original diagnosis and independent of each other and the subsequent histopathologic re-evaluation.

Immunohistochemistry (IHC)

Table 1 presents the markers used and the conditions for the IHC staining. Immunohistochemical markers evaluated included known lineage-specific markers (cytokeratins, S100, pan melanoma markers, desmin, and myogenin), markers of neuroendocrine differentiation in sinonasal neoplasms (chromogranin, synaptophysin and CD56), neurofilament, and the basal cell marker p63. All samples were blindly evaluated as follows: 0, negative for staining; 1+, less than 10% staining; 2+, 10 to 49% staining; and 3+, 50% or more positive tumor cells. All markers were evaluated for cytoplasmic staining except for p63 and myogenin, which were graded for nuclear expression, and CD99, for which membranous staining was also noted.

Cell Lines

The TC-71 cell line, a Ewing's sarcoma cell line containing the EWS-FLI1 translocation, the RB-SJH30 cell line, a rhabdomyosarcoma with translocation PAX3-FRHK, t(2;13), and the H720 cell line, a lung small cell carcinoma cell line that expresses hASH1, were used as positive controls for their corresponding transcripts. TC71 cells were maintained in a

modified Eagle's medium vitamin solution, combined with nonessential amino acids, L-glutamine, and 10% fetal bovine serum. The H720 cells were maintained in Dulbecco's modified Eagle's medium (DMEM-F12) combined with L-glutamine and 10% FBS. RNA from the RB-SJH30 cell line was provided by The University of Nebraska Medical Center.

RNA Extraction

Total RNA was extracted from all fresh tissue samples and cell lines grown in monolayer using a standard phenol/guanidine isothiocyanate procedure (TRIZOL reagent from Invitrogen, Inc., Carlsbad California). Briefly, 200 mg of fresh tissue frozen from each sample was homogenized in 1 ml of TRIZOL reagent, and the insoluble material was removed after centrifugation. To the clear homogenate, 0.2 ml of chloroform was added for phase separation by centrifugation. The aqueous phase was carefully transferred into an Eppendorf tube and the RNA precipitated with 0.5 ml of isopropyl alcohol. Following centrifugation, the remaining RNA was washed with 1 ml of 75% ethanol, centrifuged, briefly air-dried, and re-dissolved in 100 µl of diethylpyrocarbonate-treated water. RNA concentration was measured via optical density using the Beckman Coulter DU 60 spectrophotometer. RNA was extracted from 10-µm paraffin sections of tumor utilizing the Optimum FFPE Isolation Kit (Ambion Diagnostics, Austin, TX) per the manufacturer's instructions, and the optical density was recorded as previously described.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For each sample, 0.5 µg of total RNA was used in a final volume of 50 µl composed of nuclease-free water, 10 µl of AMV/Tfl 5× reaction buffer, 1 µl of dNTP mix, 50 pmol of both upstream and downstream primers, 2 µl of 25 mM MgSO₄, 1 µl of AMV reverse transcriptase (5 u/µl), and 1 µl of Tfl DNA polymerase (5 u/µl), using the Promega RT-PCR kit and following the manufacturer's instructions. The primer sets for the fusion gene transcripts and hASH gene are shown in Table 2. The RT-PCR protocol for EWS-FLI1, PAX-FKHR, and hASH1 amplification was one cycle at 45°C (45 minutes), one cycle at 94°C (2 minutes), 40 cycles at 94°C (30 seconds), 60°C (60 seconds), 68°C (120 seconds), and a final extension at 68°C (7 minutes). RT-PCR was performed for the EWS-FLI1 and PAX-FKHR fusion genes and the hASH1 gene using primers of previously published sequences (11, 18, 24). The PAX-FKHR primers detect PAX3 or PAX7 translocations with FKHR. Phosphoglycerate kinase (PGK), a housekeeping gene, was used to confirm adequate mRNA and RT-PCR conditions. RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide.

Results

Immunohistochemical Analysis (IHC)

The IHC staining results for sinonasal and control tumors (for comparison) are presented in Table 3 and patterns of expression for primitive tumors in Fig. 1.

