

RECEPTOR TYROSINE KINASES IN SINONASAL UNDIFFERENTIATED CARCINOMAS—EVALUATION FOR EGFR, c-KIT, AND HER2/neu EXPRESSION

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Abstract: *Background.* Our objective was to identify the expression of epidermal growth factor receptor (EGFR), c-KIT (CD117), and HER2/neu in sinonasal undifferentiated carcinoma (SNUC).

Methods. Immunohistochemistry for c-KIT (CD117), EGFR, and HER2/neu was performed on paraffin-embedded tissue from SNUC cases. A search for activating mutations in *c-kit* exons 9, 11, 13, and 17 or gene amplification was undertaken by high-resolution DNA melting curve analysis and fluorescence in situ hybridization (FISH) for *c-kit* and chromosome 4, respectively.

Results. By immunohistochemistry, 9 of 11 cases (81.8%) were diffusely (4+) positive for c-KIT, 3 of 11 cases (27.3%) were positive for EGFR, and none of the cases were positive for HER2/neu. Neither activating mutations nor gene amplification of *c-kit* were detected in any of the 8 assessable tumors.

Conclusion. c-KIT is frequently expressed in SNUC. However, the overexpression is not due to activating mutations or gene amplification. © 2009 Wiley Periodicals, Inc. *Head Neck* 31: 919–927, 2009

Keywords: sinonasal undifferentiated carcinoma; c-KIT; HER2/neu; epidermal growth factor receptor; activating mutations

Sinonasal undifferentiated carcinoma (SNUC) is a rare tumor with less than 100 cases reported.¹ Histologically, it is composed of nests, trabeculae, and sheets of cells that have medium to large nuclei with prominent nucleoli, small amounts of eosinophilic cytoplasm, high mitotic and apoptotic indices, and often prominent lymphatic invasion. Squamous, glandular, or other forms of differentiation are absent (see Figure 1).

SNUCs arise in the nasal cavity and paranasal sinuses and are usually locally advanced at presentation. Presenting symptoms include nasal obstruction, epistaxis, proptosis, visual disturbances, and pain, with the duration of symptoms typically being short (weeks to months).^{1,2} Despite multimodality therapy, including surgical resection, chemotherapy, and radiation, the prognosis for patients with SNUC is poor. It is almost uniformly fatal, with many patients dying within a year of diagnosis.^{3–6}

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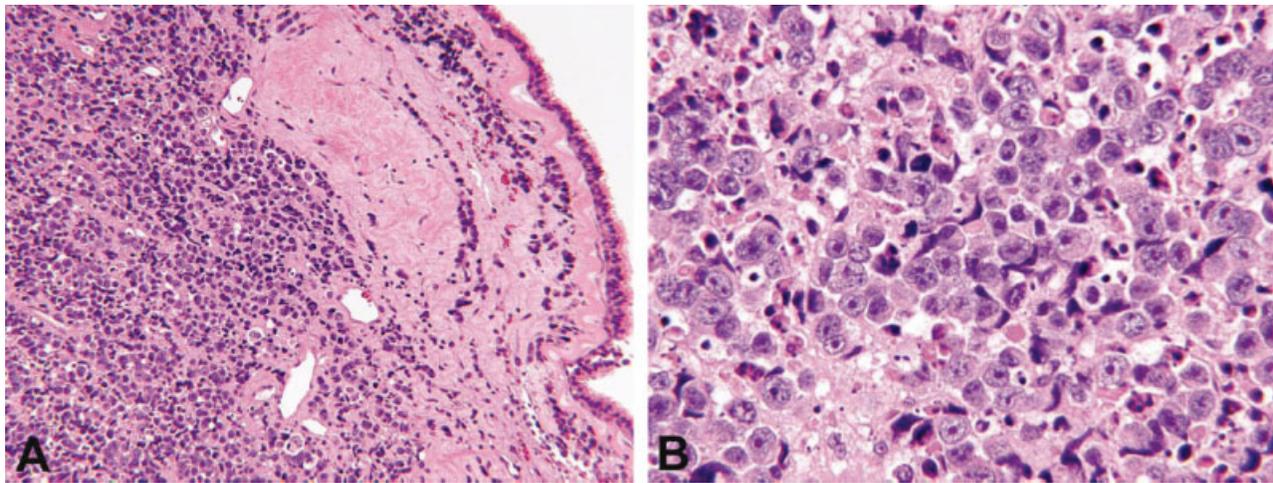


