Featured Article

Detection of Overexpressed and Phosphorylated Wild-Type Kit Receptor in Surgical Specimens of Small Cell Lung Cancer

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ABSTRACT

Purpose: The combinations of various chemotherapeutic drugs currently used to treat advanced small cell lung cancer (SCLC) led to similarly poor survival outcomes, which is why new molecular biology approaches are needed to design and select targeted therapies.

Experimental Design: Thirteen stage I SCLC surgical specimens were screened for *c-Kit* gene mutations by sequencing whole cDNA and for KIT receptor expression/activation by immunoprecipitation and Western blotting. Both the paraffin-embedded and frozen materials were analyzed by immunocytochemistry, and the stem cell factor cognate ligand was assessed by retrotranscription PCR.

Results: In all cases, we showed the presence of wild-type KIT receptors by analyzing the entire coding sequence, which together with the detection of the cognate ligand stem cell factor, supports the establishment of an autocrine loop. In addition, the KIT receptor was activated/phosphorylated. The immunoprecipitation/Western blotting data fit the observed immunophenotype. Interestingly, comparison of the level of KIT expression was at least 10 times higher in the tumoral specimens than the normal reference lungs.

Conclusions: The KIT molecular profile derived from the analysis of SCLC surgical specimens shows that wild-type KIT is overexpressed and phosphorylated in the presence of stem cell factor. This finding, which is consistent with pathological KIT activation driven by an autocrine loop, is particularly interesting in the light of the recent development of new tyrosine kinase inhibitory drugs, which are highly effective in blocking wild-type KIT receptors.

INTRODUCTION

Small cell lung cancer (SCLC) accounts for 15 to 25% of all new cases of lung cancer. Forty percent of patients present limited disease at diagnosis, whereas the remaining 60% show extensive disease/neoplastic growth. These tumors have a highly aggressive clinical course and can be distinguished from non-SCLC by their rapid doubling time, high growth fraction, and early metastatic development.

Although SCLC is highly sensitive to chemotherapy and radiotherapy, the small number of long-term survivors show that it is rarely curable with the currently available drug combination regimens mainly based on etoposide and cisplatin/carboplatin (1–3). For this reason, new molecular biology approaches to the disease are needed to select targeted therapies.

Among the biological agents with a targeted mechanism of action, the pharmacological interruption of KIT signaling alone or with standard therapy could be of therapeutic benefit. KIT is a type III tyrosine kinase receptor belonging to the platelet-derived growth factor receptor family, the ligand of which is stem cell factor, also known as steel factor.

Immunophenotype-based analyses have showed that the KIT oncoprotein is variably expressed in SCLC (4, 5) and that KIT expression correlates with an unfavorable prognosis (6), although this was not confirmed in a recent study (7).

Preclinical investigations indicate that >70% of SCLC cell lines express both *Kit* and stem cell factor mRNA, thus suggesting an autocrine loop regulation of the receptor (8, 9). This mechanism of action has also been observed in SCLC surgical specimens (10) analyzed in Northern blot experiments. The recent detection of a wild-type *Kit* exon 11 (11), one of the mutation hot spots of the gene, reduces the likelihood of ligand-independent activation through gain of function mutations, which are commonly described in many hematopoietic disorders (12), seminomas (13), and gastrointestinal stromal tumors (14), but supports the hypothesis of an autocrine loop as the most probable cause of KIT activation.

It is widely accepted that imatinib (Glivec, Novartis, Basel, Switzerland) can inhibit KIT tyrosine kinase activity and is particularly effective when KIT receptors are present in a mutated form, as in gastrointestinal stromal tumors carrying mutations in the juxtamembrane domain. In models in which KIT is activated by an autocrine loop, the switching off of the receptor

(detected through its dephosphorylation) is reported after imatinib treatment in SCLC and Ewing's cell lines (15, 16). More recently, the new compound, SU11248, has been found to be more effective than imatinib in slowing down cell growth in SCLC cell lines in an autocrine loop-activated KIT model (17). Although all of the demonstrations of KIT activation derive from SCLC cell lines, there is little evidence of its activation in such tumors as fresh material is required for the detection of KIT receptor phosphorylation.

