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Frequent mono-allelic loss associated with deficient PTEN expression in imatinib-resistant gastrointestinal stromal tumors

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Insufficiency of phosphatase and tensin homolog (PTEN) occurs in numerous tumor types and has been implicated as a resistance mechanism to receptor tyrosine kinase-targeted therapies in human cancer. In this study, we have performed a comprehensive molecular and immunohistochemical characterization of PTEN in 58 imatinib-naïve and 54 imatinib-treated gastrointestinal stromal tumors (GISTs). The findings were correlated with clinicopathological data. At the genomic level, *PTEN* was affected mainly by mono-allelic loss, which was significantly less frequent in imatinib-naïve *vs* imatinib-resistant tumors (9% *vs* 39%, $P<0.001$). Neither *PTEN* mutations nor *PTEN* promoter hyper-methylation were found. By immunohistochemistry, PTEN depletion was clearly related to GIST progression. Low PTEN protein expression was common (50%) and often paralleled with total immunonegativity in imatinib-resistant tumors. The abnormal PTEN protein expression correlated with *PTEN* loss at the genomic level ($P=0.001$). In addition, the effect of small interfering RNA (siRNA) *PTEN* knockdown on KIT signaling was examined in GIST-T1 and GIST430 cell lines, in the absence or presence of a dual PI3K/mTOR inhibitor NVP-BEZ235, alone or in combination with imatinib. In both cell lines, siRNA silencing of *PTEN* resulted in the substantial upregulation of PI3K-AKT and MAPK pathways. The MAPK hyperactivation was further potentiated by NVP-BEZ235 in the imatinib-sensitive GIST-T1 cells; yet, this effect was counteracted efficiently by combined treatment. In the imatinib-resistant GIST430 cells, neither NVP-BEZ235 alone or in combination with imatinib yielded sufficient inhibition of hyper-phosphorylated MAPK and downstream intermediate S6 protein. In conclusion, depleted PTEN expression associated with mono-allelic *PTEN* loss occurs frequently in imatinib-resistant GIST and might serve as a biomarker for stratifying patients for optimal treatment. *In vitro*, the PTEN insufficiency leads to hyperactivation of AKT and MAPK pathways in tumor cells. Novel therapies targeting multiple components of the integrated KIT receptor signaling pathways in imatinib-resistant GIST warrant further studies.
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The phosphatase and tensin homolog (PTEN) is a phosphatase able to convert membrane-associated phosphatidylinositol 3,4,5-triphosphate back to phosphatidylinositol 4,5-bisphosphate; thereby it negatively regulates the signaling transduction of the PI3K/AKT pathway.[1] After *TP53*, *PTEN* represents the second most frequently mutated tumor-suppressor gene in cancer. PTEN inactivation has a role in several human neoplasms, including glioblastoma, endometrial, prostate, colon, and breast carcinoma. In some of these tumors it has been

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demonstrated that PTEN deficiency is associated with advanced tumor stage and therapeutic resistance, especially to targeted therapies of receptor tyrosine kinases and their pathways.[2] In breast cancer, *PTEN* loss confers resistance to the anti-HER2 antibody trastuzumab.[3] Similarly, it promotes resistance to EGFR tyrosine kinase inhibitors in glioblastoma, colon, and lung cancer.[4–6]

In gastrointestinal stromal tumors (GISTs), KIT and PDGFRA are fundamental therapeutic targets; however, the majority of GIST patients eventually develop resistance to imatinib mesylate and to other receptor tyrosine kinase inhibitors currently applied in the clinic.[7] This resistance is mainly due to the re-activation of KIT signaling by the acquisition of secondary *KIT* mutations. The PI3K/AKT/mTOR signaling pathway represents a crucial driving force for the growth, survival, and progression of GIST.[8] In human malignancies, PTEN inactivation or insufficiency constitutively activates this pathway.[9] Thereby, GIST patients with PTEN deficiency could benefit from alternative therapies targeting the PI3K/AKT/mTOR pathway. In line with this concept, an oral mTOR inhibitor everolimus (RAD001, Novartis Pharmaceuticals) has been tested in combination with imatinib in phase I-II clinical trials for patients with imatinib-resistant GIST, pointing to a potential therapeutic benefit of the combined administration.[10] Recent preclinical *in vivo* studies performed on GIST xenografts, using PI3K inhibitors in combination with imatinib, indicated synergistic and long-lasting effects, and suggested that PTEN inactivation could have an impact on the response to this therapy.[11,12] Yet, currently no studies have been performed to investigate the range of PTEN abnormities in imatinib-resistant GIST patients.