FIGURE 1. Histologic features of SNUC (hematoxylin–eosin stained sections). **(A)** Low-power image (original magnification $\times 100$) showing sheets of cells beneath respiratory epithelium. **(B)** High-power image (original magnification $\times 400$) of tumor cells with medium- to large-sized nuclei, prominent nucleoli, small amounts of eosinophilic cytoplasm, and numerous pyknotic (apoptotic) nuclei.

Given the limited success of current therapeutic options, patients with SNUC could benefit greatly from additional treatment types.

Transmembrane tyrosine kinase (TK) receptors play key roles in tumorigenic pathways involving growth, adhesion, and migration.⁷ Overexpression of TK receptors occurs in a variety of human tumors including epidermal growth factor receptor (EGFR) in lung and colon carcinomas, HER2/neu (a member of the EGFR family) in breast carcinomas, and c-KIT in most gastrointestinal stromal tumors (GISTs).^{8–12} Moreover, the expression of these TKs is of great interest to oncologists, given the growing availability of specific pharmacologic inhibitors. Anti-TK receptor therapies targeting EGFR (cetuximab/Erbitux), HER2/neu (trastuzumab/Herceptin), and c-KIT (imatinib/Gleevec) are already in common use for several malignancies. Furthermore, specific activating mutations in the *c-kit* gene have been shown to correlate with treatment response to imatinib.^{13,14} However, these TKs have not been examined in SNUC, either at the gene or protein levels. Our aim was to evaluate the expression of EGFR, c-KIT (CD117), and HER2/neu in SNUC as potential therapeutic targets.

PATIENTS AND METHODS

Case Selection. The study was approved by the Human Studies Committee of Washington Uni-

versity. The surgical pathology files at Barnes-Jewish Hospital/Washington University were searched for patients diagnosed with SNUC between 1988 and 2006. Hematoxylin–eosin (H&E)-stained slides from primary tumors that had available corresponding paraffin-embedded tissue were retrieved and reviewed by 2 of the study pathologists (R.D.C. and J.S.L.). The diagnosis of SNUC was based on the WHO classification of head and neck tumors and, as such, was confirmed by the presence of the following histologic features: nests, trabeculae, and/or sheets of cells with medium to large sized nuclei, prominent nucleoli and small amounts of eosinophilic cytoplasm, as well as frequent apoptotic and/or mitotic figures.¹ Cases with any histologic evidence of squamous, glandular, adenoid cystic, or other forms of differentiation were excluded. Immunohistochemistry had been performed on all tumors as part of their routine work up at the time of diagnosis. The results were reviewed for consistency with the diagnosis of SNUC. Cytokeratin (AE1/AE3) staining was required and was present in all cases. Neuron-specific enolase positivity plus the absence of staining with melanocytic markers (HMB-45 or Melan-A), hematopoietic markers (CD45), and muscle markers (myoglobin) was consistent with SNUC. Some of the tumors showed focal, weak S-100 positivity. The neuroendocrine markers synaptophysin and chromogranin-A were evaluated in all cases and were negative in all except 2, both of which showed only weak, focal staining.

Immunohistochemistry. Immunohistochemistry was performed on representative 4- μ m sections cut from formalin-fixed, paraffin-embedded tissue blocks using a polyclonal rabbit antibody to c-KIT (Dako, Carpinteria, CA; 1:200 dilution), a prediluted monoclonal rabbit antibody to HER2/neu (4B5) (Ventana Medical Systems, Tucson, AZ), or a monoclonal mouse antibody to EGFR (Zymed Laboratories, San Francisco, CA; 1:80 dilution). The HER2/neu and c-KIT staining was performed on a Ventana Benchmark automated immunostainer (Ventana Medical Systems) according to standard protocols. EGFR staining was performed on a Dako automated immunostainer (Dako), also according to standard protocols. The binding of primary antibodies was assessed by the LSAB plus Dako detection kit for EGFR. Appropriate positive and negative controls were included. Staining was scored as follows: 0 = no staining; 1+ = less than 5% of cells staining; 2+ = 5% to 25%; 3+ = 25% to 50%; 4+ = 50% to 100%. The staining was graded separately by 2 study pathologists (R.D.C. and J.S.L.) and discrepancies were resolved by joint review.

