Response to Sunitinib Malate in Advanced Alveolar Soft Part Sarcoma

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Abstract

Purpose: Alveolar soft part sarcoma (ASPS) is a rare, chemoresistant soft tissue sarcoma. ASPS harbors the t(17-X) (p11.2;q25) translocation, resulting in the ASPACR1-TFE3 fusion protein, causing MET autophosphorylation and activation of downstream signaling. The tumor vascular pattern prompted us to use sunitinib malate (SM), a tyrosine kinase inhibitor with antiangiogenic properties.

Experimental Design: Since July 2007, five patients with progressive metastatic ASPS have been treated with continuous SM 37.5 mg/d on a named basis. Four patients are evaluable for response. In four cases, cryopreserved material was available. Upstream and downstream targets of receptor tyrosine kinase (RTK) pathways, as well as mechanisms of activation, were investigated by biochemical profiles, including human phospho-receptor RTK antibody arrays and immunoprecipitation/Western blotting, molecular analyses, immunohistochemistry, and fluorescence *in situ* hybridization analyses.

Results: After 3 months, two patients had RECIST (response evaluation criteria in solid tumor) partial response, as well as positron emission tomography response and subjective improvement. One had a RECIST stable disease. One progressed and stopped treatment. One patient is still responding after 12 months. The upstream analysis showed activation of all the platelet-derived growth factor receptor (PDGFR) family members, as well as epidermal growth factor receptor, MET families, and RET. Vascular endothelial growth factor receptors (VEGFR1 and VEGFR2) were activated only in one case. The downstream target analysis showed strong activation of phosphatidylinositol 3-kinase/AKT, extracellular signal-regulated kinase 1/2, and mTOR and its targets (S6K and S6). The absence of any upstream mTOR effector deregulation and the presence of RTK cognate ligands support an autocrine-paracrine activation loop mechanism.

Conclusion: SM may have antitumor activity in ASPS, possibly through a mechanism involving PDGFR and RET. The role of MET, epidermal growth factor receptor, and mTOR, as well as PDGFR inhibition, needs to be further explored.

Alveolar soft part sarcoma (ASPS) is a very rare sarcoma (incidence 0.5/100,000/year) of uncertain histogenesis, mainly affecting deep soft tissues of the extremities of young patients (15-35 years; ref. 1). ASPS has a high metastatic potential to the lungs, bone, liver, soft tissue, and brain. Most patients who metastasize cannot be cured and have a definitely poor prognosis in spite of a prolonged natural history. Chemotherapy has long been known to be inactive in ASPS (1–3). Reports of tumor responses to IFN have been only anecdotal (4–6). Histologically, ASPS consists of epithelioid, poorly cohesive tumoral cells arranged in nests, accompanied by prominent capillary vascularity. ASPS carries a translocation t(X;17)(p11.2;q25) resulting in the ASPACR1-TFE3 fusion gene (previously designated ASPL-TFE3; ref. 7). Two recent preclinical investigations (8, 9) pointed out the role of unregulated TFE3 overexpression in the context of ASPSCI-TFE3 fusion transcript, which leads to activation of MET and its downstream signaling that may represent potential targets of receptor tyrosine kinase (RTK) inhibitors.

In the lack of any therapeutic alternative in a group of progressive advanced ASPS resistant to IFN, the peculiar vascular pattern of this disease prompted us to use sunitinib malate (SM; SU11248, Sutent), a known RTK inhibitor with antiangiogenic properties approved for treatment of GIST and renal cancer (10–14). SM is an RTK inhibitor with direct antitumor and antiangiogenic activity targeting platelet-derived growth factor receptor (PDGFR), KIT, FTL3, M-CSF, all

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Translational Relevance

We investigated the upstream and downstream tyrosine kinase–related pathways in alveolar soft part sarcoma through phospho-receptor tyrosine kinase antibody arrays, immunoprecipitation/Western blotting, mutational analyses, immunohistochemistry, and fluorescence *in situ* hybridization. In all cases, the upstream analysis showed activation of the platelet-derived growth factor receptor family, as well as epidermal growth factor receptor, MET families, and RET. Vascular endothelial growth factor receptors (VEGFR1 and VEGFR2) were activated only in one case. The downstream target analysis showed a strong activation of phosphatidylinositol 3-kinase/AKT, extracellular signal-regulated kinase 1/2, and mTOR and its targets without any upstream mTOR effector deregulation. No evidence of mutations and gain or loss of genes were found, whereas a consistent presence of cognate ligands was promptly detected. These findings give a rationale to the use of sunitinib malate, because sunitinib malate is a receptor tyrosine kinase inhibitor with direct antitumor and antiangiogenic activity by targeting the platelet-derived growth factor receptor family and vascular endothelial growth factor receptors, as well as RETreceptor.

belonging to the PDGFR family, vascular endothelial growth factor receptors (VEGFR) and RET (15–17). We have also studied the biochemical-molecular-cytogenetic profile of four primary ASPSs to gain deeper insights into the RTK expression/activation status, the upstream and downstream signaling, and their activation mechanisms.