In this study, we have performed a comprehensive molecular and immuno-histochemical characterization of *PTEN* in a heterogeneous cohort of GIST, to assess the incidence and the nature of PTEN malfunction during GIST progression and during the course of imatinib therapy. Furthermore, we have investigated the impact of small interfering RNA (siRNA) *PTEN* knockdown on KIT signaling in imatinib-sensitive and imatinib-resistant GIST cell lines treated with a dual PI3K/mTOR inhibitor alone or in combination with imatinib, in order to better understand the functional consequences of PTEN insufficiency in GIST cells.

Materials and methods

Pathologic GIST specimens, from patients who underwent a biopsy or surgical resection of their tumor at the University Hospitals of Leuven, Belgium, and at the Department of Soft Tissue/Bone Sarcoma and Melanoma M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland, were retrieved from the Departments of Pathology of both institutions. Patients with advanced GIST were treated with imatinib in a dose 400–800 mg per day. The majority of these patients acquired resistance to imatinib, which was clinically defined as progressive tumor growth that occurred after an initial period >6 months on imatinib during which the patient had responsive disease. Both response and progression have been objectively assessed according to RECIST criteria. Clinical information was obtained from the databases and from review of medical charts. The local institutional ethics board of both participating institutions approved the study.

In total, 112 specimens from imatinib-naïve ($n=58$, 49 primary and 7 metastatic) and imatinib-treated ($n=54$; 48 imatinib-progressive and 6 imatinib-responsive) GIST were available for the analysis (Table 1).

The pathologic material was examined and the diagnosis of GIST was confirmed using hematoxylin and eosin staining and CD117 (KIT) immunohistochemistry on formalin-fixed, paraffin-embedded tissues, according to standard procedures. *KIT*/*PDGFRA* genotyping was done as reported.[13]

PTEN Copy Number Loss, Mutational Analysis, and Methylation Status

Dual-color interphase fluorescence *in situ* hybridization (FISH) was performed on 85 paraffin-embedded specimens, using LSI *PTEN*(10q23)/CEP10 Probe (Abbott Laboratories, Green Oaks, IL, USA), as described.[12] The array comparative genomic hybridization (aCGH) data from 54 GIST included in this study were published before by our group.[14,15]

Mutational analysis of the entire coding sequence of *PTEN* (ENST00000371953-exons 1–9) was performed as described.[12] The primer sequences are listed in Table 2.

The methylation status of the *PTEN* promoter (CpG Island 101755) was evaluated using the EpiTect Methyl qPCR Assay (Qiagen) according to the manufacturer's protocol. Universal Methylated Human DNA Standard (Zymo Research, Freiburg, Germany) and Human Genomic DNA (Roche, Basel, Switzerland) were used as positive and negative controls, respectively.

PTEN Expression Analysis

For the reverse-transcriptase quantitative PCR (RT-qPCR), total RNA (1 μg) was reverse transcribed with SuperScript III (Life Technologies, Carlsbad, CA, USA). The cDNA product was amplified using qPCR MasterMixPlus for SYBR®Green I without UNG (Eurogentec, Seraing, Belgium) in a Light Cycler 480 (Roche). The endogenous reference *GADPH* gene and the normal stomach tissues were used as references to normalize the results. *PTEN*

Table 1 Clinical, histopathologic, and molecular findings of 112 GIST under study