c-kit Mutation Analysis and Sequencing. Unstained slides were cut from the same paraffin blocks used for immunohistochemistry and mounted in 5- μ m sections on Superfrost slides. The area of tumor on the H&E slides was identified on the unstained slide and circled with an indelible fine tip pen. The unstained slides were deparaffinized and rehydrated by successive washes first in xylene, and then in 95%, 70%, 50%, and 30% ethanol. After final rinsing in deionized H₂O, the slides were dried under an infrared lamp (Fisher Scientific, Pittsburgh, PA) for 5 minutes. The appropriate area of tumor tissue was microdissected off the slides with a scalpel and incubated in 50 to 100 μ L of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Tween 20, and 1.0 mg/mL proteinase K overnight at 37°C. The following morning, the samples were incubated in a boiling H₂O (95°C in Salt Lake City) bath for 10 minutes to inactivate the proteinase K. After cooling on ice, the samples were diluted in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. Microdissection was not done in 1 case for which a tissue block was not available. An archived roll of paraffin tissue cut from the original block was used instead.

The samples were then subjected to polymerase chain reaction (PCR) with appropriate *c-kit*

exon 9, 11, 13, or 17 primers as previously described.¹⁵ PCR was performed in a total volume of 20 μ L in a Light Cycler capillary cuvette. The reaction mixture contained a 50 mmol/L concentration of Tris hydrochloride (pH 8.5), a 3 mmol/L concentration of magnesium chloride, 0.5 mg/mL of bovine serum albumin, a 200 μ mol/L concentration each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate, a 600 μ mol/L concentration of deoxyuridine triphosphate, a 0.5 μ mol/L concentration of primers, 1 μ L of diluted KlenTaq polymerase (1 μ L of cold-sensitive KlenTaq polymerase incubated with 10 μ L of enzyme diluent), 1 U of uracil *N*-glycosylase (Amperase), and the dye LCGreen I (1 \times). PCR was performed on a light cycler (Roche Diagnostics) with an initial denaturation for 10 minutes at 95°C (to denature the uracil glycosylase), followed by 45 cycles that consisted of a denaturation step at 95°C for 3 seconds, a temperature transition rate of 20°C/s to an annealing step at 58°C (exons 9 and 11), 62°C (exon 13), or 55°C (exon 17) for 10 seconds, and then a temperature transition rate of 1°C/s to 75°C for 0 seconds during which elongation occurs. Genomic DNA was used as a control. All samples were run in triplicate.

The PCR products were heated momentarily to 95°C and then cooled to 40°C. The samples were then transferred to the HR-1, a high-resolution DNA melting analysis instrument (Idaho Technology). Melting curve analysis was performed as previously described.¹⁶

For DNA sequencing, 100 μ L of deionized water was added to the appropriate PCR-amplified exon (20 μ L). For 3 μ L of the diluted amplified exon, the forward or reverse primer (3.2 pmol in 4 μ L) was added. The sequence for the forward and reverse strands was obtained from the DNA sequencing core facility at the University of Utah. Analysis of the sequence was performed with the use of DNA Sequencer 4.1.4 software (Gene Codes, Ann Arbor, MI).

Fluorescence In Situ Hybridization. Dual-color fluorescence in situ hybridization (FISH) experiments were performed as previously reported.^{17,18} Thin sections (5–6 μ m) from the same formalin-fixed, paraffin-embedded blocks used for PCR-based testing and immunohistochemistry were mounted onto poly-L-lysine-coated slides. Normal brain was used as a negative control and a glioblastoma with *c-kit*

Table 1. Clinical and treatment characteristics.