Materials and Methods

Biochemical, molecular, and cytogenetic analyses were done on four ASPS primary tumors operated at our institution and whose cryopreserved material was available. In all cases, diagnosis were confirmed by positive immunostaining for TFE3 and by interphase fluorescence *in situ* hybridization (FISH) using a probe for 17q25.3 mapping telomeric to ASPL in combination with a centromere 17–specific probe (Table 1A and B).

Patients treated in this series had to have an unresectable progressive ASPS during the previous 3 mo according to RECIST (response evaluation criteria in solid tumor; ref. 18). A performance status (Eastern Cooperative Oncology Group) of ≤3 and an adequate bone marrow and organ function were also requested. Patient's written informed consent to a nonconventional medical treatment, selected in the lack of alternative therapies known to be effective in the disease, was required, and all patients were aware that there was no previously reported proof of antitumor activity and efficacy of SM in ASPS. The ethical committee approved the use of the drug in all cases.

Treatment. Patients were treated with continuous 37.5 mg of SM once daily, within a compassionate use program. Treatment was continued as long as there was no disease progression. Treatment was withheld for hematologic grade of ≥3 adverse events and for nonhematologic grade of ≥2 adverse event (defined according to the National Cancer Institute Common Toxicity Criteria, version 3.0) and restarted after recovery to grade of <2 in case of hematologic or grade of <1 in case of nonhematologic.

Evaluation. At baseline, all patients were evaluated with a complete history and physical examination, a complete blood count and serum chemistry (comprehensive of ANC, platelet, haemoglobin, creatinine, bilirubin, SGOT, SGPT, ALP, LDH, cholesterol, triglycerides, FT3, FT4, TSH, CPK) and full cardiologic assessment. Adverse events, serum chemical analyses, and blood count were monitored after 2 wk from treatment start and then monthly, but in case of problems. Required imaging studies before treatment included a whole body computed tomography scan and a computed tomography scan and magnetic resonance imaging of the sites of disease; baseline positron emission tomography (PET) scan was done in all cases. Scanning was repeated after 6 wk from treatment start then every 3 mo.

Efficacy assessment. Response to treatment was assessed with the use of RECIST criteria and PET response, according to the currently available European Organization for Research and Treatment of Cancer 1999 tumor response criteria (18, 19).

Analyses of frozen surgical material

Phosphorylation of RTK array. Expression of phosphorylated growth factor RTKs was detected using the Proteome Profiler Array kit (R&D Systems). The procedures were done according to the manufacturer's protocol using 2 mg protein lysate per array.

Total protein extraction, immunoprecipitation, and Western blotting. Total proteins were extracted and immunoprecipitated with specific antibodies, as previously reported (20). Experimental condition for PDGFRA, PDGFRB, and epidermal growth factor receptor (EGFR) immunoprecipitation and subsequent Western blotting were already reported (20). Rabbit polyclonal anti-MET (20 μL, Santa Cruz Biotechnology) and rabbit polyclonal anti-RET (5 μL, Santa Cruz Biotechnology) were used for MET and RET immunoprecipitation. For Western blotting, the same MET antibody and the anti–P-RET (Santa Cruz Biotechnology) were used. The downstream signaling analysis was done by direct Western blotting, loading 20 μg of total protein per lane, using the following antibodies and the dilutions listed in Table 1C.

Molecular analysis. Mutational analysis of EGFR, PDGFRA, and PDGFRB was done, as previously described (20), whereas RET mutations were analyzed according to Borrello et al. (21). Phosphatidylinositol 3-kinase (PI3K) hot spot mutations were studied, as reported in Moroni et al. (22), whereas RAS (K/N and H) and BRAF were investigated according to Frattini et al. (23). PTEN was analyzed using the primers and experimental conditions listed in Table 1D.

Total RNA was extracted and reverse-transcribed, as previously described (24). Expression of PDGFRB and PDGFRA ligands was done according to Tamborini et al. (20). MET ligand (HGF) and RET ligands (GDNF and NTN) were amplified using the specific primers listed in Table 1D. EGFR ligands (EGF and TGFA) were analyzed by real-time PCR using the specific probes listed in Table 1D.

Analysis of formalin-fixed paraffin-embedded material

Immunohistochemistry. Immunohistochemistry was done on 2 μm formalin-fixed and paraffin-embedded sections using specific antibodies directed against TFE3, EGFR, PDGFRB, PDGFRA, MET, RET, and KIT. The details of the antibodies are shown in Table 1A.

FISH analysis. FISH analysis was done on paraffin-embedded sections, in which representative areas were selected under microscopic control, as previously described (25), using the probes described in Table 1B.

The analysis for PDGFRA and PDGFRB has already been reported (20). The corresponding gene status was evaluated, as previously described (disomic, low polysomy, high polysomy; refs. 26, 27).