			Primary tumor						PTEN loss		PTEN expression			
No.	*Age (years)*	*Gender*	*Primary site*	*Size (mm)*	*MI (per 50 HPF)*	*Type of tissue at the time of surgery*	*Primary genotype*	*Secondary mutation*	*aCGH*	*FISH*	*RT-PCR*	*IHC*	*PTEN methylation*	*PTEN mutation*
1	65	M	Colon	25	35	IM-PD	KIT 11 p.D579del	Not detected	ND	Yes	0.1	0	Negative	ND
2	59	F	Stomach	100	52	IM-PD	KIT 11 p.Q556_V559delinsH	KIT 13 p.V654A	ND	Yes	0.38	0	Negative	Negative
3	54	F	Intraabd	NA	NA	IM-PD	WT	KIT ampl	ND	Yes	ND	0	Negative	ND
4	42	M	Stomach	100	NA	IM-PD	KIT 11 p.W557_K558del	KIT 14 p.T670I	ND	Yes	ND	0	Negative	Negative
5	47	F	Sm Int	10	7	IM-PD	KIT 11 p.V559D	KIT; p.D820G	ND	Yes	0.55	0	Negative	Negative
6	49	M	Stomach	65	120	IM-PD	KIT 11 p. K558_G565delinsR	Not detected	ND	Yes (homo)	0.04	0	ND	ND
7	51	M	Sm Int	12	>10	IM-PD	KIT 11 p.K550_K558del	KIT 17 p.D820Y	ND	Yes	0.1	0	Negative	ND
8	70	M	Stomach	NA	NA	IM-PD	KIT 11 p.W557_K558del homo	KIT 14 p.T670I	ND	No	ND	0	Negative	ND
9	39	M	Duod	NA	NA	IM-PD	KIT 11 p.W557_K558del	Not detected	ND	Yes	ND	0	Negative	ND
10	55	M	Colon	NA	NA	IM-PD	KIT 11 p.V569_L576del	Not detected	ND	Yes	0.01	0	Negative	Negative
11	47	M	Sm Int	NA	38	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	0.95	1	Negative	Negative
12	59	M	Sm Int	NA	NA	IM-PD	KIT 11 p.L576P	KIT 17 p.D820Y	ND	Yes	0.43	1	Negative	Negative
13	40	M	Intraab	240	25	IM-PD	KIT 11 p.V559G	KIT 13 p.V654A	Yes	Yes	ND	1	Negative	Negative
14	67	M	Sm Int	40	60	IM-PD	KIT 11 p.K550_K558delinsQ	KIT 17 p.D820Y	ND	No	1.75	1	Negative	ND
15	45	M	Sm Int	60	28	IM-PD	KIT 11 p.W557_T574del homo	KIT 17 p.N822K	ND	Yes	ND	1	Negative	Negative
16	57	F	Stomach	150	NA	IM-PD	KIT 11 p.P573_T574dup	KIT 13 p.V654A	ND	Yes	ND	1	Negative	Negative
17	45	M	Sm Int	9	40	IM-PD	KIT 11 p.N567_L576delinsI	Not detected	ND	No	2.5	1	Negative	ND
18	52	F	Sm Int	NA	25	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	Yes	0.54	1	Negative	Negative
19	45	M	Sm Int	35	75	IM-PD	KIT 11 p.W557_K558del	KIT 17 p.D816G	No	No	1.34	1	Negative	ND
20			Colon meta	13	20	Met	KIT 11 p.W557_K558del	ND	No	No	ND	1	Negative	ND
21	59	F	Sm Int	8	3	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	Yes	ND	1	Negative	ND
22	33	F	Stomach	Multiple	46	IM-PD	WT	Not detected	ND	Yes	0.3	1	Negative	ND
23	46	M	Sm Int	NA	NA	IM-PD	KIT 11 p.K558delinsNP	Not detected	ND	Yes	ND	1	Negative	ND