Epithelial Markers

Pan-cytokeratin was strongly expressed in 12 (23%) of the 52 tumors; five of these co-expressed cytokeratin 7 and included rhabdomyosarcoma, SNUCs, and neuroendocrine

carcinomas (Table 3). Combined morphologic and phenotypic evaluation identified cytokeratin staining in all SNUCs, in five of the six neuroendocrine carcinomas, and in one each for Ewing's sarcoma and rhabdomyosarcoma (Fig. 2). The cytokeratin-negative neuroendocrine carcinoma (by original classification) expressed neuroendocrine markers and was reclassified as an olfactory neuroblastoma.

Neuroendocrine Markers

Neuroendocrine markers (chromogranin, synaptophysin, and CD56) were collectively expressed in 22 (42.3%) of the 52 tumors. Synaptophysin was positive in all 10 olfactory neuroblastomas, 4 of the 6 originally diagnosed neuroendocrine carcinomas, and 1 each of the 10 of Ewing's and rhabdomyosarcomas. Chromogranin was positive in 6 of 10 olfactory neuroblastomas, and 2 of 6 neuroendocrine carcinomas while the CD56 expression was noted in 9 of 10 olfactory neuroblastomas, 4 of 10 rhabdomyosarcomas, 4 of 6 neuroendocrine carcinomas, and 2 of the 11 sinonasal melanomas. Co-expression of 2 or 3 of these markers was found in the majority of neuroblastomas and neuroendocrine carcinomas (Table 3). A tumor originally diagnosed as neuroendocrine carcinoma was positive for keratin and neuron-specific enolase (a nonspecific marker) during initial diagnostic work-up; however, it was negative for all 3 neuroendocrine markers in this analysis and was reclassified as a SNUC. Chromogranin was the most specific ($= 1.0$) and the least sensitive ($= 0.53$) marker for neuroendocrine tumors. Synaptophysin was the most sensitive ($= 0.93$) and specific ($= 0.95$) marker for these tumors. CD56 was the least specific (specificity $= 0.84$ and sensitivity $= 0.87$) of this group of markers.

Skeletal Muscle Markers

Ten (19.2%) of the 52 tumors were positive for desmin and 8 (15.4%) co-expressed nuclear myogenin staining. The combined evaluations of the histologic and IHC results confirmed the diagnosis of rhabdomyosarcomas in 10 tumors. One tumor showed mixed epithelioid and spindled cell features and showed nuclear myogenin positivity; this tumor also manifested high keratin expression.

Melanocytic Markers

S100 was expressed in 11 (21.2%) of 52 tumors. Morphologic evaluation showed S100 expression in sinonasal melanomas (6 of 11) and showed focally dispersed positive cells in olfactory neuroblastomas (5 of 10). Pan-melanoma markers (Mart-1, HMB45, and tyrosinase cocktail) were expressed in 11 (21.2%) of 52 cases; all 11 of these cases were phenotypically sinonasal melanomas.

Miscellaneous New Markers

CD99 is a sialomucin glycoprotein adhesion molecule expressed variably in different cell types. It has been used as a marker in the diagnosis of Ewing's/PNET tumors. CD99 expression was identified in 11 (21.2%) of the 52 tumors. Phenotypic evaluation showed that 7 were Ewing's sarcomas with strong membranous and cytoplasmic staining, 3 were Ewing's sarcomas with faint cytoplasmic staining classification (2 confirmed with a positive EWS-FLI1 transcript) 1 with cytokeratin express reclassified or SNUC, and 1 was diagnosed

as SNUC. Neurofilament, a neuroectodermal marker, was expressed in 30 (57.7%) of 52 cases. These included rhabdomyosarcoma, Ewing's sarcoma, olfactory neuroblastoma, and SNUC. p63, a marker for primitive basal cells, was present in 10 (19.2%) of 52 tumors. Occasional positivity for p63 was seen in all tumor types except for all melanomas (Table 3).

Molecular Markers

a) EWS-FLI1 Transcript—The EWS-FLI1 fusion transcript was detected in 4 (9.3%) of the 43 fresh frozen tumor specimens. Integrated results revealed all four tumors to be among the 10 histologically and immunohistochemically confirmed as Ewing's/PNET (Fig. 3, Table 4). In two of these tumors, the diagnosis of Ewing's/PNET was suspected; but because of the faint CD99 expression, definitive diagnosis was not rendered. To test whether archival materials would yield similar results, additional mRNA extractions from three EWS-FLI1-positive Ewing's sarcomas with matching paraffin blocks were also analyzed for the fusion transcript. Only 1 of the 3 tumors was positive, and 2 failed to amplify the fusion gene with RNA integrity confirmed by PGK amplification. To confirm the molecular finding of the fresh tissue, FISH analysis was performed on slides from archival paraffin tissues on both tumors with positive transcript on fresh tissue but negative on archival. Positivity of the translocation was confirmed in one tumor by FISH while the second tumor failed to hybridize and was considered uninformative.