Results

As of June 2008 and from July 2007, five patients (patients A-E; Table 2) with advanced ASPS resistant to IFN have been

Table 1. Antibodies and their working dilutions are listed for immunochemistry (A) and Western blotting (C) analyses and all the primers and amplification conditions, including real-time PCR, are reported (D) for molecular investigations and for all the probe FISH employed in the study (B)

A

Antibody	Clone	Company	Dilution	Antigen retrieval	Positive control
EGFR	K1492	Dako	The procedures were done according to the manufacturer's protocol		
PDGFRB	sc-339	Santa Cruz Biotec	1:100	6 min at 95°C 5 mmol/L citrate buffer, pH 7	Human breast carcinoma
PDGFRA	sc-338	Santa Cruz Biotec	1:200	6 min at 95°C 5 mmol/L citrate buffer, pH 6	Exon 18 *PDGFRA* mutated GIST
MET	sc-10	Santa Cruz Biotec, CA, USA	1:50	15 min at 95°C 1 mmol/L EDTA buffer, pH 8	Human breast carcinoma
RET	3F8	Vector Laboratories	1:20	15 min at 95°C 1 mmol/L EDTA buffer, pH 8	Small intestine
CD117	A4502	Dako	1:50	6 min at 95°C 5 mmol/L citrate buffer, pH 6	Exon 11 *c-kit* mutated GIST
TFE3	SC-5958	Santa Cruz Biotec	1:250	6 min at 95°C 1 mmol/L EDTA buffer, pH 8	Pediatric renal adenocarcinoma

B

Gene	Probe	Company
PTEN	LSI PTEN/CEP10 dual-color probe	Vysis
EGFR	LSI EGFR/CEP7 dual-color probe	Vysis
MET	LSI D7S552 (7q31) Spectrum Orange/7Spectrum Green Probe	Vysis
RET	BAC clone RP11-231C18 labeled with Spectrum Green dUTP coupled with CEP10 Spectrum Orange SO	Dr. M. Rocchi (Resources for Molecular Cytogenetic, University of Bari)
ASPL	BAC clone RP11-525L23 labeled with Spectrum Orange dUTP coupled with CEP17 Spectrum Green SO	Invitrogen

C

Antibody	Clone	Company	Dilution	Positive control
P Akt	4058S Ser473	Cell Signaling	1:1000	A431 cell line
Akt	9272	Cell Signaling	1:1000	A431 cell line
P MAPK	4376S Thr 202/Tyr204	Cell Signaling	1:1000	A431 cell line
MAPK	9102	Cell Signaling	1:1000	A431 cell line
P m-TOR	2971S Ser2448	Cell Signaling	1:1000	A431 cell line
m-TOR	2972	Cell Signaling	1:1000	A431 cell line
P p70S6K	9234S Thr389	Cell Signaling	1:1000	A431 cell line
p70S6K	9202	Cell Signaling	1:1000	A431 cell line
P S6	2215L Ser 240/244	Cell Signaling	1:2000	A431 cell line
S6	2217	Cell Signaling	1:1000	A431 cell line
PTEN	9559	Cell Signaling	1:2000	A431 cell line
Actin	A2066	Sigma	1:5000	A431 cell line

D

Gene		Primers	PCR conditions
PTEN	exon 5 fw	5′-TGCAACATTTCTAAAGTTACCTACT-3′	96°C 8 min; 96°C 30 s, 55°C 30 s, 72°C 1 min: 40 cycles; 72°C 7 min
	exon 5 rev	5′-GAGGAAAGGAAAAACATCAAAAA-3′	
	exon 6 fw	5′-TTTTTCAATTTGGCTTCTCTTTTT-3′	
	exon 6 rev	5′-TGTTCCAATACATGGAAGGATG-3′	
	exon 7 fw	5′-CAGTTAAAGGCATTTCCTGTG-3′	
	exon 7 rev	5′-TTTTGGATATTTCTCCCAATGAA-3′	
	exon 8 fw	5′-TGTCATTTCATTTCTTTTTCTTTTC-3′	
	exon 8 rev	5′-AAGTCAACAACCCCCACAAA-3′	
	exon 9 fw	5′-TCATGGTGTTTTATCCCTCTTG-3′	
	exon 9 rev	5′-TGAGTCATATTTGTGGGTTTTCA-3′	
HGF	fw	5′-GGGAAATGAGAAATGCAGCCAG-3′	96°C 8 min; 94°C 1 min, 58°C 1 min, 72°C 1 min: 5 cycles; 94°C 30 s, 59°C 30 s, 72°C 1 min: 35 cycles; 72°C 5 min
	rev	5′-AGTTGTATTGGTGGGTGCTTC-3′	
GDNF	fw	5′-TGAAGTTATGGGATGTCGTGG-3′	96°C 8 s; 95°C 1 min, 62°C 30 s, 72°C 1 min: 40 cycles; 72°C 5 min
	rev	5′-TACTTTGTCACTCACCAGCCTT-3′	
NTN	ex fw	5′-GCTGTCCATCTGGATGTGTC-3′	95°C 5 min; 95°C 15 s, 62°C 15 s, 72°C 1 min: 40 cycles; 72°C 5 min
	ex rev	5′-AAGGACACCTCGTCCTCGTAG-3′	
	int fw	5′-GAGAGGGCCTGCTTCTCGA-3′	95°C 5 min; 95°C 15 s, 58°C 15 s, 72°C 1 min: 40 cycles; 72°C 5 min
	int rev	5′-GAACAGCACCGTCTCGTCGGA-3′	