24	64	M	Sm Int	40	15	IM-PD	KIT 9 p.A502_Y503dup	Not detected	No	No	ND	1	ND	ND
25	65	M	Rectum	NA	47	IM-PD	KIT 11 p.W557_V559delinsF	Not detected	ND	Yes	0.31	1	Negative	Negative
26	38	F	Sm Int	90	9	IM-PD	KIT 11 p.Q556_E561delinsQ	Not detected	No	No	0.97	2	Negative	ND
27	49	M	Colon	75	15	IM-PD	KIT 11 p.M552_E554del	BRAF V600E	ND	No	ND	2	Negative	Negative
28	72	M	Rectum	NA	4	IM-PD	KIT 11 p.K558N	BRAF V600E	ND	No	ND	2	ND	ND
29	58	M	Colon	>10	>10	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	1.05	2	ND	ND
30	63	F	Sm Int	12	10	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	0.93	2	Negative	ND
31	56	F	Stomach	95	10	IM-PD	WT	Not detected	ND	No	1.8	2	ND	ND
32	56	M	Sm Int	10	14	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	1.21	2	Negative	Negative
33	43	M	Stomach	8	15	IM-PD	KIT 11 p.W557_K558del hom	KIT 13 p.V654A	ND	No	1.56	2	Negative	ND
34	55	F	Sm Int	70	11	IM-PD	KIT 11 p.W557R	KIT 17 p.N822K	No	No	1.19	2	Negative	Negative
35	51	M	Sm Int	15	0	IM-PD	KIT 11 p.W557_K558del	KIT 17 p.N822K	ND	No	1.25	2	ND	ND
36	76	M	Sm Int	NA	55	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	0.53	3	Negative	ND
37	50	F	Stomach	NA	52	IM-PD	WT	Not detected	No	No	3.79	3	ND	ND
38	41	M	Colon	38	14	IM-PD	KIT 9 p.A502_Y503dup	Not detected	No	No	1.23	3	ND	ND
39			Colon	80	2	Met	KIT 9 p.A502_Y503dup	ND	No	ND	1.23	3	Negative	Negative
40	44	M	Intraab	NA	25	IM-PD	KIT 9 p.A502_Y503dup	KIT 13 p.V654A	ND	No	1.36	3	Negative	Negative
41	12	F	Stomach	Multiple	25	IM-PD	WT	Not detected	No	No	ND	3	ND	ND
42	22	F	Stomach	230	51	IM-PD	KIT 11 p.I563_Q575del	KIT 17 p.D820Y	ND	No	ND	3	ND	ND
43	41	F	Sm Int	150	17	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	Yes	0.49	3	ND	ND
44	66	F	Stomach	50	126	IM-PD	PDGFRA 18 p.D842V	Not detected	ND	No	3.16	3	ND	ND
45	56	M	Sm Int	10	100	IM-PD	KIT 11 p.E554_D572del	KIT 13 p.V654A	ND	No	1.9	3	ND	ND
46	54	M	Sm Int	16	20	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	1.63	3	Negative	Negative
47	56	M	Sm Int	2	10	IM-PD	KIT 11 p.V560E homo	Not detected	ND	No	0.86	3	ND	ND
48	73	M	Sm Int	10	60	IM-PD	KIT 11 p.V560D	KIT 13 p.V654A	ND	No	ND	3	ND	ND
49	37	F	Stomach	NA	NA	IM-PD	WT	Not detected	ND	Yes	0.21	3	ND	ND
50	43	F	Sm Int	6	15	IM-PD	KIT 9 p.A502_Y503dup	Not detected	ND	No	1.6	3	Negative	Negative
51	50	M	Sm Int	7	2	IM-RESP	KIT 11 p.M552_W557del	Not detected	ND	No	2.08	1	Negative	Negative