Here, we provide the first description of molecular analyses of frozen surgical specimens taken from 13 patients with stage I SCLC and show the presence of wild-type stem cell factor and KIT receptors by the sequencing of all of the coding sequence of *c-Kit* cDNA, which supports the existence of an autocrine loop. Moreover, immunoprecipitation and Western blot experiments showed that KIT receptors are at least 10 times more expressed in fresh tumors than normal lung, as well as being phosphorylated/activated. Finally, KIT expression was confirmed by immunophenotyping fixed and frozen material.

MATERIALS AND METHODS

Patients. We analyzed cryopreserved material from surgical specimens taken from 13 patients with stage I SCLC; a section of each tumoral specimen was stained with H&E to verify the absence of normal lung tissue. The normal lung tissues came from wide surgical resections of four patients with a different tumoral histotype not included in the study.

Positive Controls. The human megakaryoblastic leukemia M07e cell line (kindly provided by Professor L. Pegoraro) was kept in culture as described by Brizzi *et al.* (18) and used as a positive control for *c-Kit* and stem cell factor mRNA expression.

In immunoprecipitation and Western blot experiments, a KIT/Δ559 cell line obtained by transfecting NIH3T3 with a c-Kit plasmid carrying the deletion of residue 559 was used as a positive control for KIT phosphorylation and expression.

RNA Extraction and Reverse Transcription-PCR. Total RNA was extracted by the RNAzol method (Gibco BRL/Life Technology, Inc., Paisley, UK), and 1 μg was reverse-transcribed with Superscript reverse transcriptase (Gibco BRL/Life Technology, Inc.) with oligo dT and random examers as recommended by the manufacturer: 30 μL of sterile 10 mmol/L Tris (pH 8)-1 mmol/L EDTA were added to 20 μL of total volume of cDNA obtained from each sample. All of the samples were tested for cDNA integrity by amplifying the β-actin housekeeping gene.

Our reference was Kit National Center for Biotechnology Information GenBank sequence accession number X-06182.

The PCR conditions with AmpliTaq Gold were as follows: 40 cycles at 94°C for 30 seconds, annealing for 30 seconds, 72°C for 1 minute.

For the stem cell factor analyses, PCR amplification was carried out with the following primers (19): stem cell factor (forward), 5′-ATTCAAGAGCCCAGAACCCA-3′, and stem cell factor (reverse), 5′-CTGTTAACCAGCCAATGTACG-3′ under the following conditions: 40 cycles at 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 1 minute.

The amplification reaction products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. The PCR results were confirmed by two to four repeat amplification procedures.

All of the sequence reactions were carried out with an automated sequencing system (377 DNA Sequencer, ABI PRISM PE, Applied Biosystems, Foster City, CA), according to standard protocols.

Protein Extraction. Proteins were extracted from tissue samples stored at −80°C by homogenization at 4°C in lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 10%glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L $Na_4 P_2O_7$, and 100 mmol/L NaF) supplemented with protease and phosphatase inhibitors (Cocktail Inhibitors I and II, Sigma, St. Louis, MO).

Lysis was performed by frequent vortexing, and the lysates were then cleared by centrifugation at 13,000 rpm at 4°C for 30 minutes and measured with a Bio-Rad protein assay.

Table 1 c-Kit amplification primers

Name	Position	Temperature of annealing (°C)	Fragment
Kit a (forward): 5′-CTGCACTTGGGCGAGAGC-3′	−81 from the ATG	59	1
Kit a (reverse): 5′-AGAACAATGCAGACAGAGCCG-3′	561		
Kit b (forward): 5′-CAGACCCAGAAGTGACC-3′	416	54	2
Kit b (reverse): 5′-ACTGCTGGTGTTCAGGTTTGG-3′	1042		
Kit c (forward): 5′-GCAAATGTCACAACAACC-3′	895	52	3
Kit c (reverse): 5′-ATTCAACCGTGCCATTGTGC-3′	1471		
Kit d (forward): 5′-AACTGAGCAGAGATGC-3′	1335	50	4
Kit d (reverse): 5′-TGATTACCAAGGTAACTCAGG-3′	1928		
Kit e (forward): 5′-TGAGGCAACTGCTTATGG-3′	1812	55	5
Kit e (reverse): 5′-GCTCCCAAAGAAAAATCCCATAGG-3′	2584		
Kit f (forward): 5′-GAGTTGGCCCTAGAGTTAGA-3′	2281	63	6
Kit f (reverse): 5′-CCTGGAGTTGGATGCAAGTT-3′	3029		
Kit g (forward): 5′-AACGAGATTAAAAGGCACC-3′	1137	53	7
Kit g (reverse): 5′-AGCTACGAT TACGAAACC-3′	1609		
Kit h (forward): 5′-ACCCTGTTCACTCCTTTG-3′	1558	53	8
Kit h (reverse): 5′-TTACAGCGACAGTCATGG-3′	1867		
Kit i (forward): 5′-TGAGGCAACTGCTTATGG-3′	1821	53	9
Kit i (reverse): 5′-ACAACATAAGAAACTCCAG-3′	2195		

NOTE. The primers used for the amplifications of whole c-Kit cDNA.