Ligands		Probes	Real-time PCR conditions
EGF	Hs00153481_M1	Applied Biosystem	50°C 2 min; 95°C 10; 95°C 15 s, 60°C 1 min: 40 cycles
TGFA	Hs00608187_M1		

treated with continuous SM at 37.5 mg/d. Among them, four are still on therapy, whereas one has stopped treatment after 3 months for progressive disease. Four are evaluable for response, whereas one has just started his treatment. Pretreatment frozen surgical specimens from primary tumor were available for two of four patients (patients A and B). Two additional specimens again from primary tumor were assessed, from patients F and G not treated in this series.

Patients

Patient characteristics are listed in Table 2 [mean age/range, 35 years/22-58 years; female/male, 1/6; site: five lower limbs and two upper limbs; locoregional/metastatic: 0/7, with involvement of the lung (7), liver (4), soft tissue (3), bone (2), brain (2)]. The WHO performance status of three of them was $\geq$2. All patients had been heavily pretreated with one or more surgical procedures (7), radiotherapy (5), and medical therapy (7). Three of them were symptomatic for pain and functional impairment requiring constant treatment. All patients had progressed within 3 mo before starting treatment.

The mean duration of treatment was 5 mo (range, 1-12 months). All patients received SM of 37.5 mg/d, according to a continuous dosing regimen. In all patients stopping their treatment for toxicity, the drug was then restarted at the same dose level.

Overall, SM was well tolerated. The major nonhematologic toxicities include fatigue (one case, G2), hypothyroidism (one case, G2), and nausea and vomit (G2). The most common hematologic toxicities were neutropenia (three patients, no G3-4), chronic anemia (one patient, no G3-4), and thrombocytopenia (1 patient, no G3-4). No treatment interruptions for G3 toxicity were required. No dose reductions were required due to adverse event.

Response

Clinical findings. As shown in Table 3, four patients (A-D) are evaluable for response. According to RECIST, after 1 month of treatment, two patients (A and B) had a partial response, one patient (D) was stable, and one patient (C) was slightly progressive. After 3 months, patients A and B were still responding, patient D was stable, and patient C had a progressive disease and stopped his treatment. Patient A is still responding after 12 months of treatment, with a slow continuous decrease in size and PET uptake. Patient B progressed after 9 months, although without a RECIST progressive disease, so that he is still on therapy. PET confirmed response in patient B. Computed tomography scan responses are shown in Figs. 1 and 2.

Patients A and B reported improvement in symptoms. Interestingly, patient A had an increase of subcutaneus lesions during treatment interruption, and a decrease once on therapy again.

Pathology/biology

1. Upstream targets. Because SM blocks the activity of multiple receptors, we evaluated the expression profile of different RTKs in primary tumor of four ASPS patients. Two of these patients received SM (patients A and B). This analysis showed a heterogeneous activation profile. As shown in Fig. 3A, we observed a high level of activation of members belonging to the PDGFR family (PDGFRB, Flt3, M-CSFR) and EGFR family (EGFR, Her4), followed by HGFR family (HGFR and MSPR) and VEGFR family (VEGFR1 and VEGFR2). The activation of Axl/Dtk, FGFR3, and IR seems to be less relevant, because drugs against these receptors are lacking at present.

Confirmatory immunohistochemistry and biochemical analyses

Immunohistochemistry. To confirm the data derived from the phospho RTK antibody arrays, we did immunostaining for EGFR, PDGFRB and MET, extending the analysis to the remaining members of PDGFR family (PDGFRA and CD117) and RET on formalin-fixed paraffin-embedded representative selected sections.

EGFR resulted null in all but one case, which scored as intermediate (case F; data not shown). PDGFRB was expressed in all cases, but one result was not evaluable (case B, data not shown). MET was immunoreactive in all the four cases, as shown in Fig. 3B (*left*).

PDGFRA (data not shown) and RET (Fig. 3B, *right*) were expressed in all the four cases, whereas CD 117 resulted null (data not shown).

RET was investigated because of MET overexpression, which might be related to upstream signals generated by RAS or RET activation (and by possible other oncogenes; ref. 28).

The discrepancy between phospho RTK antibody arrays and Western blotting is possibly due to the different efficacy of the applied antibodies.

Biochemical analysis: immunoprecipitation and Western blotting. Expression and phosphorylation status of EGFR, PDGFRA, PDGFRB, MET, and RET receptors were assessed by immunoprecipitation/Western blotting on the residual protein extracts used for phospho RTK antibody arrays.