Table 1 (Continued)

No.	Age (years)	Gender	Primary tumor: Primary site	Primary tumor: Size (mm)	Primary tumor: MI (per 50 HPF)	Type of tissue at the time of surgery	Primary genotype	Secondary mutation	PTEN loss: aCGH	PTEN loss: FISH	PTEN expression: RT-PCR	PTEN expression: IHC	PTEN methylation	PTEN mutation
52	34	F	Stomach	6	10	IM-RESP	WT	Not detected	ND	No	ND	2	Negative	ND
53	47	F	Sm Int	12	1	IM-RESP	KIT 11 p.K558_G565delinsR	Not detected	ND	ND	2.43	1	ND	ND
54	33	M	Sm Int	>10	12	IM-RESP	KIT 11 p.V559_Y568delinsDND	Not detected	ND	Yes	ND	1	Negative	ND
55	19	F	Stomach	25	10	IM-RESP	PDGFRA ex4	Not detected	ND	No	ND	2	Negative	ND
56	49	F	Sm Int	NA	NA	IM-RESP	KIT 11 p.W557_K558del	Not detected	ND	No	1.75	2	Negative	Negative
57	74	M	Stomach	90	200	Met	KIT 11 p.V560D	ND	Yes	Yes	0.4	1	Negative	Negative
58	63	F	Sm Int	NA	21	Met	KIT 9 p.A502_Y503dup	ND	ND	No	0.82	1	Negative	Negative
59	61	F	Liver, primary unknown	NA	55	Met	PDGFRA 18 p.R841_M844delinsR homo	ND	Yes	ND	ND	2	ND	ND
60	61	M	Sm Int	80	5	Met	KIT 17 p.N822K	ND	No	No	2.23	2	Negative	Negative
61	78	M	Sm Int	280	5	Met	KIT 11 p.W557_V559delinsF	ND	No	No	0.74	1	Negative	ND
62	25	F	Stomach	170	31	High	PDGFRA 18 p.D842_H845del homo	ND	No	No	ND	1	ND	ND
63	48	F	Stomach	70	14	High	PDGFRA 18 p.D842V	ND	No	ND	ND	1	ND	ND
64	61	M	Sm Int	38	7	High	KIT 9 p.A502_Y503dup	ND	No	No	1.02	1	ND	ND
65	68	M	Sm Int	76	8	High	KIT 11 p.M552_E554delinsK	ND	No	No	ND	1	Negative	Negative
66	57	M	Stomach	>10	23	High	KIT 11 p.W557_V559delinsF	ND	No	No	1.45	1	ND	ND
67	65	M	Sm Int	150	17	High	KIT 9 p.A502_Y503dup	ND	No	ND	ND	2	Negative	Negative
68	65	F	Stomach	180	60	High	KIT 11 p.W557_V560delinsF	ND	No	No	2.0	3	ND	ND
69	69	M	Mesent	120	21	High	KIT 11 p.V560D	ND	ND	Yes	ND	3	Negative	Negative
70	48	F	Stomach	50	37	High	KIT 11 p.K550-V555del	ND	ND	No	1.33	3	ND	ND
71	59	M	Sm Int	35	7	High	KIT 11 p.V560_L576delinsD	ND	No	ND	ND	3	Negative	Negative
72	78	M	Sm Int	55	12	High	KIT 11 p.V560D	ND	ND	No	1.03	3	ND	ND
73	72	M	Oesoph	20	11	High	KIT 11 p.K558_V559delinsN homo	ND	No	No	1.0	3	ND	ND
74	44	F	Stomach	160	4	High	KIT 11 p.W557R	ND	Yes	Yes	0.84	3	ND	ND
75	69	M	Stomach	40	11	Intermed	PDGFRA 18 p.D842_H845del	ND	No	ND	ND	2	ND	ND
76	83	F	Stomach	85	7	Intermed	KIT 11 p.V560D	ND	ND	No	1.06	2	ND	ND
77	75	F	Stomach	110	1	Intermed	PDGFRA 18 p.D842V	ND	No	ND	ND	2	ND	ND
78	69	M	Stomach	>100	4	Intermed	KIT 11 p.L576_R588dup	ND	No	ND	0.96	2	ND	ND
79	51	M	Stomach	130	2	Intermed	PDGFRA 18 p.D842V	ND	No	ND	ND	2	ND	ND
80	76	F	Stomach	30	17	Intermed	PDGFRA 18 p.D842V	ND	ND	ND	0.76	2	Negative	Negative
81	53	M	Duod	55	5	Intermed	KIT 11 p.L576P	ND	No	No	1.59	3	ND	ND
82	68	F	Duod	30	4	Intermed	KIT 11 p.V560A	ND	No	ND	1.39	3	ND	ND
83	67	F	Stomach	40	25	Intermed	KIT 11 p.W557R	ND	No	No	1.58	3	ND	ND
84	50	F	Sm Int	50	5	Intermed	KIT 9 p.A502_Y503dup	ND	No	No	4.08	3	ND	ND
85	36	F	Stomach	35	60	Intermed	KIT 11 p.W557-K558del homo	ND	No	No	ND	3	ND	ND
86	47	F	Duod	60	2	Intermed	KIT 11 p.P577_R588dup; p.L589S	ND	No	ND	ND	3	Negative	Negative
87	77	F	Eosoph	100	5	Intermed	KIT 9 p.A502_Y503dup	ND	ND	ND	ND	3	ND	ND
88	85	M	Stomach	60	5	Low	KIT 11 p.V559A	ND	ND	No	1.43	2	ND	ND
89	61	M	Stomach	55	5	Low	KIT 11 p.V554D	ND	No	No	2.29	2	ND	ND
90	53	M	Stomach	60	5	Low	KIT 11 p.V559D	ND	ND	gain of chr. 10	1.74	2	ND	ND
91	49	M	Stomach	75	4	Low	PDGFRA 18 p.D842V	ND	No	No	ND	2	Negative	Negative
92	64	M	Stomach	75	6	Low	KIT 11 p.W557_K558del	ND	ND	ND	0.74	2	ND	ND
93	62	M	Stomach	90	5	Low	PDGFRA 18 p.D842V	ND	No	ND	ND	2	Negative	Negative
94	72	M	Sm Int	48	3	Low	KIT 11 p.V560D	ND	No	No	0.83	2	ND	ND
95	46	F	Stomach	50	5	Low	KIT 11 p.K558_G565delinsN	ND	Yes	No	ND	2	Negative	Negative
96	80	F	Stomach	55	3	Low	KIT 11 p.581-590insKWEFPRNRLS	ND	ND	No	ND	2	Negative	Negative
97	55	M	Stomach	50	5	Low	PDGFRA 14 p.N659K	ND	No	ND	ND	3	ND	ND
98	68	M	Stomach	30	6	Low	KIT 11 p.Q556_I563del	ND	No	No	1.55	3	ND	ND
99	44	M	Stomach	80	5	Low	PDGFRA 18 p.D842V	ND	No	ND	ND	3	ND	ND

