Clinical and Molecular Evidence for c-kit Receptor as a Therapeutic Target in Neuroblastic Tumors

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ABSTRACT

Purpose: Clinicobiological characteristics of neuroblastic tumor (NT) expressing c-kit tyrosine kinase receptor and/or its ligand, stem cell factor (SCF), are debated. This study aimed at investigating the clinicobiological features of primary NTs expressing c-kit and/or SCF in order to define the clinical relevance of selective therapeutic targeting.

Experimental Design: c-Kit and SCF expression was studied in 168 NTs using immunohistochemistry and in 106 of 168 using Northern blot. Quantitative determination of c-kit expression in 54 additional NTs was also done using real-time reverse transcription-PCR. Correlations between c-kit and SCF expression and clinicobiological features were analyzed using χ² test, univariate, and multivariate regression analyses.

Results: c-Kit protein was detected in 21 of 168 NTs (13%) and its mRNA in 23 of 106 NTs (22%). SCF protein was shown in 30 of 106 NTs (28%) and its mRNA in 33 of 106 NTs (31%). No mutations in exon 11 of c-kit gene were identified. By univariate analysis, c-kit and SCF expression correlated with advanced stage, MYCN amplification, and 1p36 allelic loss. Cox simple regression analysis showed that overall survival probability was 17% in the c-kit–positive subset versus 68% in the negative (P < 0.001), 43% in the SCF-positive subset versus 78% in the negative (P < 0.001). When using real-time reverse transcription-PCR, significant levels of c-kit mRNA were found in 35 of 54 NTs (65%), but the correlations with clinicobiological features were no longer documented.

Conclusions: c-Kit expression can be detected in the majority of primary NTs. High levels of expression are preferentially found in tumors with unfavorable clinicobiological variables. c-Kit may represent a useful therapeutic target in a subset of otherwise untreatable NTs.

INTRODUCTION

Peripheral neuroblastic tumor (NT), defined as embryonal tumor of the sympathetic nervous system deriving from neural crest precursors, is the most common extracranial childhood solid malignancy. NTs are very heterogeneous in terms of biological and clinical characteristics. In a subset of NTs, the spontaneous regression or maturation of neoplastic cells is widely documented but incompletely understood (1). Maturation of neoplastic cells is at least partially regulated by signals through neurotrophins and their receptors (2–4). In contrast, other NTs are highly malignant and aggressive, and poorly responsive to current treatments. The main unfavorable prognostic factors are age >1 year at diagnosis, advanced clinical stage, and genotypic alterations such as MYCN amplification, loss of heterozygosity (LOH) for the chromosomal region 1p36 (1p36 LOH), gain of the long arm of chromosome 17, and diploid/tetraploid cellular DNA content (5–8).

Recently, better understanding of growth factor/receptor signaling pathways in human cancers and development of small molecules able to selectively block the activity of protein tyrosine kinase (PTK) receptors has raised the opportunity of investigating the expression of these receptors in human tumors and its possible relation to their outcome. Among these kinases, c-kit encodes a transmembrane receptor (CD117) endowed with a tyrosine kinase component. The ligand for c-kit is the stem cell factor (SCF) and the SCF/c-kit pathway is important for the control of hematopoiesis, gametogenesis, and melanogenesis (9). Constitutive activation of c-kit by gain-of-function somatic mutations has been shown in a number of human malignancies, including gastrointestinal stromal tumors (GIST; ref. 10), mastocytosis (11), acute myelogenous leukemia (12), and testicular seminoma (13). In addition, paracrine and/or autocrine
activation of c-kit has been shown to occur during transformation and progression in several other malignancies including small cell lung cancer (14), neuroblastoma (NB; ref. 15), colorectal cancer (16), Ewing’s family tumors (17), and ovarian cancer (18) among others. Furthermore, in GISTs, a correlation among malignancy, disease recurrence, shortened survival, and gain-of-function mutations mainly in exon 11 of c-kit gene has also been reported (19–21).

The role of c-kit and SCF in relationship to the clinical characteristics and outcome of NTs is still very controversial. Some reports showed no significant correlation between SCF/c-kit expression and clinical characteristics or outcome (15, 22), whereas we reported that c-kit and SCF proteins are preferentially expressed in NB tumors amplified for MYCN (23). Other authors subsequently identified c-kit expression to be associated with favorable clinical characteristics and outcome (24). We also show that c-kit and SCF signaling is active in promoting in vitro NB cell line proliferation that can be selectively inhibited by treatment with imatinib mesylate (STI-571, Gleevec; ref. 23). Similarly, it has also been reported that imatinib mesylate inhibits in vivo tumor growth of c-kit/PDGFR-positive NB xenografts (25).

In targeted cancer therapeutics, the significance of immunohistochemical analysis of human malignancies has expanded to also provide information on treatment eligibility. Therefore, it is critical to validate immunohistochemical findings by comparing them to those obtained by quantitative molecular analyses. This prompted us to investigate extensively and using multiple detection techniques the frequency and clinicobiological features of primary NTs expressing c-kit and/or SCF, which may benefit by treatment with selective tyrosine kinase inhibitors.