EGFR and PDGFRB were expressed and activated in all the four samples. PDGFRA was also expressed but with a lower phosphorylation level (data not shown).

MET and RET were expressed and phosphorylated in all the four samples with a weaker activation level in samples G and B for MET (Fig. 3B, *left*) and sample F for RET (Fig. 3B, *right*).

Taken together, with the exception of EGFR (finding otherwise not unexpected; ref. 25), there was a good correlation between immunohistochemistry and biochemical analysis and between this latter and phospho RTK antibody array results.

2. Downstream targets

Biochemical analyses (immunoprecipitation/Western blotting). As PI3K/AKT cascade, coimmunoprecipitation experiments with PDGFRA, MET, RET, EGFR, and PDGFRB showed in all the samples the presence of p85, the regulatory subunit of PI3K, which is consistent with an upstream RTK-mediated activation of p85 (Fig. 3C, *top left*, coimmunoprecipitation of p85 with PDGFRB and EGFR).

Regarding RAS/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, ERK1/2 showed high levels of expression and activation (Fig. 3C, *intermediate and bottom left*).

Furthermore, in keeping with the literature pointing out that not only PI3K/AKT but also RAS/ERK1/2 triggers mTOR (29), we observed mTOR expression and activation in all the four samples (Fig. 3C, *top right*), and the same was true for its downstream targets, the p70S6K and the ribosomal protein S6 (Fig. 3C, *intermediate and bottom right*).

Overall, both the PI3K/AKT and ERK1/2 pathways were activated.

Mutational analysis. p110, the catalytic subunit of PI3K, PTEN, RAS, and BRAF were sequenced, and no mutation was observed.

Table 2. Patient characteristics

	Age (age at the time of diagnosis), y	Gender	Performance status	Site of primary tumor	Site of metastasis at the time of diagnosis
Patient A	58 (49)	M	2	Right forearm	None
Patient B	28 (24)	M	1	Right arm	Lung
Patient C	24 (24)	M	3	Right leg	Lung, liver, soft tissue, bone, brain
Patient D	31 (26)	M	0	Right thigh	Lung
Patient E	25 (23)	M	0	Right leg	Lung
Patient F	55 (52)	M	2	Left high	Lung
Patient G	22 (20)	F	1	Left thigh	Lung

FISH and Western blotting analysis of PTEN. Because PTEN, the negative regulatory effector of PI3K/AKT pathway, resulted as wild type, we did FISH analysis of this suppressor gene, which showed disomic pattern in three samples and low polysomy in one. Moreover, Western blotting analysis showed that this gain of copy number did not affect the amount of PTEN protein.

Overall, all the downstream effectors of upstream mTOR did not show any alterations.

3. Assessment of mechanisms of activation of upstream targets. Because the strong activation of PI3K/AKT and RAS/ERK1/2 pathways in the absence of any upstream mTOR effector deregulation supported an upstream RTK-driven activation, we looked for possible mechanisms of activation.

Mutational analysis. EGFR, PDGFRA, PDGFRB, and RET were sequenced, and no mutation was observed. We omitted MET gene sequencing because no mutation was detected in the series investigated by Tsuda (9).

FISH analysis. EGFR, PDGFRA, PDGFRB, MET, and RET were analyzed by FISH, and a disomic pattern was observed in all cases.

Reverse transcription–PCR and real-time PCR analyses for assessment of ligand expression. PDGFA, PDGFB, HGF, GDNT, and NTN (Fig. 3D, *top*), as well as EGF and TGFA (Fig. 3D, *bottom*), turned out to be expressed in all cases.

Taken together, the absence of mutations and gene copy number alterations in the presence of cognate ligand expression is consistent with an autocrine/paracrine activation loop.

Discussion

We have been treating five patients with progressive, heavily pretreated, advanced ASPS, resistant to IFN, with continuous SM. Among the four patients evaluable for response, two showed a RECIST partial response, as well as a PET response. One had a RECIST stable disease, whereas the other progressed. Although very preliminary, these observations point to some effectiveness of SM in ASPS. Noteworthy, dimensional responses took place. This is of great interest in a tumor resistant to chemotherapy (1–3). Furthermore, in one case, SM was active for long. These findings prompted us to investigate the upstream and downstream tyrosine kinase–related pathways of four patients whose frozen material from primary tumor was available through phospho RTK antibody array, immunoprecipitation/Western blotting, molecular analyses, immunohistochemistry, and FISH. In all cases, the upstream analysis showed activation of the PDGFR family, as well as EGFR, MET families, and RET. VEGFR1/VEGFR2 were not activated, but in one case; in particular, they were not activated in the two patients with dimensional responses. The downstream target analysis showed a strong activation of PI3K/AKT, ERK1/2, and mTOR and of its targets (S6K and S6) in the absence of any upstream mTOR effector deregulation.