Immunoprecipitation and Western Blotting. Equal amounts of protein lysates underwent immunoprecipitation by incubation with 2 μL of mouse monoclonal antibody Ab-3 (K45) (NeoMarkers, Union City, CA) directed against the c-KIT receptor, even if conjugated to its receptor (stem cell factor; ref. 20).

Immunocytochemistry. Immunoperoxidase phenotyping was performed on representative sections of the corresponding paraffin-embedded tumoral specimens fixed in neutral buffered formalin with CD117 antibodies provided by Dako (A4502, Dako, Carpinteria, CA, 1:50 diluted; epitope 963–973) and Santa Cruz Biotechnology (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 diluted; epitope 959–973) with heat-induced epitope retrieval. The slides were developed with 3,3′-diaminobenzidine.

A gastrointestinal stromal tumor molecularly characterized for KIT mutations was used as a positive control.

RESULTS

Molecular Analysis: Mutational Status of the c-Kit Gene. In all 13 cases, entire c-Kit cDNA was analyzed by amplifying 9 different overlapping regions with specific primer pairs, and the PCR products were directly sequenced. After aligning our sequencing data with the National Center for Biotechnology Information kit sequence (accession number X06182), we detected the alternative splicing of exon 9 (giving rise to the two c-Kit isoforms differing by 12 bp in length), and exon 15 (which, by deleting 3 bp, led to the Ser^{715} deletion) in all of the samples.

No canonical mutations affecting c-Kit mutation hot spots were observed. Three cases (6, 13, and 15) showed the previously reported (21) amino acid substitution mol/L 541 L in exon 10.

Furthermore, 10 cases showed the D745E substitution in exon 16. This amino acid exchange was also observed in 14 unrelated tumors and two normal tissue samples (data not shown) and probably represents an unreported KIT receptor polymorphism.

Stem Cell Factor Expression. The expression of the stem cell factor c-Kit ligand was analyzed by reverse transcription-PCR, the efficiency of which was assessed by amplifying the β-actin housekeeping gene. All of the samples showed a homogenous and highly comparable PCR products (Fig. 1B).

Four cases were negative for stem cell factor expression because no bands were observed on the agarose gel. Nine
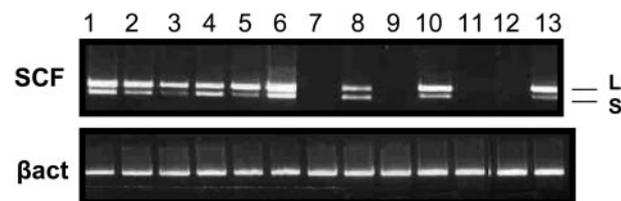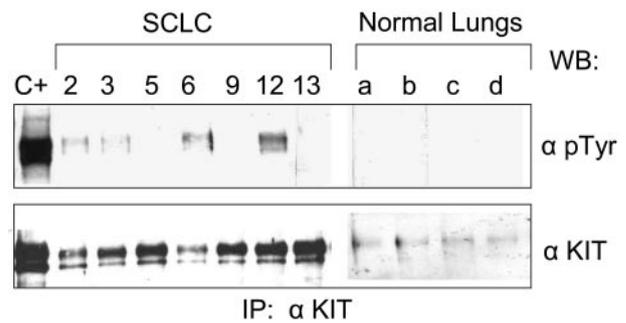

Fig. 2 KIT receptor expression and phosphorylation. For each sample, 800 μg of total protein extracts were immunoprecipitated with αKIT antibodies, run on gel, and blotted with the indicated antibodies. C+, NIH3T3 cell line, transfected with the KIT/Δ559 mutant (carrying the deletion of residue 559) and overexpressing a constitutively phosphorylated KIT receptor (positive control). SCLC, the numbers correspond to the cases listed in Table 1. Samples *a–d* are normal lungs.

samples were positive (69.2%), with two bands of the expected molecular weight of 494 bp (L) and 409 bp (S) (Fig. 1A). L-Stem cell factor was more expressed than S-stem cell factor in cases 3, 5, 10, and 13.