**MATERIAL AND METHODS**

**Patients.** Tumor samples from primary site were obtained by surgery from 168 children with previously untreated NT admitted between 1979 and 1999 to the Department of Pediatrics at the University La Sapienza, the Division of Oncology at Bambino Gesù Children’s Hospital and the Division of Oncology at RLC NHS Trust Alder Hey. No selection criteria were applied except for the availability of adequate tumor tissue for the analyses.

Patients were 95 males and 73 females ages from 1 day to 180 months (median, 28). Primary site was adrenal in 73 patients, abdominal nonadrenal in 54, thoracic in 36, and cervical in 5. Tumors were classified according to the International Neuroblastoma Pathology Classification (INPC; ref. 26): 127 (76%) as NB, 29 (17%) as ganglioneuroblastoma (GNB), and 12 (7%) as ganglioneuroma (GN; Table 1). The final clinicopathologic diagnosis fulfilled the International Criteria for Neuroblastoma Diagnosis (27). The 156 patients with NB or GNB were staged according to the International Neuroblastoma Staging System: 22 patients were at stage I, 31 at stage II, 29 at stage III, 57 at stage IV, and 17 at stage IVS. Treatment after biopsy was modulated on conventional clinical variables such as stage and age at diagnosis, regardless of molecular features such as MYCN amplification or del 1p36.3. Briefly, in patients at stage I or II, the tumor was surgically excised followed by adjuvant chemotherapy only in a minority of cases. Patients with stage III (unresectable primary) were given neoadjuvant chemotherapy to reduce the tumor bulk, followed by surgery and adjuvant chemotherapy. Patients with stage IV received similar treatment to stage III but, in addition, some of them underwent autologous stem cell rescue after achieving complete remission. Stage IVS patients remained untreated unless life-threatening symptoms and/or diffuse disease progression occurred.

As of January 2003, the median follow-up for the 156 patients with NB or GBN was 56 months (range, 1 day to 272 months). In 88 patients, either disease-free (n = 83) or alive with disease (n = 5) at that time, a median follow-up of 84 months (range, 4-272 months) was observed, with all but four children having been followed for >18 months. In 68 patients, either dead of complications (n = 3) or dead of disease (n = 65), a median follow-up of 14 months (range, 1 day to 120 months) was recorded.

An additional series of 54 NTs (NBs or GNB) were obtained from the Italian Childhood Solid Tumor Tissue Bank at National Cancer Institute (IST) in Genoa and solely analyzed by real-time reverse transcription-PCR (RT-PCR) analysis. They were staged according to the International Neuroblastoma Staging System as follows: 9 patients were at stage I, 10 at stage II, 9 at stage III, 22 at stage IV, and 4 at stage IVS. MYCN amplification was assessed at IST (S.C. and G.P.T.) by using double-color fluorescence in situ hybridization on interphase nuclei and was shown in 9 of 54 tumors (17%). Two patients were lost to follow-up. Of the remaining 52 patients, 32 were disease-free, 2 alive with disease, 17 dead of disease, and 1 dead of complications, after a median follow-up of 37 months (range, 22 days to 182 months). No significant differences between these 54 cases and the previous 156 NB/GNB cases were shown regarding stage and MYCN amplification.

Institutional written informed consent was obtained from the patient’s parents or legal guardians. The study underwent ethical review and approval according to local institutional guidelines.

**Tumor Sample Handling.** A part of the tumor samples was formalin-fixed and paraffin-embedded for histology and immunohistochemical analysis; a part was snap frozen in liquid nitrogen and stored at −80°C until immunohistochemical and molecular analyses were done. Frozen tumor aliquots for mRNA extraction and cryostat sections for immunohistochemical analysis and c-kit amplification or del 1p36.3. Briefly, in patients at stage I or II, the tumor was surgically excised followed by adjuvant chemotherapy only in a minority of cases. Patients with stage III (unresectable primary) were given neoadjuvant chemotherapy to reduce the tumor bulk, followed by surgery and adjuvant chemotherapy. Patients with stage IV received similar treatment to stage III but, in addition, some of them underwent autologous stem cell rescue after achieving complete remission. Stage IVS patients remained untreated unless life-threatening symptoms and/or diffuse disease progression occurred.

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic, clinical, and molecular features of the 168 NT cases according to the INPC histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Age at diagnosis &gt;1 y</td>
</tr>
<tr>
<td>Neuroblastoma (n = 127)</td>
<td>80 (63%)</td>
</tr>
<tr>
<td>Ganglioneuroblastoma (n = 29)</td>
<td>23 (79%)</td>
</tr>
<tr>
<td>Ganglioneuroma (n = 12)</td>
<td>12 (100%)</td>
</tr>
</tbody>
</table>

NOTE: Abbreviations: NA, not applicable; DOD, dead of disease.
investigations were only available in 106 of 168 cases (75 NBs, 22 GNBs, and 9 GNs).