Because this pattern of activation is consistent with an RTK-driven upstream activation, we looked for the possible mechanisms of activation by molecular and FISH analyses. No evidence of mutations and gain or loss of genes was found, whereas a consistent presence of cognate ligands was promptly detected. This is in keeping with multiple RTK-driven autocrine/paracrine loops encompassing RTKs fitting with the target profile of SM, in addition to EGFR and MET. These findings give a rationale to the use of this compound, because SM is an RTK inhibitor with direct antitumor and antiangiogenic activity by targeting the PDGFR family, VEGFR, and RET receptor (15–17). Moreover, an activation of all these receptors, but KIT, was shown at upstream level; notably, a ligand-dependent activation of PDGFRA, PDGFRB, and RET was also observed. Remarkably, the lack of a role of KIT has been already reported (30). Overall, our results support the hypothesis that the antitumor activity of SM in ASPS may be mainly mediated by PDGFRs, whereas the inhibition of VEGFR, in keeping with available studies (8), seems to be less relevant.

RET is an SM target. Furthermore, an alternative way to fusion protein in inducing MET overexpression is represented by the presence of a deregulated RAS or RET, as reported in another tumor type (28). As in our model RAS is structurally normal, we investigated RET and provided evidence that RET was phosphorylated; thus, the possibility of MET overexpression induced by RET signaling could been envisioned.

Hence, our data regarding MET suggest that, in addition to the unregulated TFE3 fusion protein expression, as shown by preclinical assays (8, 9), the presence of an activated RET might also concur to the activation of MET together with a potent activation of the detected HGF ligand (28). Both mechanisms may contribute to evoke the strong activation of PI3K/AKT and ERK1/2 pathways. Furthermore, considering the pivotal role of HGF/MET system in angiogenesis (31) and the peculiar

Table 2. Patient characteristics (Cont'd)

Site of metastasis before starting sunitinib	Presence of symptoms before starting sunitinib	Previous surgery/ radiation therapy/ chemotherapy	Previous treatment with IFN	Treatment with sunitinib	Material for biochemical analysis
Lung, liver, soft tissue, bone	Y	Y/Y/Y	Y (progression)	Y	Y
Lung, liver, soft tissue, brain	Y	Y/Y/Y	Y (progression)	Y	Y
Lung, liver, soft tissue, bone, brain	Y	Y/Y/N	Y (progression)	Y	N
Lung, liver	N	Y/N/Y	Y (SD followed by progression)	Y	N
Lung	N	Y/N/Y	Y (progression)	Y	N
Lung	Y	Y/Y/Y	Y (SD followed by progression)	N	Y
Lung	N	Y/Y/N	Y (SD followed by progression)	N	Y

vascular pattern of ASPS, our data strongly support a therapeutic role of MET inhibitors. Currently, no data on the activity of SM on MET are reported. However, SU5416, another RTK inhibitor chemically related to SM with similar functional groups, has been shown to inhibit activation of HGF/MET in hepatocarcinoma, suggesting that SM might be involved even in HGF/MET down-signaling switch off (32).

Regarding EGFR, owing to the constant coexpression of PDGFRB and EGFR and the good response of the two patients treated with SM, both carrying this coexpression, we cannot rule out that EGFR could also be activated through a cross-talk with activated PDGFR in PDGFR-addicted tumors (33). In fact, the cross-activation phenomenon through intrafamily (34), as well as interfamily, heterodimerization (28) is not unusual among RTKs.

Table 3. Results

	Treatment with sunitinib	Duration of treatment	Hematologic toxicity	Non hematologic toxicity	Evaluable for response	Symptoms improvement	CT/MRI response (RECIST)	PET response	RTK expression profile
Patient A	Y	12 mo, ongoing	Neutropenia (G2); thrombocytopenia (G1); anemia (G1)	Fatigue (G2), hypothyroidism (G2)	Y	Y	Partial response after 6 wk confirmed at 3-6-9-12 mo	Y	PDGFRB, MCSFR, RET, MET
Patient B	Y	10 mo, ongoing	Neutropenia (G1)	Nausea/vomit (G2)	Y	Y	Partial response after 6 wk confirmed at 3 and 6 mo; slight progression after 9 mo (RECIST SD)	Y	PDGFRB, MCSFR, FLT3, RET, MET family
Patient C	Y	3 mo, stopped	None	None	Y	N	Slight progression after 6 wk (RECIST SD), progressive disease after 3 mo	N	
Patient D	Y	4 mo, ongoing	Neutropenia (G1)	None	Y	Not applicable	SD after 4 wk, slight response after 3 mo (RECIST SD)	No change	
Patient E	Y	1 mo, ongoing	None	None	N (too early)	Not applicable	Too early	Too early	
Patient F	N			Not applicable					PDGFRB, MCSFR, RET, MET
Patient G	N			Not applicable					PDGFRB, MCSFR, FLT3, RET, MET family