b) PAX-FKHR Transcript—A cell line control, an alveolar rhabdomyosarcoma with known PAX-FKHR translocation, was positive for the transcript. All fresh frozen tumor tissues including the eight rhabdomyosarcomas were evaluated and were negative for the transcript. Morphologic analysis did not identify the alveolar subtype among the eight rhabdomyosarcomas.

c) hASH1 Transcript—The hASH1 mRNA transcript was detected in 11 (26.2%) of 42 tumors (one tumor failed to amplify). Formalin-fixed archival tumor specimens tested for the hASH1 transcript for comparison were positive in only 1 of the 4 positive tumors from fresh materials. Integrated analysis showed that the positive tumors included 3 olfactory neuroblastomas, 4 neuroendocrine carcinomas, 1 SNUC, 1 rhabdomyosarcoma, and 1 mucosal melanoma (Fig. 4).

Summary of Integrated Analysis

The combined light microscopic re-review immunophenotyping and molecular findings led to the confirmation of the diagnosis in 49 (94.2%) of the 52 tumors, reclassification of 3 neoplasms, and the dismissal of aberrant high keratin expression by molecular analysis in 1. In the latter case the tumor was diagnosed as Ewing's sarcoma based solely on morphology, but owing to the intense expression of cytokeratin, SNUC diagnosis was entertained. Fresh tissue from the tumor was available and analysis for the EWS-FLI-1 fusion transcript and was negative. One neuroendocrine carcinoma lacking cytokeratins was morphologically consistent with and was reclassified as an olfactory neuroblastoma. Another case of neuroendocrine carcinoma was initially diagnosed based on positive staining to neuron-specific enolase; however, with the lack of reactivity to our panel of neuroendocrine

markers, the diagnosis was changed to SNUC. The case with aberrant cytokeratin expression and myogenin remained classified as a rhabdomyosarcoma.

Discussion

The results of this study indicate that the integration of histopathologic features with a panel of selected lineage-specific markers allow for early efficient diagnosis and classification of poorly differentiated neoplasms of sinonasal and skull base sites. Employing a prioritized series of markers in a logarithmic order, the diagnoses were confirmed in the majority of tumors, settled in a few disputed cases, and reclassified in others. The initial application of limited but highly specific markers led to the separation of carcinomas and melanomas from other primitive small cell tumors. A second tier of complimentary lineage-related markers supported the initial diagnosis in most tumors and confirmed the histogenesis of few of the disputed cases with aberrant expressions. Although few tumors were reclassified, the study results provide an integrative and organized approach for the application of a limited set of markers to optimize the timely diagnosis for multimodality management of these patients.

As expected, aberrant expression of certain histogenetic markers has led to differential diagnostic uncertainties. In this series, keratin expression was detected in 2 tumors originally diagnosed as Ewing's sarcoma and rhabdomyosarcoma. In the Ewing's case, the initial diagnosis was most likely based on the detection of CD99 in tumor cells [27]. The uniform and strong expression of cytokeratin and the weak expression of CD99 in this tumor led to its reclassification to a SNUC. This finding further underscore the non-specific diagnostic nature of CD99 as reported in several tumor studies and the collective use of complementary markers [1,4,28,29,30]. However, the recent finding of the role of CD99 in the biological evaluation of certain tumors indicates that further studies are needed [31]. The keratin expression in the rhabdomyosarcoma case was negated by the positive detection of multiple skeletal muscle markers [30,32].

Interestingly, the expression of the neurofilament (NF), neuronal derived marker, was observed in the majority of olfactory neuroblastoma, rhabdomyosarcomas, Ewing's sarcoma, and SNUC, as previously reported [32]. These results and those of others underscore the limited usefulness of this marker in the differential diagnosis of small round cell tumors [33–35]. In contrast to previous studies, our data show a lower incidence of S100 expression in sinonasal melanomas [1]. This discrepancy could be attributed to the use of a small core of tissue and the variable processing differences of the assembled blocks. Accordingly, we advocate concurrent use of multiple melanoma markers (tyrosinase, HMB-45, Melan-A). Similarly, although S100 may play a role in the evaluation of olfactory neuroblastomas, it is limited and must be used in conjunction with other complementary markers [36,37].