Table 1 (Continued)

No.	Age (years)	Gender	Primary tumor: Primary site	Primary tumor: Size (mm)	Primary tumor: MI (per 50 HPF)	Primary tumor: Type of tissue at the time of surgery	Primary genotype	Secondary mutation	PTEN loss: aCGH	PTEN loss: FISH	PTEN expression: RT-PCR	PTEN expression: IHC	PTEN methylation	PTEN mutation
100	71	M	Stomach	55	1	Low	PDGFRA 18 p.I843_D846del	ND	No	No	ND	3	Negative	Negative
101	83	M	Stomach	50	4	Low	KIT 11 p.573_574dup; T574dup; Q575_R586dup	ND	No	ND	2.51	3	Negative	ND
102	73	F	Stomach	85	1	Low	KIT 11 p.T574_R586insK	ND	No	ND	ND	3	Negative	Negative
103	77	F	Stomach	60	4	Low	PDGFRA 12 p.D561V	ND	No	No	ND	3	ND	ND
104	60	M	Stomach	18	2	Very low	KIT 11 p.D572_D579dupinsL	ND	No	ND	1.2	2	ND	ND
105	82	F	Stomach	20	4	Very low	KIT 11 p.W557R	ND	No	ND	ND	2	ND	ND
106	70	F	Stomach	25	0	Very low	KIT 11 p.L576P	ND	ND	NO	0.97	3	ND	ND
107	75	M	Stomach	30	3	Very low	PDGFRA 14 p.N659K	ND	No	ND	ND	3	ND	ND
108	64	F	Stomach	25	2	Very low	KIT 11 p.W557R	ND	No	ND	2.99	3	ND	ND
109	54	M	Stomach	15	0	Very low	KIT 11 p.P573_H580ins	ND	No	ND	1.8	3	ND	ND
110	66	F	Stomach	35	5	Very low	PDGFRA 18 p.D842_H845del	ND	No	NO	ND	3	Negative	Negative
111	73	F	Stomach	45	4	Very low	PDGFRA 18 p.D842V	ND	No	ND	ND	3	ND	ND
112	63	F	Stomach	25	2	Very low	PDGFRA 18 p.D842V	ND	No	ND	1.33	3	ND	ND

Abbreviations: ampl, amplification; Duod, duodenum; homo, homozygous; IM-PD, progressive disease on imatinib; IM-RESP, responsive to imatinib; Intraabd, intraabdominal; Intermed, intermediate risk; Met, metastasis; NA, not available; ND, not done; Oesophag, oesophagus; Sm Int, small intestine.

expression was considered abnormal if the ΔΔCt *PTEN/GADPH* value was <0.6.