Case no.	Age, y	Sex	Tumor status	Stage	Treatment	Patient status	Survival, mo
1	70	M	Primary	T4N0M0 (IVB)	Chemo/Rad + Salvage surgery	AWD	63
2	83	M	Primary	T4N1M0 (IVA)	Surgery + Chemo/Rad	DWD	10.5
3	67	F	Primary	T4N0M0 (IVB)	Chemo/Rad	AWD	34
4	78	F	Primary	T4N0M0 (IVB)	Unresectable*	DWD	3
5	35	M	Recurrent	Unknown	Chemo/Rad + Salvage surgery	DWD	48.5
6	51	M	Primary	T4N0M0 (IVA)	Surgery (x3) + Chemo/Rad	DWD	58
7	33	M	Recurrent	T4N0M0 (IVA)	Salvage surgery + Chemo	DWD	11
8	52	F	Primary	T3N0M0 (III)	Surgery + Chemo/Rad	FOD	7
9	42	M	Primary	T4N0M0 (IVB)	Surgery [†] + Chemo/Rad	DWD	44.5
10	57	M	Recurrent	T4N0M0 (IVA)	Chemo/Rad	DWD	13
11	48	M	Primary	T4N0M0 (IVA)	Rad	DWD	14

Abbreviations: Chemo, chemotherapy; Rad, radiation; AWD, alive with disease; DWD, dead with disease; FOD, free of disease.

*Adjuvant therapy unknown.

[†]Incomplete resection.

amplification was used as a positive control. After deparaffinization, the sections were subjected to “antigen retrieval” using “steam cooking” in citrate buffer for 20 minutes, followed by a 20-minute cool-down period and a 5-minute wash with distilled water. After pepsin digestion at 37°C for 30 minutes and a subsequent wash in 2× SSC, the slides were allowed to air dry. Paired commercial Spectrum Orange centromere-enumerating probe 4 (CEP4; Vysis, Downers Grove, IL) and home-brew FITC-labeled c-kit (Human BAC library CTD-2027N10) were used. Dual-target hybridizations using both probes were performed in DenHyB buffer (In Situ Laboratories, Albuquerque, NM) at a dilution of 1:12. The hybridization mix (10 μL per slide) was applied to the sections, followed by simultaneous denaturing of probe and target at 90°C for 13 minutes. Hybridization was performed overnight at 37°C in a humidified chamber. Posthybridization washes in 50% formamide/1× SSC (5 minutes) and 2× SSC (5 minutes) were performed at room temperature, and the slides were again allowed to air dry. Nuclei were then counterstained with 4′6-diamidino-2-phenylindole-2 HCl (0.5 μL/mL), and the sections were viewed under an Olympus BX60 fluorescent microscope with appropriate filters (Olympus, Melville, NY).

For each hybridization, 100 nonoverlapping nuclei were assessed for numbers of orange and green signals. An interpretation of monosomy was made when >50% of the nuclei harbored 1 orange signal. Polysomy or gains were defined by the presence of at least 10% of the nuclei with >2 CEP4 signals. High-level polysomy was defined by the presence of at least 40% of the

nuclei with ≥4 CEP4 signals.¹⁹ Gene amplification was defined by a *c-kit*/CEP4 signal ratio of >2.

RESULTS

A total of 11 cases of SNUC were retrieved from the pathology department files. Clinical and treatment characteristics are described in Table 1. The age range of the patients was broad (33–83, mean of 56 years) and there was a male preponderance (M:F of 8:3). The majority of the patients were seen with stage IV disease. Overall survival was poor with most patients dying with disease within 4 years despite multimodality therapy in most cases. Table 2 summarizes the immunostaining results. Nine of the 11 cases (81.8%) were diffusely (4+) and strongly positive for c-KIT with a membranous pattern (see Figure 2). In contrast, the c-KIT staining

Table 2. Results of immunohistochemistry.

Case no.	c-KIT	EGFR	HER2/neu
1	4+, strong	0	0
2	4+, strong	0	1+, weak
3	4+, strong	0	2+, weak
4	4+, strong	2+, weak	0
5	4+, strong	2+, weak	0
6	4+, strong	0	0
7	4+, strong	0	0
8	4+, strong	0	0
9	4+, strong	0	0
10	0	0	0
11	0	4+, strong	2+, weak

Notes: 0 = no staining; 1+ = staining in less than 5% of the cells; 2+ = staining in 5% to 25% of the cells; 3+ = staining in 25% to 50% of the cells; 4+ = staining in 50% to 100% of the cells.