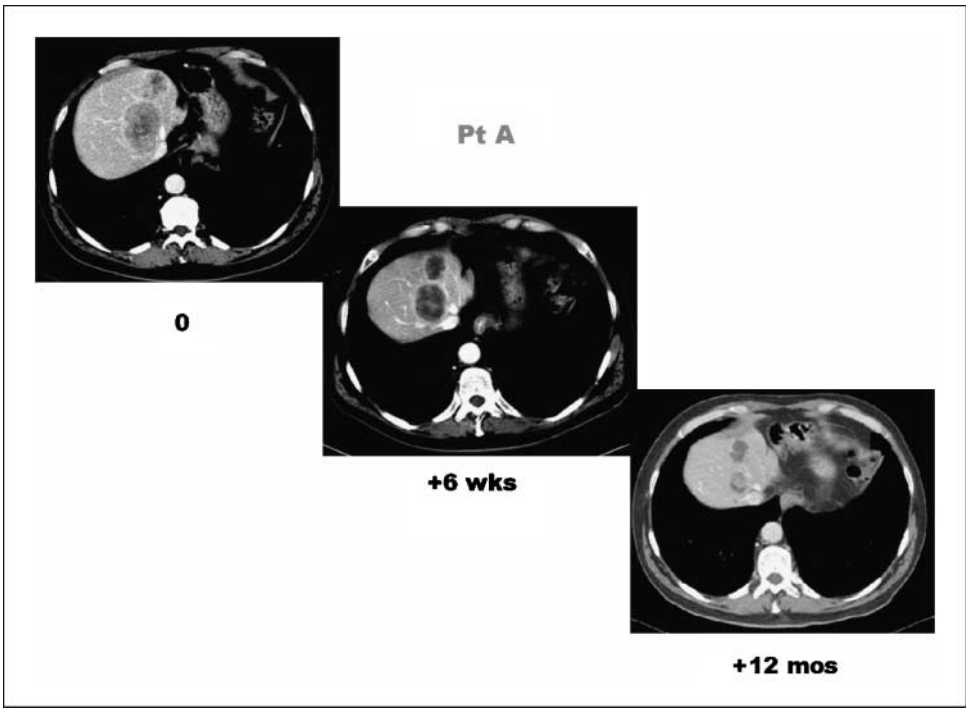

Fig. 1. Patient A. Liver metastasis from right forearm primary ASPS: response to sunitinib.

Finally, the strong activation of downstream signaling supports the possibility that a combination of upstream tyrosine kinase and downstream mTOR inhibitors could result in an enhanced tumor growth control. This combination could also avoid the possible rapamycin-mediated enhancement of PI3K/AKT activation induced by silencing the S6K-mediated down-regulation of PI3K/AKT, i.e., the so-called negative feedback loop that might result in an attenuation of the effects of mTOR inhibition (29).

In summary, our clinical observation, as well as biomolecular data provided herein, may open a new opportunity for ASPS treatment, to be prospectively evaluated in a case setting lacking any conventional medical treatment.

Disclosure of Potential Conflicts of Interest

S. Stacchiotti, P. Casali, and A. Marrari have received commercial grant support from Pfizer.

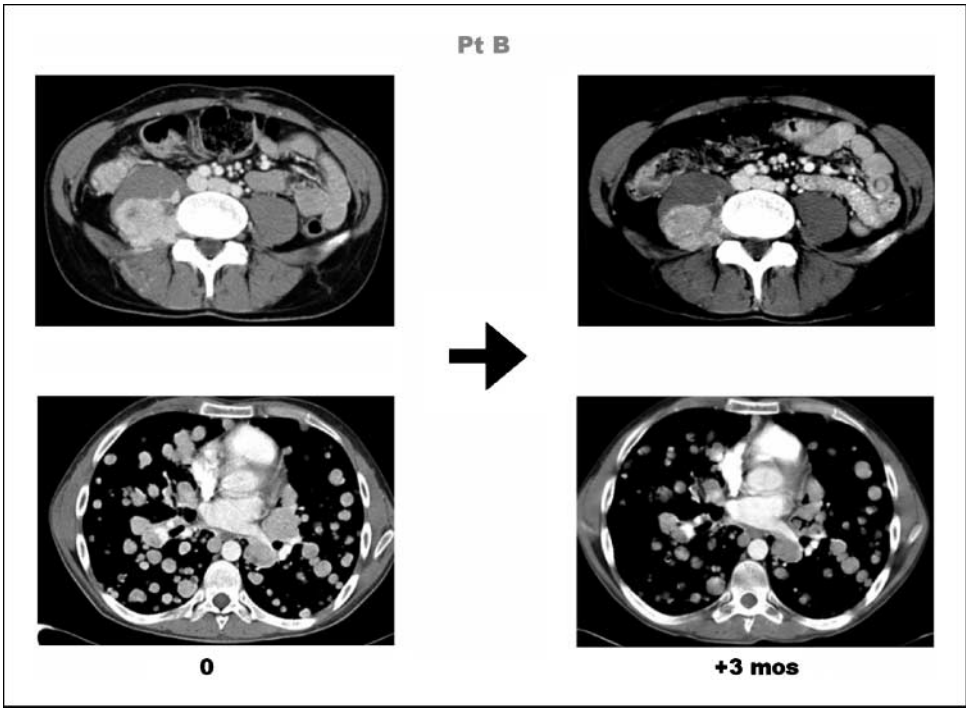

Fig. 2. Patient B. Soft tissue and lung metastasis from right arm primary ASPS: response to sunitinib.