Comparison of the total amount of stem cell factor in the samples revealed that the level of expression was not homogenous and, given the same retrotranscription efficiency, we concluded that stem cell factor was differently expressed in our tumoral cases.

Biochemical Analysis: KIT Expression and Phosphorylation. Total protein extracts obtained from seven SCLC patients for whom frozen material was available, four normal lungs (used as controls to detect basal c-Kit expression), and a cell line expressing the constitutively phosphorylated KIT protein KIT/Δ559 (positive control) were analyzed by immunoprecipitation and Western blotting for KIT receptor expression and phosphorylation. The extracts were immunoprecipitated with a monoclonal α-KIT antibody that recognizes the KIT receptor, even if it is conjugated to stem cell factor. Hybridization with a polyclonal α-KIT antibody revealed the 145 and 125 kDa KIT isoforms, respectively, corresponding to the mature fully glycosylated and the partially glycosylated receptor. The two forms were detected in the positive control and all of the SCLC cases (Fig. 2), but the level of expression was at least 10 times less in the normal tissue.

Hybridization of the same membrane with the α-pTyr antibody revealed a band of 145 KDa corresponding to the phosphorylated mature KIT receptor in four of the seven tumoral samples, thus demonstrating the presence of an autocrine loop.

Correlation between Stem Cell Factor and Activated KIT Receptor. In four of the seven cases analyzed for KIT expression by immunoprecipitation experiments, the KIT receptor was phosphorylated, and the presence of its stem cell factor ligand correlated with its activated form. In two cases, the lack of phosphorylation was associated with the presence of stem cell factor and one case (number 10 in Table 2) showed no KIT activation or stem cell factor mRNA. These data indicate the presence of an autocrine loop as the mechanism responsible for KIT activation.

Fig. 1 SCF expression. 1 μL of cDNA was used as a template for each stem cell factor (SCF) and β-actin gene amplification reaction; 10 μL of PCR were loaded on the 2% agarose gel. In the case of SCF amplification, two bands of the expected molecular weight were detected: L = 494 bp and S = 409 bp.

Table 2 Summary of molecular and biochemical data from SCLC surgical specimens

	Molecular analysis		Biochemical analysis		Immunocytochemistry		
Case	*c-Kit* sequence	Stem cell factor	KIT phosphorylation	KIT expression	Paraffin (Dako)	Paraffin (Santa Cruz)	Frozen (Santa Cruz)
1	wt	+	nd	nd	+ c	+ c	+m
2	wt/M541L	+	+	+	+m	+ c	nd
3	wt *	+	+	+	+m	+ c	+m
4	wt *	+	nd	nd	+m	+m	nd
5	wt *	+	−	+	+ c	+ c	+m
6	wt */M541L	+	+	+	+ c	+ c	+m
7	wt *	−	nd	nd	−	−	+m
8	wt *	+	nd	nd	+m	nv	+m
9	wt	−	−	+	+m	+ c	+m
10	wt *	−	nd	nd	+ c	+ c	+m
11	wt *	−	nd	nd	+ c	+ c	ne
12	wt *	+	+	+	+m	+ c	+m
13	wt */M541L	+	−	+	+ c	+ c	+m

Abbreviations: nd, not done; ne, not evaluable; wt, wild-type in all 21 exons and with alternative splicings of exons 9 and 15. *, with the D745E substitution in exon 16; + c, cytoplasm reactivity; + m, membrane-associated reactivity.

Immunocytochemical Screening. All but one case was immunoreactive to both Dako and Santa Cruz Biotechnology antibodies. However, as shown in Table 2, half of the cases presented Dako antibody reactivity mainly restricted to the membrane (Fig. 3, *A* and *B*), and all but one processed with the Santa Cruz Biotechnology antibody showed cytoplasmic and only occasionally partial membrane decoration (Fig. 3*C*). It is worth noting that membrane-restricted reactivity to the same antibody was observed in all of the cases immunophenotyped on frozen sections 7 years ago (4), when the slides were developed with carbazol (Fig. 3*D*); unfortunately, we could not repeat the analysis with the Dako antibody because no more frozen material was available.