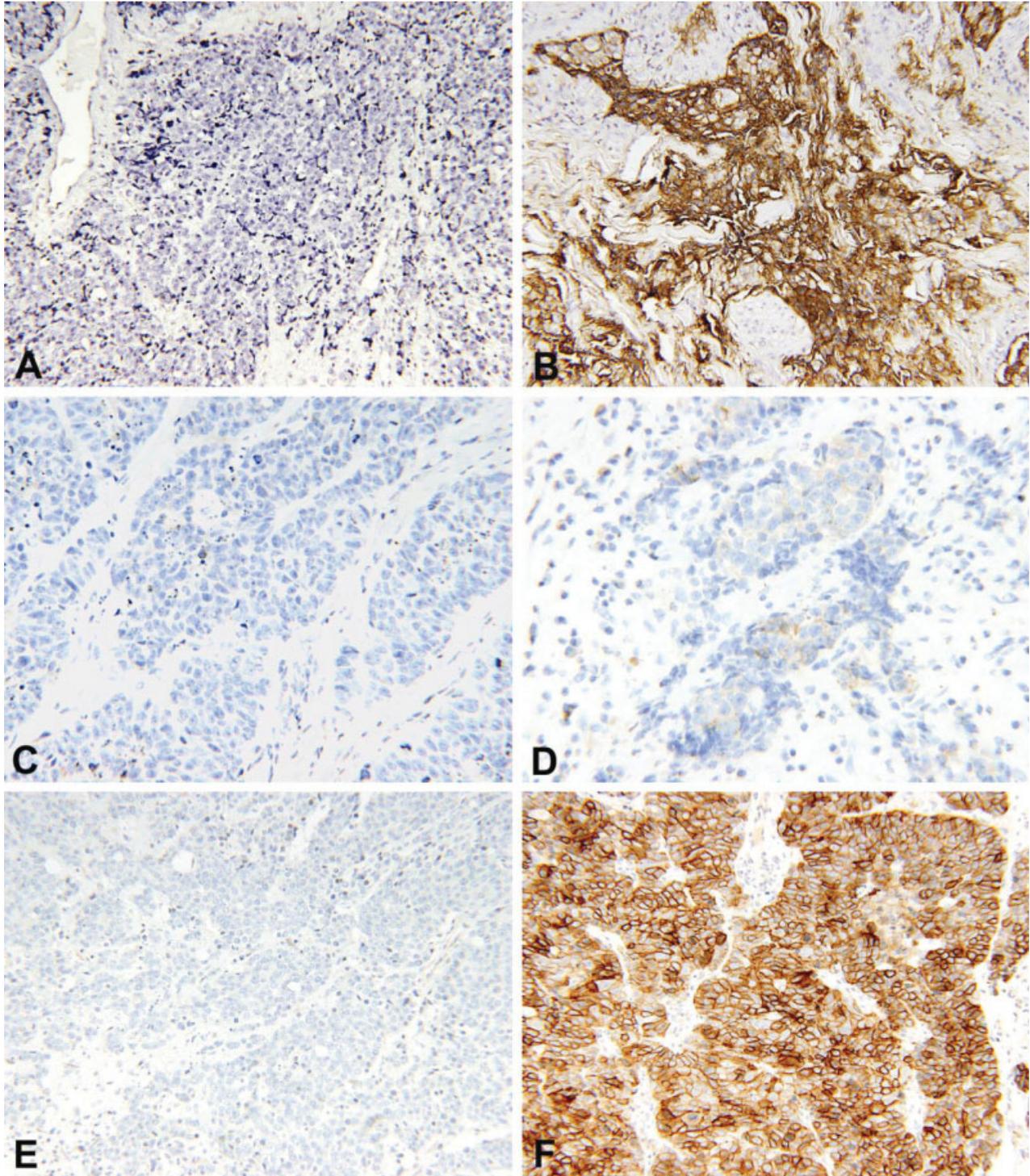


FIGURE 2. Examples of immunohistochemistry results: (A) Negative EGFR staining (original magnification $\times 100$); (B) Strong and diffuse (4+) EGFR positivity (original magnification $\times 100$); (C) Negative HER2/neu staining (original magnification $\times 200$); (D) Weak, focal (2+) HER2/neu staining (original magnification $\times 200$); (E) Negative c-KIT staining (original magnification $\times 100$); (F) Strong, diffuse (4+), membranous c-KIT positivity (original magnification $\times 200$).