The PTEN protein expression was assessed by immunohistochemistry in 112 GIST using anti-PTEN antibody (DAKO, Glostrup, Denmark; dilution 1:1000) previously validated on GIST xenografts with a known *PTEN* status.[12] Scoring for PTEN staining was semi-quantitative, based on the proportion and intensity of positive neoplastic cells (Figure 1). It was performed in parallel by two observers using a four-tier system, ranging from 3+ (>50% immune-reactive cells and strong intensity equal to that of the vascular endothelium; Figures 1a and b), 2+ (>50% immune-reactive cells and weak intensity of staining; Figures 1c and d), 1+ (<50% immune-reactive cells, referred to as reduced staining; Figure 1e) to 0 (completely negative; Figure 1f). The categories 0 and 1+ were considered to be abnormal.

In vitro siRNA PTEN Experiments

In vitro studies were performed on imatinib-sensitive GIST-T1 (carrier of *KIT*-V560_Y579del mutation) and imatinib-resistant GIST430 (carrier of primary *KIT*-V560_L576del and secondary *KIT*-V654A mutations) cell lines.

The compounds, imatinib mesylate and the dual PI3K/mTOR inhibitor NVP-BEZ235,[16] were purchased from Sequoia Research Products (Pangbourne, UK).

The siRNA experiments were performed by the use of predesigned stealth RNAi duplexes against human PTEN (PTEN Validated Stealth RNAi: PTENHSS183790+PTENHSS183791+PTENHSS183792; Invitrogen). Transient transfection was done in six-well plates at a density of 10^6 cells/plate with use of metafectene transfection reagent (Biontex, Planegg, Germany), according to the manufacturer's instructions. After 96 h, medium was exchanged for the one supplemented with dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA) only, or 100 nM NVP-BEZ235, or 200 nM imatinib, or the combination of both drugs in a given concentration. After 2 h of treatment, the cell pellets were collected for protein analysis by western blotting. The antibodies against total KIT and total PTEN (DAKO), total MAPK (Invitrogen), phospho-KIT, phospho-AKT, total AKT, phospho-MAPK, phospho-S6, total S6 (Cell Signaling, Beverly, MA), and B-actin (Sigma-Aldrich) were applied.

Statistics

For statistical analysis of the qPCR data, the Mann–Whitney *U*-test was applied. For analyses of the frequency of the genomic *PTEN* losses and the abnormal PTEN expression by immunohistochemistry, the χ^2 or Fisher exact tests were applied.

Table 2 Primers for *PTEN* mutational analysis and quantitative RT-PCR

Target	Sequence (5′→3′)
Amplicons for PTEN mutation analysis	
PTEN exon 1	F: TTCCATCCTGCAGAAGAAGC
	R: CTACGGACATTTTCGCATCC
PTEN exon 2	F: AGTATTCTTTTAGTTTGATTGCTGCAT
	R: CACAAAGTATCTTTTTCTGTGGCTTA
PTEN exon 3	F: GAAAATCTGTCTTTTGGTTTTTCTTG
	R: TGGACTTCTTGACTTAATCGGTTT
PTEN exon 4	F: TCACATTATAAAGATTCAGGCAATGT
	R: GTATCTCACTCGATAATCTGGATGACT
PTEN exon 5	F: CCTGTTAAGTTTGTATGCAACATTTCT
	R: TCCAGGAAGAGGAAAGGAAAA
PTEN exon 6	F: AATGGCTACGACCCAGTTACC
	R: TCAAATGCTTCAGAAATATAGTCTCCT
PTEN exon 7	F: AATCCATATTTCGTGTATATTGCTGA
	R: CACCTGCAGATCTAATAGAAAACAAA
PTEN exon 8	F: TGTCATTTCATTTCTTTTTCTTTTCTT
	R: AAGTCAACAACCCCCACAAA
PTEN exon 9	F: TGTTCATCTGCAAAATGGAATAAA
	R: CACAATGTCCTATTGCCATTAAAA
Amplicons for quantitative RT-PCR	
PTEN ex 6/7	F: CAATGTTCAGTGGCGGAACTT
	R: TGAATTGGAGGAATATATCTTCACCTT
PTEN ex 6-7/7	F: TGGCGGAACTTGCAATCC
	R: TGGGTCCTGAATTGGAGGAA
GADPH	F: TGACACTGGCAAAACAATGCA
	R: GGTCCTTTTCACCAGCAAGCT

Differences with *P*-value <0.05 were defined as statistically significant. The software STATISTICA (Stat Soft, USA—version 9.0) was used for statistical calculations.