The KIT expression detected by immunocytochemistry fitted with the immunoprecipitation/Western blotting experiments in the seven analyzed cases.

DISCUSSION

Here, we describe the molecular profile of KIT receptors in 13 surgical specimens taken from patients with stage I SCLC, as indicated by the entire *c-Kit* cDNA sequence and the presence of the stem cell factor cognate ligand revealed by reverse transcription-PCR. When cryopreserved material was available, KIT receptor activation and expression were analyzed by immunoprecipitation and Western blot experiments and compared with those of normal lung. Finally, the immunoprecipitation/Western blotting results were correlated with the immunophenotyping (immunocytochemistry) of the corresponding samples.

The whole KIT receptor coding region was sequenced, and no canonical mutations affecting the *c-Kit* mutation hot spots were detected.

Exon 9 and exon 15 alternative splicing was observed in all cases. The M 541 L substitution in exon 10 detected in three cases has previously been reported in some patients with chronic myelogenous leukemia, and because it has been observed in the normal population and the normal tissue of chronic myelogenous leukemia patients, it is probably a polymorphic variant (21).

Another substitution involving exon 16 (D 745 E) was

detected in 10 of our 13 cases. This conservative change did not involve any conserved region of the receptor and may be another polymorphic variant as it was also detected in 14 unrelated tumoral samples and two normal tissue samples.[6]

KIT ligand stem cell factor mRNA was expressed in nine of the 13 cases (69%). This is in line with preclinical data indicating up to 70% KIT receptor activation in SCLC cell lines.

This PCR amplification reaction detects two biologically active stem cell factor isoforms corresponding to the soluble (L) and membrane-associated ligand (S). Because it lacks exon 6, the latter encodes membrane-bound stem cell factor, whereas the L isoform encodes a protein containing a cleavage site that makes the ligand susceptible to posttranslational proteolysis, thus yielding a soluble KIT ligand (22, 23). The two bands were expressed at the same intensity in all but four of the samples, in which the soluble form was more expressed (numbers 3, 5, 10, and 13). In addition, although we are aware that this technique is not a quantitative means of assessing differently expressed mRNAs, we can say that the expression of stem cell factor was different in the tumoral samples and also speculate that the different stem cell factor isoforms and the different amounts of stem cell factor in our tumoral samples may differently stimulate the receptor, even if the KIT phosphorylation observed in our immunoprecipitation/Western blotting experiments was comparable in all of the positive samples.

Our biochemical experiments were aimed at verifying whether stem cell factor could activate KIT by assessing the phosphorylation status of the receptor and showed an activated KIT receptor whenever stem cell factor was present, except in two cases in which an unsuitable snap-frozen procedure may have damaged the material. In one case with no stem cell factor, an expressed but nonphosphorylated receptor was immunoprecipitated, thus supporting the hypothesis that an autocrine loop is responsible for KIT activation.
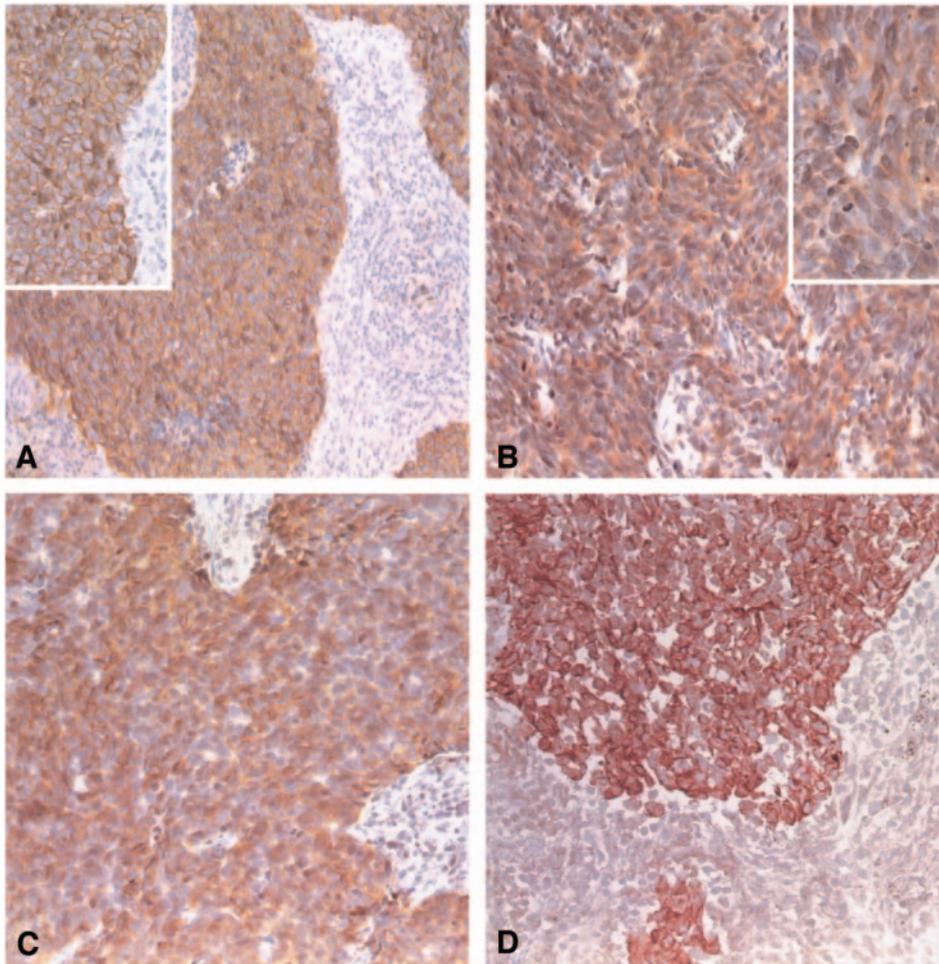
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[6] Unpublished data.