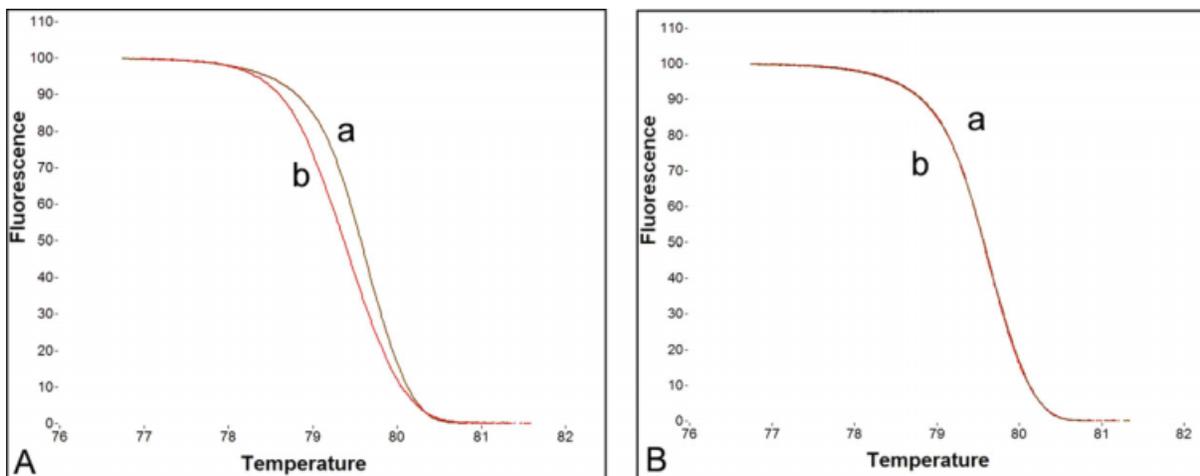


FIGURE 3. *c-kit* exon 17 high-resolution melting amplicon analysis. **(A)** *c-kit* exon 17 polymorphism in 1 tumor. The DNA melting curve from the tumor (b) is distinctly different than normal genomic DNA (a). The abnormal curve indicates a *c-kit* exon 17 abnormality, but direct DNA sequencing showed that this was due to a nucleotide polymorphism (I981I) and not an activating mutation. **(B)** *c-kit* exon 17 mutation negative tumor. The DNA melting curve from the tumor (b) is identical to normal genomic DNA (a) and the melting curves are superimposable.

pattern in gastrointestinal stromal tumors (GISTs) is usually strong and pancytoplasmic, although membranous staining may be seen in epithelioid GISTs. A perinuclear dot-like staining pattern may also be seen in some GISTs. Only 3 of the 11 cases (27.3%) were positive for EGFR with a membranous and cytoplasmic pattern (Figure 2). According to Dako EGFR pharmDx interpretation guide (<http://www.dako.com/>), any membranous staining is considered positive. However, of the 3 cases, only 1 was strongly and diffusely (4+) positive. The other 2 showed focal staining (2+ or 5% to 25% of cells). Three cases showed weak, focal (2+ in 2 cases and 1+ in 1 case) HER2/neu staining with an incomplete membranous and cytoplasmic pattern (see Figure 2). According to the FDA guidelines for scoring HER2/neu immunohistochemistry in breast carcinoma, the staining observed in the 3 cases would correspond to a score of 1+, which is considered negative, or not indicative of HER2/neu amplification.²⁰

Outcome (in months) was analyzed according to c-KIT expression using Pearson's product correlation. There was no statistically significant association between patient status and c-KIT expression in this small cohort (Pearson's coefficient = .249; $p = .48$).

Amplifiable DNA was obtained from 8 of the 11 cases. No mutations were detected in *c-kit* gene exons 9, 11, 13, or 17. An abnormality was found in exon 17 in case 11 by melting curve

analysis (see Figure 3). However, sequencing of the exon showed the base change to represent a polymorphism (I981I [ATC→ATT]).