**Immunohistochemistry.** All 168 tumors were investigated for c-kit protein expression on paraffin-embedded sections, whereas 106 of them were also analyzed for c-kit and SCF protein expression on cryostat sections.

Five-micrometer paraffin sections were deparaffinized, rehydrated and incubated with rabbit anti-human c-kit PoAb A4502 (Dako, Glostrup, DK) and mouse antiphosphotyrosine MoAb 4G10 (Upstate Biotechnology, Lake Placid, NY). The antibodies were used at 1:50 dilution and no antigen retrieval technique was employed. After repeated washing with PBS, sections were incubated with avidin-biotin complex kit (avidin-biotin complex peroxidase, Dako). The reaction product was revealed by 0.2% hydrogen peroxide and 0.6% 3,3-diaminobenzidine (Sigma, St. Louis, MO). Slides were counterstained with Mayer’s hematoxylin. As positive controls for c-kit expression, paraffin sections from a case of GIST were used.

Four-micrometer cryostat sections were acetone-fixed and incubated with the anti-human c-kit MoAb 1.D29.3D6 (Boehringer Mannheim Biochemica, Mannheim, Germany) and the anti-human SCF MoAb 10E5 (28). Indirect avidin-biotin immunoperoxidase staining was done (Vectastain, Vector Laboratories, Burlingame, CA). Slides were counterstained with Mayer’s hematoxylin. Sections incubated with isotype matched control immunoglobulins were used as controls.

**Southern Blot Analysis.** Genomic DNA was extracted according to Blin and Stafford (29). MYCN copy number was determined by Southern blot as previously described (30) and samples were classified MYCN-amplified when densitometric readings exceeded at least thrice those of nonamplified controls (5). 1p36 LOH was assessed by Southern blot as described elsewhere (31) and it was defined as the complete or almost complete disappearance of one band. MYCN amplification was shown in 27 of the 156 NBs and GNBs (17%) and in none of the 12 GNs. Likewise, 1p36 LOH was found in 36 of the 156 NBs and GNBs (23%) and in none of the GNs.

**Northern Blot Analysis.** Frozen tumor aliquots of 106 cases were investigated for both c-kit and SCF mRNA expression using Northern blot analysis. Total RNA was extracted using the RNAwiz extraction kit (Ambion, Austin, TX), blotted on nylon membranes and hybridized as previously described (32, 33). The probes used for the analyses were human c-kit cDNA (5100-bp full-length cDNA) and human SCF cDNA (900-bp cDNA fragment). A human β-actin fragment (34) was used for normalization. After hybridization and washes, filters were exposed to X-ray films in the presence of intensifier screens at 70°C.

**DNA Analysis for c-kit Mutations.** DNA extraction for detection of c-kit mutations was done on paraffin blocks of 18 cases of NT, of which nine were c-kit positive and nine c-kit negative with comparable histologic, clinical, and molecular variables. As positive controls, inflammatory tonsils were used.

Ten-micrometer paraffin sections were deparaffinized with xylene, dehydrated with ethanol, and incubated in extraction buffer (Tris 50 mmol; 0.1% Tween 20 Detergents; Phenol:water) containing 100 μg/mL proteinase K overnight at 48°C. The proteinase K was inactivated by a 10-minute incubation at 95°C followed by cooling at −20°C. The resulting lysate was centrifuged in a microcentrifuge for 5 minutes to pellet debris.

DNA in the tissue extract was amplified by PCR using oligonucleotide primer pairs designed to amplify a 213-bp fragment of c-kit exon 11. The sequence for the forward primer was 5′-CCAGAGTGCTCTAATGACTGAGAC-3′, and the sequence for the “reverse” primer was 5′-AGCCCCGTGTTTCA-TACTGACC-3′. Reactions were carried out in a 25 μL volume containing 2 μL of the nucleic acid extract, 0.5 μL of each primer, 1.0 μL of deoxyribonucleotide triphosphate, 2.5 μL PCR-buffer and 0.5 μL Enzyme (Quiagen Taq PCR Core Kit, Hilden, Germany). Amplification consisted of the following: 1 cycle at 94°C for 5 minutes; followed by 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute; and concluded with 1 cycle at 72°C for 7 minutes. The amplification products were size fractioned for gene Scan Analysis (Abi Prism 310).

**Real-Time RT-PCR.** Total RNA (0.5 μg) from each tumor was reverse transcribed in cDNA using the retroscript kit (Ambion). Quantitative real-time PCR was carried out to detect β-actin expression that was used to normalize the amount of cDNA of each sample. β-Actin primers were 5′-CCTTCAAACCCCCCCAGCCA-3′ and 5′-ACCCCTCTGTA-GATGGGCAC-3′.