On the other hand, failure to express specific markers led to the reclassification of histogenetically diverse entities. In this study, an initially diagnosed neuroendocrine carcinoma failed to express any of the cytokeratins and was reclassified to neuroblastoma. Paradoxically, an equivocal diagnosis of neuroendocrine carcinoma based on reactivity to NSE, a marker with low specificity [34,35], was confirmed by a better and complementary

neuroendocrine marker. In contrast, an initially diagnosed neuroendocrine carcinoma was reclassified to SNUC because of the lack of reactivity to new and specific neuroendocrine markers. The diagnosis of this entity, however, should be distinguished from the morphologically similar nasopharyngeal carcinoma undifferentiated type for therapeutic purposes. The latter entity typically exhibits an integral lymphocytic component and positivity to Epstein-Barr virus. In our experience synaptophysin is a reliable marker with high sensitivity and good specificity. As an example, an instance of non-specific expression was observed in a rhabdomyosarcoma and Ewing's sarcoma [32,38]. The panel used correctly classified rhabdomyosarcoma by dual reactivity to desmin and myogenin, while the reactivity to synaptophysin in the Ewing's sarcoma was considered non-specific, especially in the lack of positivity to chromogranin.

Other entities that must be considered in the differential diagnosis of poorly differentiated sinonasal malignancies are basal cell carcinoma and lymphoproliferative disorders. The positivity for pan-keratins and CK7 along with diffuse p63 nuclear expression is helpful, along with the patient's clinical history (results not shown). Although an uncommon occurrence, clinical history of prior treated skin lesion aids in the correct interpretation of this entity. Similarly, lymphomas, also rare at this site, manifest infiltrative sheet-like growth for which CD45 immunohistochemical staining should aid in this classification. In a tumor negative for the panel of markers proposed, CD45 should be performed to evaluate this rare possibility. Since CD56 is expressed in hematopoietic pathologies, its diagnostic value in the differentiation of neuroendocrine tumors should be kept in mind [5,39–41].

Morphologic and clinical correlation may also be necessary for resolving problematic cases in which further molecular analysis may establish the diagnosis [22]. The application of molecular analysis revealed the detection of the EWS-FLI1 fusion transcript was restricted to Ewing's sarcomas and PNET. This is highlighted by the positive detection of these transcripts in two cases where the diagnosis on light microscopic evaluation and markers bases was equivocal and were found to be positive for the transcript, thus confirming the diagnosis. It is worth noting, however, that matched fresh and archival analyses showed that fresh tumor specimens are more reliable in the detection of the transcript than archival materials. Moreover, in situ hybridization may in some instances complement archival molecular analysis to verify the presence of the translocation.

The results of our PAX-FKHR fusion transcript analysis, however, were negative in all tumors, including rhabdomyosarcomas. Since all the rhabdomyosarcomas in our cohort were of the embryonal subtype, the diagnostic and biologic significance of this transcript, which is limited to the alveolar subtype, cannot be determined in this study. However, alveolar rhabdomyosarcomas may manifest a solid growth pattern, and the molecular evaluation of the PAX-FKHR translocation should be performed, since more aggressive treatment is required. The hASH1 transcript was expressed in a variety of our neural and non-neural tumors. We observed, in addition to neuroendocrine tumors, hASH1 expression in SNUC, rhabdomyosarcoma, and sinonasal melanoma. Our findings, therefore, are at variance with the study suggesting that expression of these markers is limited to neuroblastoma and contend that their utility, [24] exclusive of the IHC evaluation, is minimal.

In summary, our study indicates that a structured approach comprising IHC analysis combined with morphologic assessment allows for better evaluation of sinonasal and skull base neoplasms (Fig. 3). Using a carefully selected panel (Fig. 5) of keratin, synaptophysin, desmin, and melanoma markers is the most practical and economic approach to accurately classify these tumors [6]. Molecular markers may help substantiate the classification and diagnosis of these tumors, particularly Ewing's/PNET, for which no lineage-specific marker is known. Additionally, EWS-FLI1 and PAX-FKHR may be valuable for future triaging of patients for targeted therapy and prognostication [14,15,19,21,22].

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Fig. 1.