Eight of the 11 tumors were informative for fluorescence in situ hybridization (FISH) analysis. The 3 noninformative tumors were the same 3 from which amplifiable DNA for mutation analysis could not be obtained. Polysomy 4 was detected by FISH in 4 of 8 tumors with high-level polysomy in 2 cases (see Figure 4). However, true gene amplification was not detected in any of the cases.

DISCUSSION

Expression of c-KIT is virtually a defining feature of GISTs, observed in 95% of tumors. In approximately 60% to 70% of GISTs, expression of c-KIT is due to activating mutations in the *c-kit* gene.^{13,21–23} Activating mutations are most frequently observed in exon 11 and include in-frame deletions, point mutations, and internal tandem duplications. Mutations in exons 9, 13, and 17 have also been reported but are rare.^{14,23,24} The presence and type of activating mutations has been shown to correlate with tumor response to specific TK inhibitors.^{25,26} For example, exon 11 mutations predict the best response to imatinib in GISTs.^{13,14}

A subset of GISTs that lack *c-kit* activating mutations and show reduced or absent c-KIT reactivity by immunohistochemistry have more

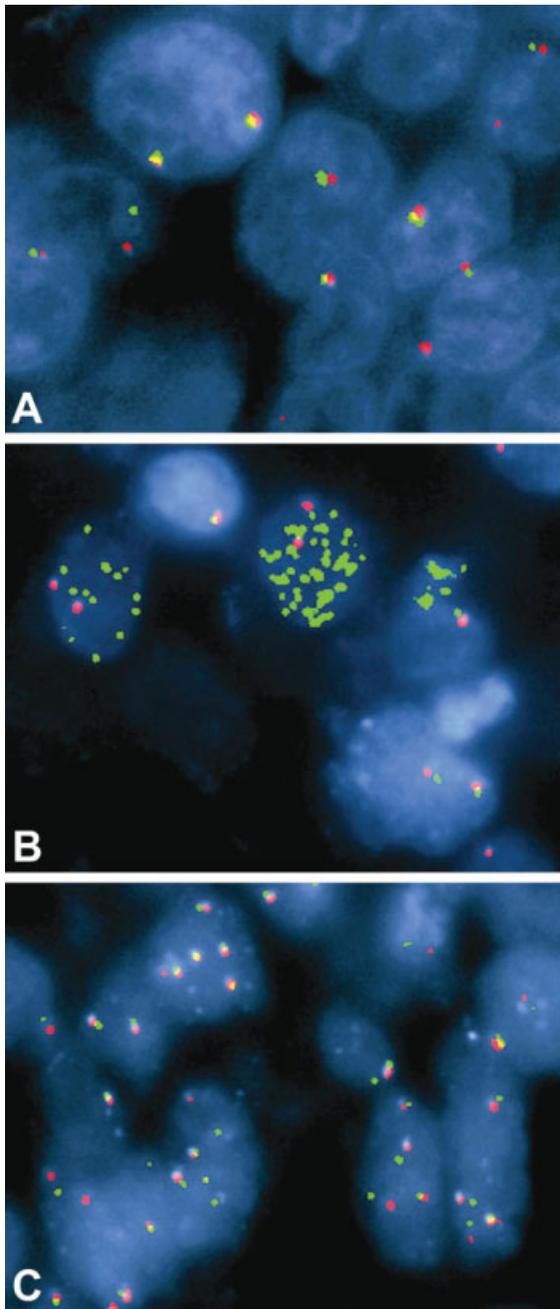


FIGURE 4. Results of FISH performed with Spectrum Orange centromere-enumerating probe 4 and FITC-labeled *c-kit* (original magnification $\times 1000$). (A) Negative control, no amplification. (B) Positive control, *c-kit* amplification. (C) Polysomy 4 in a SNUC.

recently been shown to harbor activating mutations in the platelet-derived growth factor receptor alpha (*PDGFRA*) gene instead. *PDGFRA* is a receptor TK that is highly homologous to *c-KIT* and activates many of the same downstream signaling pathways.²⁷ However, based on in vitro studies, the majority of