Equal amounts of cDNA from each sample were amplified using the following primers to detect c-kit: 5′-ATTTCCTTCTGGTGTTGCTCTAC-3′ and 5′-GGGCCACCGGGCAT-TATA-3′. Reaction linearity was checked by running serial dilutions of cDNA from HTLA230 human NB cell line taken as positive control (23). Two independent experiments were carried out in triplicate using the ABI Prism 7000 cycler (Applied Biosystems, Foster City, CA) with the Sybr green fluorochrome. Results were analyzed by the ABI Prism 7000 SDS software (Applied Biosystems).

**Statistical Analysis.** The correlation between expression of c-kit and SCF and histologic, clinical, and molecular features were analyzed using χ2 test. Univariate and multivariate regression analyses according to the Cox proportional hazard model (35), Kaplan-Meier survival curves (36), and log-rank significance tests were carried out using the software package SPSS 7.0 for Windows (SPSS, Inc., Chicago, IL). Concordance rate, Cohen’s κ statistics value between 0 and 1 (0 = no agreement, 1 = total agreement) and P referred to the significance of concordance were calculated with the above-mentioned software package.

**RESULTS.**

One hundred sixty-eight NTs were investigated for the expression of c-kit by immunohistochemistry on paraffin sections, of which 106 were also evaluated for c-kit and SCF protein expression by immunohistochemistry on cryostat sections and for mRNA by Northern blot. Quantitative real-time RT-PCR analysis of c-kit expression was done in an additional 54 NTs. The demographic, clinical, and molecular features of the initial 168 cases according to the histologic category are listed in Table 1.

**c-Kit Protein Expression by Immunohistochemistry.** In frozen and paraffin sections, c-kit immunostaining was investigated using two different anti-human c-kit antibodies (MoAb 1.D29.3D6 and PoAb A4502, respectively), and immunolabeling was evaluated in a simple-blind trial fashion by two independent observers (P.G.N. and S.U.).
On frozen sections, immunoreactivity for c-kit protein was shown in 18 of 106 NTs (17%). The large majority of tumor cells were c-kit positive, had neuroblastic morphology, and showed a mainly diffuse cytoplasmic staining pattern.

On paraffin sections, immunostaining for c-kit protein was detected in 21 out of 168 (13%) NTs (Fig. 1A). As a positive control, paraffin sections from a case of GIST were used (Fig 1B). In the positive cases, an intense cytoplasmic staining reaction with accentuation at the cell membrane was detected, mainly confined to nests of neuroblasts scattered throughout tumor tissue. In contrast to the cryostat sections, the number of c-kit–positive cells was much lower, presumably as a consequence of the different sensitivity of the technique. Twenty out of 21 c-kit–positive cases were undifferentiated/poorly differentiated NBs with high mitosis/karyorhexis index and one case had the morphologic aspects of GNB. GNs were consistently c-kit negative on both cryostat and paraffin sections.

c-Kit protein expression on paraffin sections was compared with that obtained on frozen sections. In 103 out of 106 tumors, identical results were obtained, being 87 double negatives and 16 double positives, with a concordance rate of 97% (Cohen’s $\kappa$ statistics value 0.9, $P < 0.001$ referred to the concordance of significance).

**mRNA Expression for c-kit by Northern Blot Analysis.**
mRNA expression for c-kit was detected in 23 out of 106 NTs (22%). Molecular and immunohistochemical findings were compared for each individual tumor. As expected, a trend for concomitant expression of higher levels of mRNA and immunohistochemical c-kit protein detection was shown, with a concordance rate of 94% (Cohen’s $\kappa$ statistics value of 0.82, $P < 0.001$) between mRNA and c-kit protein on paraffin sections, and of 95% (Cohen’s $\kappa$ statistics value of 0.85, $P < 0.001$) between mRNA and c-kit protein on cryostat sections.

In 99 out of 106 cases (95%) concordant results were obtained being 83 triple negatives and 16 triple positives.

**SCF Protein and mRNA Expression by Immunohistochemistry and Northern Blot Analysis.** The presence of SCF was assessed by immunohistochemistry and Northern blot analysis in 106 of 168 cases. Immunostaining for SCF on cryostat sections was observed in 30 out of 106 NTs (28%); SCF was expressed by the neuroblasts with a diffuse cytoplasmic staining pattern. By Northern blot, mRNA for SCF was detected in 33 out of 106 NTs (31%). Again, none of the nine GNs expressed protein or RNA expression for SCF. The comparison between molecular and immunohistochemical findings for each individual tumor revealed a trend for concomitant expression of SCF protein and higher levels of mRNA with a concordance rate of 97% (Cohen’s $\kappa$ statistics value of 0.93, $P < 0.001$): 73 tumors were double negatives and 30 double positives.