Histologic and immunohistochemical comparison of primitive sinonasal and skull base tumors (cytokeratin negative tumors): olfactory neuroblastoma (ONB), rhabdomyosarcoma (Rhabdo), Ewing's sarcoma (Ewings), and melanoma. A1–D1: Hematoxylin and eosin-stained sections are shown for each tumor along with markers expressed in each tumor type. A1–A3: Olfactory neuroblastoma showing diffuse expression of synaptophysin (Synp) (A2) and scattered chromogranin (Chromo) (A3). B1–B3: Rhabdomyosarcoma expressing desmin (B2) and myogenin (nuclear expression) (B3). C1–C3: Ewing's sarcoma expressing CD99 (C2) and negative for cyto-keratin (C3). D1–D3: Melanoma expressing scattered S100 (D2) and pan-melanoma (Pan-Mel) markers (Mart1, HMB45, and tyrosinase) (D3).

Fig. 2.

Discrepant immunohistochemical expression Rhabdomyosarcoma A–D: (A) Hematoxylin and eosin-stained section (H&E); (B) aberrant cytokeratin expression; (C) desmin positivity; and (D) myogenin nuclear positivity confirming diagnosis. An initial Ewing's sarcoma reclassified as SNUC E–G: (E) H&E staining, (F) keratin expression, and (G) CD99 expressing weak reactivity.

Fig. 3.

Molecular analysis for the EWS-FLI-1 fusion transcript in Ewing's sarcomas. EWS-FLI-1 transcript is present in samples 2, 3, 8, and 10. The amplified transcript of the corresponding house-keeping gene, phosphoglycerate kinase (PGK), is also shown. P= Positive control, RB-SJH30 cell line and N= Negative control, without RNA.

Fig. 4.

Molecular analysis for the hASH1 gene transcript. N = negative control without RNA; numbers 1, 6, 9, and 10 are neuroendocrine carcinomas; 2–5, 7, and 8 are olfactory neuroblastomas; P= positive control (H720 small cell carcinoma cell line); numbers 11–21 are non-neuroendocrine tumors. Tumors positive for hASH1 transcript: numbers 1, 4–10; 14 (basal cell carcinoma); 16 (rhabdomyosarcoma); 20 (melanoma); and 21 (SNUC).

Fig. 5.

Diagnostic flow chart for immunohistochemical evaluation of poorly differentiated skull-base tumors. An initial panel (A) of keratin, synaptophysin (Synap), desmin, and melanin markers allows for the classification of most neoplasms. Panel B includes confirmatory/ ancillary markers and molecular studies for rhabdomyosarcoma (Rhabdo), MyoD or Myogenin and PAX-FKHR for the alveolar type, and Ewing's Sarcoma/Peripheral Neuroectodermal tumor (EWS/PNET), CD99 and EWS-FLI1. Olfactory neuroblastoma (ONB); neuroendocrine carcinoma (NEC); squamous carcinoma (SCC); sinonasal undifferentiated carcinoma (SNUC).

Table 1**Immunohistochemistry Markers Utilized in the Tissue Microarray**

Biomarker	Clone	Dilution	Manufacturer	Retrieval	Chromogen
Pan-cytokeratin cocktail				P24 (Enzyme retrieval)	AEC
	AE1/AE3	1:50	Dako, Carpinteria, CA		
	CAM 5.2	1:50	Becton Dickinson, San Jose, CA		
	Cytokeratin MNF116	1:50	Dako, Carpinteria, CA		
	Keratin 8 and 18	1:25	Zymed, South San Francisco, CA		
CK7	OV-TL 12/30	1:100	Dako, Carpinteria, CA	P24 (Enzyme retrieval)	AEC
CD56	123 C3	1:50	Zymed, South San Francisco, CA	Citrate	AEC
Synaptophysin	SY38	1:200	Dako, Carpinteria, CA	Citrate	AEC
Chromogranin	LK 2H10	1:4000	Chemicon, Temecula, CA	Citrate	AEC
Desmin	D33	1:200	Dako, Carpinteria, CA	Citrate	AEC
Myogenin	FSD	1:125	Dako, Carpinteria, CA	TRIS	DAB
CD99	12 E7	1:50	Dako, Carpinteria, CA Santa Cruz Biotechnology, Santa	TRIS	AEC
P63	A4A	1:200	Cruz, CA	TRIS	AEC
NF	NEF	1:50	Zymed, South San Francisco, CA	Citrate	AEC
S100	A6	1:40	Biogenex, San Ramon, CA	NA	AEC
Pan-melanoma cocktail				Citrate	AEC
MART 1	AB3	1:100	Novocastra Vision, Boston, MA		
HMB45	HMB45	1:50	Dako, Carpinteria CA		
Tyrosinase	T311	1:20	Novocastra Vision, Boston, MA		

CK, cytokeratin; NA, no pretreatment; NF, neurofilament.