PDGFRA mutation-driven tumors are likely to be imatinib resistant.²⁸

In attempts to identify other tumor types that would benefit from similar inhibitor treatment, a variety of malignancies have been evaluated for *c-KIT* expression by immunohistochemistry. Tumors that are frequently *c-KIT* positive include adenoid cystic carcinoma (ACC),²⁹ seminoma,^{30–32} mast cell neoplasms,³³ acute myeloid leukemia,³⁴ chromophobe renal cell carcinoma, renal oncocytoma,³⁵ and pulmonary small cell and large cell neuroendocrine carcinomas,^{32,36} among others. Of these tumors, activating mutations have been found in seminomas, acute myeloid leukemias, mastocytosis, and sinonasal type NK/T-cell lymphomas, but they are uncommon in the other tumor types.^{37,38} *c-KIT* positivity has also been variably observed in malignant melanoma.³⁹ However, activating mutations have been reported in only about 2% of melanomas.⁴⁰

To date, *c-KIT* expression has not been investigated in SNUC. In our study, we demonstrate frequent, diffuse and strong, membranous *c-KIT* positivity. In contrast, we find that overexpression of *EGFR* is uncommon and that overexpression of *HER2-neu* does not occur.

Because of the frequency of *c-KIT* immunoreactivity, the mechanism of *c-KIT* overexpression was further explored. Activating mutations, specifically those characteristic of GISTs, were not detected. Given this finding, gene amplification was investigated as an alternative explanation for the *c-KIT* overexpression. *c-kit* gene amplification has been reported in glioblastomas and medulloblastomas, as well as in rare GISTs. However, gene amplification was not identified in any of the cases of SNUC. Polysomy of chromosome 4 was seen in 50% of the amplifiable cases. Although information regarding the molecular genetics of SNUC is lacking, this finding is consistent with a previous study that showed chromosomal abnormalities including near triploidy in a subset of SNUCs.⁴¹

Alternate mechanisms of *c-KIT* overexpression are not well characterized in other tumor types that overexpress *c-KIT* but lack activating mutations. Disruption of signaling pathways or loss of transcription factors are just a couple of mechanisms which could theoretically account for *c-KIT* overexpression in these tumors as well as in SNUC. Positive regulators of *c-KIT* expression, such as bone morphogenic protein-4 (BMP-4) and transforming growth factor- β 1 (TGF- β 1),

have been found in neural crest cells and hematopoietic cells.^{42,43} It is possible that increased expression of these proteins may secondarily increase the expression of c-KIT in tumors, although this has not been explored.

Given the lack of activating mutations, the likelihood of clinically significant responses to inhibitors in SNUC is unclear. Several small clinical trials have investigated tumor response to TK inhibitors in other tumor types that express c-KIT but lack activating mutations. These have had mixed results but have not been terribly promising overall. For example, neither clinical response nor stabilization of disease was observed with imatinib treatment in c-KIT-positive metastatic small cell lung carcinoma.^{44,45} Similarly, a single-arm, phase II clinical trial failed to detect clinical response in 15 patients with unresectable or metastatic ACC.⁴⁶ Lin et al also observed rapid progression of metastatic, c-KIT-positive ACC in 3 of 5 patients treated with imatinib.⁴⁷ On the other hand, in another phase II clinical trial of patients with recurrent or metastatic ACC and radiologic evidence of tumor progression, stabilization of disease occurred in 3 of 6 patients and a partial response was observed in 1 patient.⁴⁸ Clinical response to imatinib was also reported by Alcedo et al in 2 patients with metastatic c-KIT-positive ACC.⁴⁹ Response to imatinib has not been extensively explored in other c-KIT-positive, mutation-negative tumors in order to know whether there may be a therapeutic response.

CONCLUSION

Although c-KIT is frequently expressed in SNUC, the overexpression is not due to activating mutations or gene amplification. c-KIT overexpression without activating mutations in the *c-kit* gene is found in many other tumor types, although the mechanism of overexpression is not well understood. A few, limited clinical trials have shown mixed responses to imatinib in some of these tumors lacking activating mutations. Overall, the results from these trials are not promising. However, given the poor prognosis and limited success of current treatment options in SNUC, a clinical trial of imatinib may be warranted.

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