**Phosphorylation of the c-kit Tyrosine Kinase Pathway.**
The concomitant expression in tumor cells of c-kit and SCF proteins was shown in 14 out of 23 c-kit–positive NTs (61%) and suggested the possibility of an autocrine loop leading to the activation and phosphorylation of the tyrosine kinase pathway. The c-kit phosphorylation status was investigated by immunohistochemistry on paraffin sections obtained from nine c-kit–positive and nine c-kit–negative NTs with matched histologic, clinical, and molecular variables. Antiphosphotyrosine MoAb 4G10 recognizing phosphotyrosine was employed. As a positive control, paraffin sections from a case of GIST were used (Fig. 1D). Immunostaining for phosphotyrosine was observed in the cytoplasm of tumor neuroblasts in 6 out of 9 c-kit–positive (66%) and in 5 out of 9 c-kit–negative NTs (55%; Fig. 1C).

**Analysis of c-kit Mutations.** The presence of mutations at exon 11 of the c-kit gene was studied in seven c-kit–positive
The SCF expression correlated with adrenal primary site (P < 0.001), first year of diagnosis (>1 year), primary site (adrenal versus other sites), and INPC histologic categories.

Univariate analysis was also applied to correlate SCF protein expression on cryostat sections and the prognostic clinicobiological features. The results are summarized in Table 4. The SCF expression correlated with adrenal primary site (P < 0.05), MYCN amplification, and 1p36 LOH (P < 0.001). No statistically significant correlation was observed with regard to age at diagnosis (>1 year), primary site (adrenal versus other sites), and INPC histologic categories.

The association between expression of c-kit and/or SCF protein and unfavorable clinicobiological variables, was further confirmed when the c-kit–positive NBs and GNBs were compared with the MYCN-amplified cases (Table 2).

In summary, based on the immunohistochemical findings, c-kit and/or SCF-positive cases tended to be undifferentiated or poorly differentiated NBs, at an advanced stage, and with unfavorable molecular features such as MYCN amplification and 1p36 LOH. In only one case, c-kit protein expression was associated with GNB morphology; nonetheless, this case presented with unfavorable prognostic features, being aged 180 months at diagnosis, with an adrenal tumor at stage IV, MYCN amplified and 1p36 deleted, that died of disease 17 months after diagnosis. SCF protein expression was associated with GNB morphology in five cases (Table 4). Two of them were conventional high-risk patients, >1 year at diagnosis, and with adrenal primary: one was at stage III, with a 1p36 deleted tumor, and died of disease 23 months after diagnosis and one was at stage IV and died of disease 44 months after diagnosis. The remaining three cases were standard-risk patients. Interestingly, c-kit protein expression was never detected in any of the 17 stage IVS tumors.

Table 2 Demographic, clinical, and molecular features of the NTs other than GNs expressing either c-kit or SCF proteins in comparison to the MYCN-amplified cases

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age at diagnosis &gt;1 y</th>
<th>Gender (M/F)</th>
<th>Adrenal primary</th>
<th>INPC category (NBs/GNBs)</th>
<th>Advanced stage (III and IV)</th>
<th>MYCN amplified</th>
<th>1p36 deleted</th>
<th>Outcome (DOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit–positive NTs (21/156, 13%)</td>
<td>17 (81%)</td>
<td>16/5</td>
<td>13 (62%)</td>
<td>20/1</td>
<td>19 (90%)</td>
<td>17 (81%)</td>
<td>17 (81%)</td>
<td>17 (81%)</td>
</tr>
<tr>
<td>SCF-positive NTs (23/97, 24%)</td>
<td>14 (61%)</td>
<td>15/8</td>
<td>16 (70%)</td>
<td>19/4</td>
<td>16 (70%)</td>
<td>14 (61%)</td>
<td>15 (65%)</td>
<td>16 (70%)</td>
</tr>
<tr>
<td>MYCN-amplified NTs (27/156, 17%)</td>
<td>20 (74%)</td>
<td>17/10</td>
<td>15 (56%)</td>
<td>25/2</td>
<td>25 (93%)</td>
<td>NA</td>
<td>NA</td>
<td>27 (100%)</td>
</tr>
</tbody>
</table>

NOTE: Twelve out of 168 NTs were GNs and were excluded from this analysis since they are negative for both c-kit and SCF proteins.

Abbreviations: NA, not applicable; DOD, dead of disease.

Table 3 Univariate analysis of the correlations between c-kit protein expression (positive cases: n = 21) and prognostic variables in 156 NBs and GNBs out of 168 NTs

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age at diagnosis</th>
<th>Gender (M/F)</th>
<th>Primary site</th>
<th>INPC category (NBs/GNBs)</th>
<th>Advanced stage I-II/III-IV</th>
<th>MYCN amplified</th>
<th>1p36 LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit–positive NTs versus unfavorable variables</td>
<td>&gt;1 y, 17/103 (16%)</td>
<td>16/87 M (18%)</td>
<td>Adrenal 13/73 (18%)</td>
<td>NBs 20/127 (16%)</td>
<td>Stage III-IV 19/86 (22%)</td>
<td>Amplified 17/27 (63%)</td>
<td>LOH + 17/36 (47%)</td>
</tr>
<tr>
<td>c-kit–positive NTs versus favorable variables</td>
<td>&lt;1 y, 4/53 (7%)</td>
<td>5/69 F (7%)</td>
<td>Not adrenal 8/83 (10%)</td>
<td>GNBs 1/29 (3%)</td>
<td>Stage I-II 4S 2/70 (3%)</td>
<td>Single copy 4/129 (3%)</td>
<td>LOH — 4/120 (3%)</td>
</tr>
<tr>
<td>Statistical significance, P</td>
<td>NS</td>
<td>&lt;0.025</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE: Twelve out of 168 NTs were GNs and were excluded from this analysis because they are negative for c-kit protein.