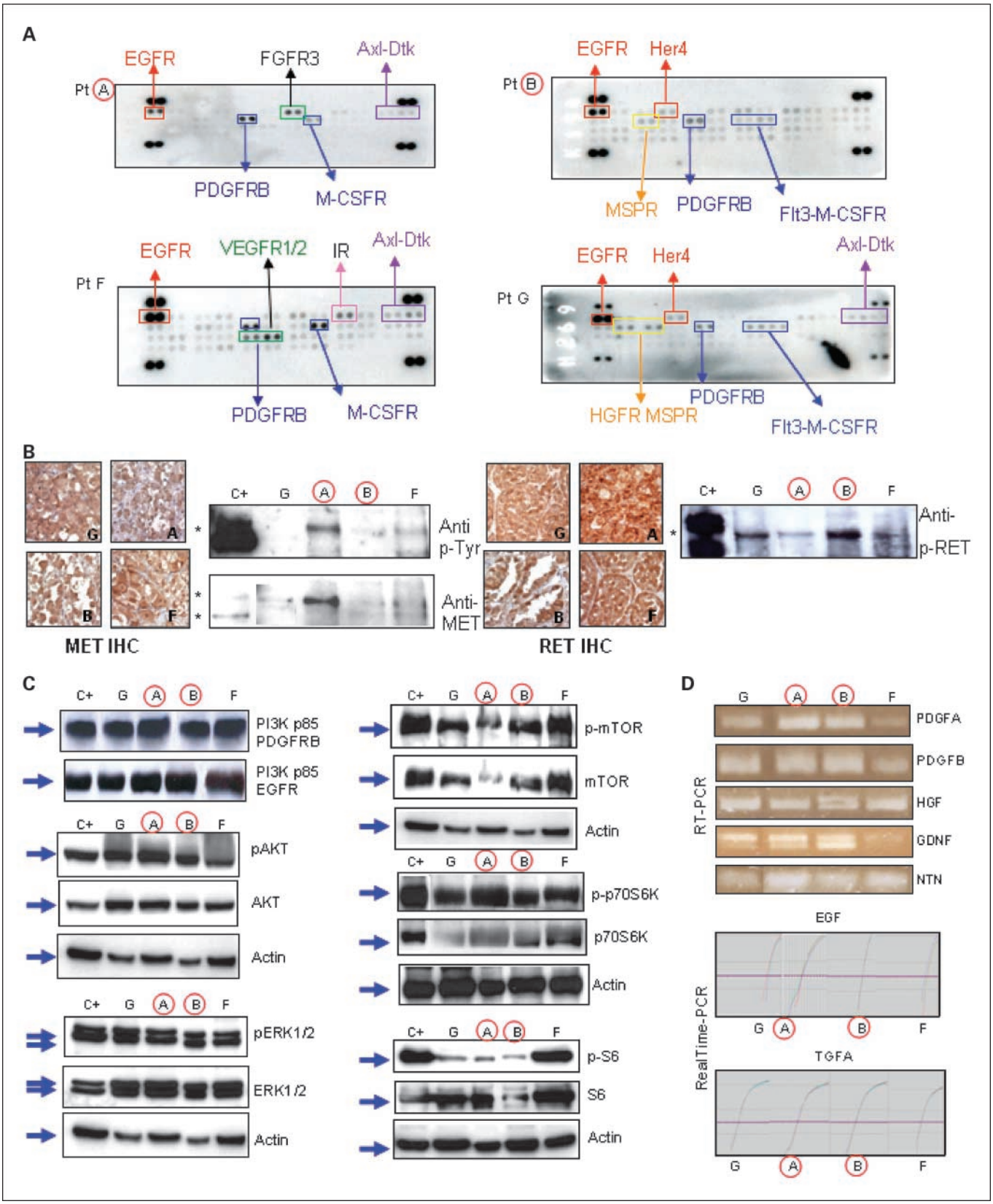

Fig. 3. *A,* spots represent the activated receptors. The double strong labeled spots at the four corners represent the positive controls. *B,* strong MET and RET immunodecoration was confirmed by immunoprecipitation/Western blotting experiments. Asterisks indicate the corresponding activated receptors (anti – p-Tyr and anti – p-Ret; *) and the receptor MET (**), respectively. *C,* head arrows indicate p85PI3K derived from coimmunoprecipitation experiments (PDGFRB and EGFR). For each of the other analyzed effectors, arrows indicate the activated (P) forms and the corresponding proteins. Actin is the quantitative experimental control. *D,* reverse transcription – PCR (*RT-PCR*) amplification products and amplification cycles (EGF and TGFA) of the named ligands.

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