Fig. 3 KIT immunocytochemistry reactivity. *A* and *B*, Dako antibody immunostaining of paraffin-embedded material showing membrane reactivity (at higher magnification in the top, left corner) and cytoplasmic reactivity (at higher magnification in the top, right corner). *C*, Santa Cruz Biotechnology antibody on paraffin-embedded material (3,3′-diaminobenzidine) showing moderate and incomplete staining of a number of cells. *D*, carbazol frozen material showing membrane decoration throughout the sample

Interestingly, KIT immunocytochemistry reactivity was mainly membrane associated, particularly on frozen material for which no antigen retrieval was used in accordance with standard immunocytochemistry criteria. This immunoreactivity pattern differs from that observed in gastrointestinal stromal tumors, which frequently show cytoplasmatic KIT decoration, sometimes with membrane strengthening. It may be interesting to verify whether the different decoration pattern of gastrointestinal stromal tumors and SCLCs is sustained by a different mechanism of KIT receptor activation. The same membrane-bound reactivity has been observed in salivary gland tumors in which a complete wild type c-Kit gene and stem cell factor were detected (24).

KIT expression was at least 10 times lower in the normal lung samples, thus additionally supporting the assumption that KIT overexpression is a relevant event in SCLC and that the KIT-driven pathway is required for tumor growth and survival. Many preclinical reports have showed that tyrosine kinase inhibitory molecules are effective in blocking KIT activation and cell growth, but their use is hampered by the need for high and clinically unachievable drug concentrations. However, a more recent study of SCLC cell lines has shown that the new compound, SU11248, is more effective than STI571 in slowing cell growth and could be successfully used in tumors in which KIT activation is sustained by an autocrine loop (17).

In the light of this, the SCLC molecular/biochemical KIT profile described here (which shows that KIT is overexpressed and phosphorylated in the presence of stem cell factor and wild-type cDNA) is important as the first evidence of activated KIT receptors in SCLC surgical specimens that can also be detected with standard immunocytochemistry techniques. Bearing in mind that the autocrine loop may be the mechanism responsible for KIT activation in SCLC, it is not surprising that imatinib has been found to have little activity in SCLC xenografts (25) and an albeit small-scale phase II study (26).

Moreover, the involvement of KIT as an early event in the pathogenesis of SCLC is still debated, and it remains to be clarified whether its activation is relevant for tumor growth and survival (27). In addition, it is well known that the deregulation of a number of other genes contributes to the growth of this complex tumor (28, 29).

For all of these reasons, the use of new molecules designed to ensure the pharmacological interruption of pathological KIT signaling may represent a promising approach to improve SCLC treatment alone or in combination with standard regimens of proven efficacy or other inhibitory molecules. Recent preclinical

studies have shown that the combination of imatinib and NVP-ADW724, a new insulin-like growth factor-I receptor inhibitor, can inhibit cell growth in SCLC cell lines coexpressing KIT and insulin-like growth factor-I receptor (30, 31).

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