Abbreviations: NS, statistically not significant; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma.
series, neither c-kit nor SCF protein expression were shown to be independent prognostic factors. Accordingly, when the survival analysis in either MYCN amplified versus MYCN single-copy cases or 1p36 deleted versus 1p36 nondeleted cases was integrated with c-kit or SCF expression, no significant difference was shown.

**Real-Time RT-PCR Analysis of c-kit mRNA Expression.** Quantitative analysis of c-kit expression was done in an additional series of 54 NTs, with or without MYCN amplification, at different clinical stage. As a positive control, we used the HTLA230 NB cell line that expresses the highest levels of c-kit among the NB cell lines tested (19). To define the lower threshold of c-kit positivity, we chose the LAN-5 human NB cell line. Although the levels of c-kit mRNA are 10 times lower in LAN-5 compared with HTLA230 cells, LAN-5 cells are still responsive in vitro to the antiproliferative activity of imatinib mesylate and express c-kit levels that can be detected by immunoprecipitation (23). Thirty-five of 54 tumors (65%) were found positive: in 28 cases (5 MYCN amplified and 23 MYCN single copy), the levels of expression were within the range delimited by the expression of LAN-5 and HTLA230 cells; in seven cases (2 MYCN amplified and 5 MYCN single copy), the levels of expression were even higher than in HTLA230 cells (Fig. 3).

No significant associations between c-kit expression determined by real-time PCR and advanced stage, MYCN amplification, and poor clinical outcome could be shown in this additional series. MYCN-amplified tumors in fact expressed detectable levels of c-kit mRNA in 78% of cases (7 c-kit–positive cases of 9 MYCN-amplified cases) compared with 62% of MYCN single-copy tumors (28 c-kit–positive cases of 45 MYCN single-copy cases).

**DISCUSSION**

This study shows that, using real-time RT-PCR, significant levels of c-kit expression are detected in a substantial number of tumors. To further explore the relationship between c-kit expression and MYCN amplification, we performed univariate and multivariate analyses of survival data. The results of these analyses are summarized in Table 4 and Table 5.

**Table 4** Univariate analysis of the correlations between SCF expression (positive cases: n = 30) and prognostic variables in 97 NBs and GNBs out of 106 NTs

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age at diagnosis</th>
<th>Gender (M/F)</th>
<th>Primary site</th>
<th>INPC category (NBs/GNBs)</th>
<th>Advanced stage I-II/III-IV</th>
<th>MYCN</th>
<th>1p36 LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF-positive NTs versus</td>
<td>&gt;1 y, 20/62 (32%)</td>
<td>20/52 M (38%)</td>
<td>Adrenal</td>
<td>NBs 24/75</td>
<td>Stage III-IV</td>
<td>Amplified</td>
<td>LOH + 16/25 (64%)</td>
</tr>
<tr>
<td>unfavorable variables</td>
<td></td>
<td></td>
<td>21/49 (43%)</td>
<td>(32%)</td>
<td>18/49 (37%)</td>
<td>14/19 (74%)</td>
<td></td>
</tr>
<tr>
<td>SCF-positive NTs versus</td>
<td>&lt;1 y, 10/35 (28%)</td>
<td>10/45 F (22%)</td>
<td>Not adrenal</td>
<td>GNBs 5/22</td>
<td>Stage I-II and IVS</td>
<td>Single copy</td>
<td>LOH – 14/72 (19%)</td>
</tr>
<tr>
<td>favorable variables</td>
<td></td>
<td></td>
<td>9/48 (19%)</td>
<td>(23%)</td>
<td>12/48 (25%)</td>
<td>26/78 (20%)</td>
<td></td>
</tr>
<tr>
<td>Statistical significance, P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**NOTE:** Nine out of 106 NTs were GNs and were excluded from this analysis because they are negative for SCF protein. Abbreviations: NS, statistically not significant; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma.