Table 2

Primers Used for RT-PCR to Detect Transcripts EWS-FLI-1, hASH1, and PGK

Primer	Sequence	Amplicon size
EWS- FLI1	TCCTACAGCCAAGCTCCAAGTC GTTGAGGCCAGAATTCATGTTA	186 bp/120 bp*
PAX -FKHR	CCGACAGCAGCTCTGCCTAC TGAACCTGCTGTGTAGGGACAG	160 bp/172 bp**
hASH1	GAGCAGGAGCTTCTCGACTTCA GATGCAGGTTGTGCGATCAC	103 bp
PGK	CAGTTTGGAGCTCCTGGAAG TGCAAATCCAGGGTGCAGTG	247 bp

* 186-bp translocation in exon 5 FLI1, 120-bp translocation in exon 6 FLI1.

** 160-bp PAX7; 172-bp PAX3.

Table 3

Expression of Immunohistochemical Markers in Skull Base and Sinonasal Tumors*

Tumor type	PanCK	CK7	CD56	Synapto	Chromo	Desmin	Myogenin	CD99	p63	NF	S100	PanMel
Ewing's sarcoma	0/9	0/9	0/9	1/9	0/9	0/9	0/9	9/9	4/9	9/9	0/9	0/9
Rhabdomyosarcoma	1/10	1/10	4/10	1/10	0/10	10/10	8/10	0/10	1/10	10/10	0/10	0/10
Neuroblastoma**	0/11	0/11	10/11	11/11	7/11	0/11	0/11	0/11	1/11	10/11	5/11 [^]	0/11
SNUC***	7/7	3/7	0/7	0/7	0/7	0/7	0/7	2/7	3/7	1/7	0/7	0/7
Neuroendocrine Ca [^]	4/4	1/4	3/4	3/4	1/4	0/4	0/4 ^{^^}	0/4	1/4	0/4	0/4	0/4
Melanoma	0/11	0/11	2/11	0/11	0/11	0/11	0/11 ^{^^}	0/11	0/11	0/11	6/11	11/11

* Final diagnoses based on immunohistochemical and morphologic assessment.

** Includes 1 reclassified neuroendocrine carcinoma without cytokeratin.

*** Includes 2 reclassified tumors, previously classified as neuroendocrine carcinoma (lacking neuroendocrine markers) and Ewing's sarcoma (diffuse strong keratin).

[^] Six tumors originally classified as neuroendocrine carcinoma, one reclassified as neuroblastoma and one to SNUC. Ca, carcinoma; Chromo, chromogranin; CK, cytokeratin; NB, neuroblastoma; NF, neurofilament; PanCK, panel of cytokeratins; PanMel, panel of melanoma markers (tyrosinase, HMB45, and Mart1); SNUC, Sinonasal undifferentiated carcinoma; Synapto, synaptophysin.[^] Sustentacular cells;^{^^} cytoplasmic staining noted. Bolded results highlight diagnostic markers for each neoplasm.

Table 4
RT-PCR Transcript Analysis of Fresh Tissues for EWS-FLI1 and hASH1 Genes in Skull Base and Sinonasal Tumors*

Transcript	Ewing's	ONB	NEC	RMS	Melanoma	SNUC	Total
EWS-FLI1	4/10	0/6	0/4	0/8	0/8	0/7	43
hASH1	0/9	4/6	4/4	1/8	1/8	1/7	42

* Results following the reclassification of 2 tumors based on cytomorphologic and immunohistochemical review (1 Ewing's sarcoma to an SNUC and 1 neuroendocrine carcinoma to an olfactory neuroblastoma). Ewing's, Ewing's sarcoma/primitive neuroectodermal tumors; ONB, olfactory neuroblastoma; NEC, neuroendocrine carcinoma; RMS, rhabdomyosarcoma; SNUC, Sinonasal undifferentiated carcinoma.