**Table 5** Simple and multiple Cox regression analyses between overall survival and prognostic factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>Cox simple regression analysis</th>
<th>Cox multiple regression analysis excluding SCF</th>
<th>Cox multiple regression analysis including SCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Survival</td>
<td>Relative risk (95% confidence interval)</td>
<td>P</td>
<td>Relative risk (95% confidence interval)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>75.38</td>
<td>1.97 (1.06-3.66)</td>
<td>0.03</td>
</tr>
<tr>
<td>≤1</td>
<td>54.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II, and IVS</td>
<td>92.86</td>
<td>12.7 (5.01-31.80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>III and IV</td>
<td>35.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYCN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not amplified</td>
<td>73.36</td>
<td>5.34 (3.12-9.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amplified</td>
<td>11.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not deleted</td>
<td>77.03</td>
<td>6.80 (4.01-11.57)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Deleted c-kit protein</td>
<td>11.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>68.41</td>
<td>4.05 (2.28-7.20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>16.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCF protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>77.61</td>
<td>3.27 (3.01-6.56)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>43.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
significance of the c-kit presence in NTs was also supported by
imatinib mesylate (23). In the present study, the possible
increase which, in turn, can be selectively inhibited by treatment

The different detection rate of c-kit expression depending on
the sensitivity of the technique employed, heavily influenced
the correlations between c-kit expression and clinical, histologic,
and molecular features of the NTs. Thus, when only the highest
levels of expression as those preferentially detected by
immunohistochemistry were considered, c-kit expression was
associated with features characteristic of the most aggressive
NTs such as advanced stage, MYCN amplification, 1p36 LOH,
and shorter overall survival. When multivariate regression
analysis was applied, c-kit protein expression was no longer an
independent prognostic factor thus indicating that its relationship
with overall survival is mediated by stage, MYCN amplification,
and 1p36 LOH. However, when even lower levels of expression
as those detected by real-time RT-PCR were included, c-kit
expression was found much more spread out to either favorable
and unfavorable subsets of tumors, and the previous associations
were no longer shown. These results may give a possible insight
into the previous conflicting reports (15, 22–24) and suggest
that c-kit by itself is not a prognostic indicator in NTs.

MYCN-amplified NTs have a typically aggressive and rapidly
progressing clinical behavior (5) that correlates with
undifferentiated or poorly differentiated morphology and
markedly increased proliferative activity (38). In these tumors,
MYCN amplification produces an excess amount of MYCN
protein, which prevents cellular differentiation and apoptosis
(39). Similarly, in NB cell lines it was reported that c-kit sustains
in vitro cell growth (15, 23). We previously showed in a limited number of primary NTs that c-kit protein is
preferentially expressed in MYCN-amplified NBs (23). More
recently, gene expression profiling has shown that c-kit is one
of the genes differentially expressed in NTs with high levels
of MYCN expression, a significantly increased c-kit expression
being associated with MYCN overexpression in metastatic
stroma-poor NBs (40). In the present study, real-time RT-PCR
analysis did not fully confirm these findings, although a trend
was observed for MYCN-amplified tumors to express signifi-
cantly higher levels of c-kit mRNA more frequently than MYCN
single-copy tumors. To the best of our knowledge, a direct
relationship between MYCN and c-kit has not been described
dthus far. However, it cannot be excluded that in neuroectodermal
tumors MYCN can act as a master transcriptional modulator
controlling directly or indirectly other genes that in turn activate
or repress other critical pathways (40). The functions of these
genes may thus complement each other, at least to some extent.
In small cell lung carcinoma for instance, it has been observed
that c-kit by itself is not a prognostic indicator in NTs.

In GISTs, gain-of-function c-kit mutations are more
common in the most aggressive lesions and are considered a
negative prognostic factor (19–21). The majority of such
mutations occur in exon 11 of c-kit gene and induce a
constitutive expression of c-kit receptor associated with the
SCF ligand–independent phosphorylation of the c-kit tyrosine
kinase domain (19–21, 42). In the present study, gain-
of-function mutations in the exon 11 of the gene could not be
shown and this is in agreement with a previous report (24). It

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**Fig. 2**  A. Kaplan-Meier survival analysis for patients expressing (c-kit+) or not expressing (c-kit−) c-kit protein. Overall survival was 17% in the first group versus 68% in the second. The difference in survival between the two groups was statistically significant (*P* < 0.001). B. same analysis as in A. carried out in patients expressing (SCF+) or not expressing (SCF−) SCF protein. Overall survival in SCF+ was 43% versus 78% in SCF− patients. The difference in survival between the two groups was statistically significant (*P* < 0.001). *n*, number of patients in each group.

---

primary NTs, accounting for 65% of the cases investigated.
However, the percentage of c-kit− positive NTs decreases to 23%
when using Northern blot, and to 17% and 13% when using
immunohistochemical analysis on cryostat and paraffin sections,
respectively. The frequency of 65% reported here is higher than
that previously reported in other studies (15, 22–24, 37), which
ranged from 8% (22) to 55% (37) of cases. Despite the high
sensitivity of the real-time RT-PCR analysis we employed, we
suggest that these levels of expression may be biologically
significant in tumor cells. In these measurements in fact, the
threshold between positive and negative cases was set up based
on the level of expression found in c-kit− positive LAN-5 NB
cell line. In *vitro*, this cell line responds to SCF with a growth
increase which, in turn, can be selectively inhibited by treatment
with imatinib mesylate (23). In the present study, the possible
significance of the c-kit presence in NTs was also supported by
the evidence that c-kit and SCF were coexpressed by tumor cells
in 61% of c-kit− positive tumors, thus implying the existence of
the autocrine SCF/c-kit loop previously described *in vitro* in NB
cell lines (15, 23).
can not be excluded however that mutations may occur but in domains other than exon 11, such as the extracellular domain encoded by exon 9 and the kinase domain encoded by exon 13, as already reported in GISTs although as rare events (43). The development of low molecular weight agents specifically aimed at selectively blocking the activity of PTK receptors has raised interest in the therapeutic potential of targeting these pathways and thus inhibiting \emph{in vivo} tumor growth (44). A prominent example is imatinib mesylate, a selective inhibitor of several structurally related tyrosine kinase receptors including c-Abl, Bcr-Abl, c-kit, and platelet-derived growth factor receptor. This agent has shown a great therapeutic potential in several human malignancies where a structurally abnormal and constitutively activated PTK is expressed, such as chronic myelogenous leukemia (45, 46), GISTs (47), and mastocytosis (48). In addition, imatinib mesylate has also shown relevant antitumor activity in malignancies where PTK receptor activation occurs following a paracrine and/or autocrine stimulation. For SCF/c-kit pathway, this is the case of small cell lung cancer (49), colorectal cancer (50), Ewing’s family tumors (51), and NB (23, 25). Previous \emph{in vitro} studies reported coexpression of c-kit and SCF in human NB cell lines, in which the inactivation of c-kit receptor using a blocking MoAb resulted in a significant decrease in tumor growth (15). A subsequent study showed that the SCF/c-kit autocrine loop plays an important role in protecting NB cells from undergoing apoptosis \emph{in vitro} (52). The possible clinical relevance of SCF/c-kit expression \emph{in vivo} however was not fully elucidated as only a small number of primary NTs were evaluated (15, 22, 37) and conflicting results on the frequency and clinico-biological features of the c-kit–positive subset were reported (23, 24). Recently, we showed in human NB cell lines that SCF/c-kit signaling is active in promoting cell proliferation that can be selectively inhibited by \emph{in vitro} treatment with imatinib mesylate (23). Subsequent studies showed that imatinib mesylate is also effective in inhibiting \emph{in vivo} tumor growth in NB c-kit–positive xenografts (25, 53). In the present study, c-kit mRNA as determined by real-time RT-PCR was found to be expressed at significant levels in 65% of NTs and in 78% of the \emph{MYCN}-amplified subset. In this subset of patients, present therapeutic protocols do not induce a satisfactory long-term survival (54). In NTs no gain-of-function mutations of c-kit have been detected thus far (ref. 24 and this study), and the paracrine/autocrine activation of this receptor may probably be a late event and may not play a pathogenic role in the development of this tumor. Moreover, whereas the concentrations of imatinib mesylate that cause 50% inhibition of GIST cells \emph{in vitro} growth (55) closely match those that induce 50% inhibition of ligand-induced phosphorylation of wild-type c-kit (pharmacologic IC\textsubscript{50}; 0.1-0.5 \textmu mol/L; refs. 51, 56), the concentrations that provoke 50% inhibition of NB cells \emph{in vitro} growth (\sim 10 \textmu mol/L; ref. 25) are approximately two logs higher of the pharmacologic IC\textsubscript{50}. The reasons for these discrepancies are not yet fully elucidated, but the existence in NB cells of additional growth stimulatory pathways in contrast to the stringent dependence of GIST cells by constitutively activated c-kit can likely play a role. However, since there is a clear evidence that the SCF/c-kit pathway partially supports NB cells proliferation and survival (15, 23, 52), it may be hypothesized that the inhibition of this kinase activity would make these tumor cells more susceptible to the growth inhibition induced by treating with conventional cytotoxic drugs, as reported in other tumors in similar conditions (57–60), and/or by targeting other pathways (61). If so, the integration of imatinib mesylate into multiagent regimens could therapeutically benefit a subset of patients selected according to the pattern of expression for PTK receptors. The potential of gene expression profiling and/or proteomic technologies in identifying molecular signatures of responsiveness to targeted agents is presently

![Fig. 3](image-url) Real-time RT-PCR analysis of 54 NTs. \textbf{Blue lines}, course obtained with RNA extracted from HTLA230 NB cell line (first blue line on the left) and with a 1:2 dilution of the same RNA. \textbf{Red line}, lower positivity threshold defined as the amount of c-kit RNA expressed in NB cell line LAN-5. Example of the data output. Each sample was run in triplicate in two independent experiments.

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under investigation (62, 63). Until these techniques will be proven to be fully reliable, we propose that in NTs in order to prospectively identify patients eligible for inclusion into trials and to retrospectively analyze relationships between pattern of expression of PTK receptors and tumor responsiveness to targeted agents, real-time RT-PCR analysis of c-kit mRNA should be the reference technique.

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Clinical and Molecular Evidence for c-kit Receptor as a Therapeutic Target in Neuroblastic Tumors

Stefania Uccini, Olga Mannarino, Heather P. McDowell, et al.


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