Results

PTEN Mono-Allelic Loss Occurs in the Progressive Stage of Disease and is Frequent in Imatinib-Treated Tumors

To assess the incidence of *PTEN* loss at the genomic level, we performed FISH analysis of 85 samples, and complemented these data with aCGH results available from 54 cases (Table 1). In total, loss of *PTEN* occurred in 26 out of 108 samples (24%); all of those tumors presented mono-allelic loss of the *PTEN* locus except for one, characterized by *PTEN* nullisomy. Concurrent analysis of *PTEN* status by FISH and aCGH indicated concordance of the results in 30 out of 31 cases; only in one specimen, FISH failed to identify a *PTEN* deletion otherwise seen by aCGH.

Within the imatinib-naïve group, *PTEN* loss was observed less frequently in very low/low/intermediate risk *vs* high risk/metastatic GIST ($P=0.03$; Table 3). Of note, the incidence of *PTEN* loss in imatinib-treated tumors was high (39%). As we did not have baseline pre-treatment tumor specimens available for comparison, we could not discriminate whether *PTEN* loss added to acquired resistance to imatinib or it was only a reflection of a more advanced stage of disease. Nevertheless, there was no correlation between the incidence of *PTEN* loss and the presence of secondary imatinib-resistant *KIT* mutations in GIST refractory to imatinib ($P=0.6$). Moreover, no significant differences were found between primary high risk/metastatic and imatinib-treated GIST ($P=0.11$). These results argue against the hypothesis that *PTEN* loss would be a direct cause of resistance to imatinib.

To further elucidate possible mechanisms responsible for aberrant PTEN expression, we performed mutational analysis of *PTEN* in 36 specimens (including 19 imatinib-progressive GIST). No somatic mutations were detected. In addition, we assessed *PTEN* promoter methylation in a total of 57 samples (including 24 imatinib-resistant GIST that lacked or expressed low level of PTEN protein by immunohistochemistry). None of the examined tumors exhibited methylation of *PTEN* promoter.

PTEN Mono-Allelic Loss Correlates with Altered PTEN Expression on Transcript and Protein Levels

By RT-qPCR, we investigated the expression of *PTEN* transcripts in 67 GIST (including 36 imatinib-resistant tumors). Abnormal *PTEN* expression was detected in 21% of samples. The low level of *PTEN* transcript expression correlated well with *PTEN* loss on the genomic level ($P=0.002$).

By immunohistochemistry, absent or reduced expression of PTEN protein was observed in 32% ($n=36$) of the tumors (Table 3). In the imatinib-naïve cohort, abnormal PTEN protein level was detected in high-risk/metastatic but not in low/intermediate-risk tumors (45% *vs* 0%; $P<0.001$). In imatinib-treated tumors, abnormal PTEN expression was common (50%); notably, all 10 completely PTEN-immuno-negative GIST were from imatinib-progressive cohort. The abnormal PTEN protein expression correlated with *PTEN* loss at the genomic level ($P=0.001$).

siRNA-Induced Downregulation of PTEN Expression in GIST Cell Lines Results in PI3K-AKT-mTOR and MAPK Pathway Activations

In the *PTEN*si GIST-T1 and GIST430 cells, the level of PTEN protein was decreased by ~50% and 80%, respectively, compared with non-*PTEN*si cells (Figure 2).

In IM-S GIST-T1, *PTEN* silencing resulted in overactivation of AKT and MAPK (by 1.6- and 1.8-fold, respectively). The AKT hyper-phosphorylation was partially reverted by 100 nM NVP-BEZ235 in both *PTEN*si and non-*PTEN*si cells (by 70% and 85%, respectively). As expected, exposure to 200 nM imatinib led to complete AKT inactivation in non-*PTEN*si cells, opposite to *PTEN*si cells, in

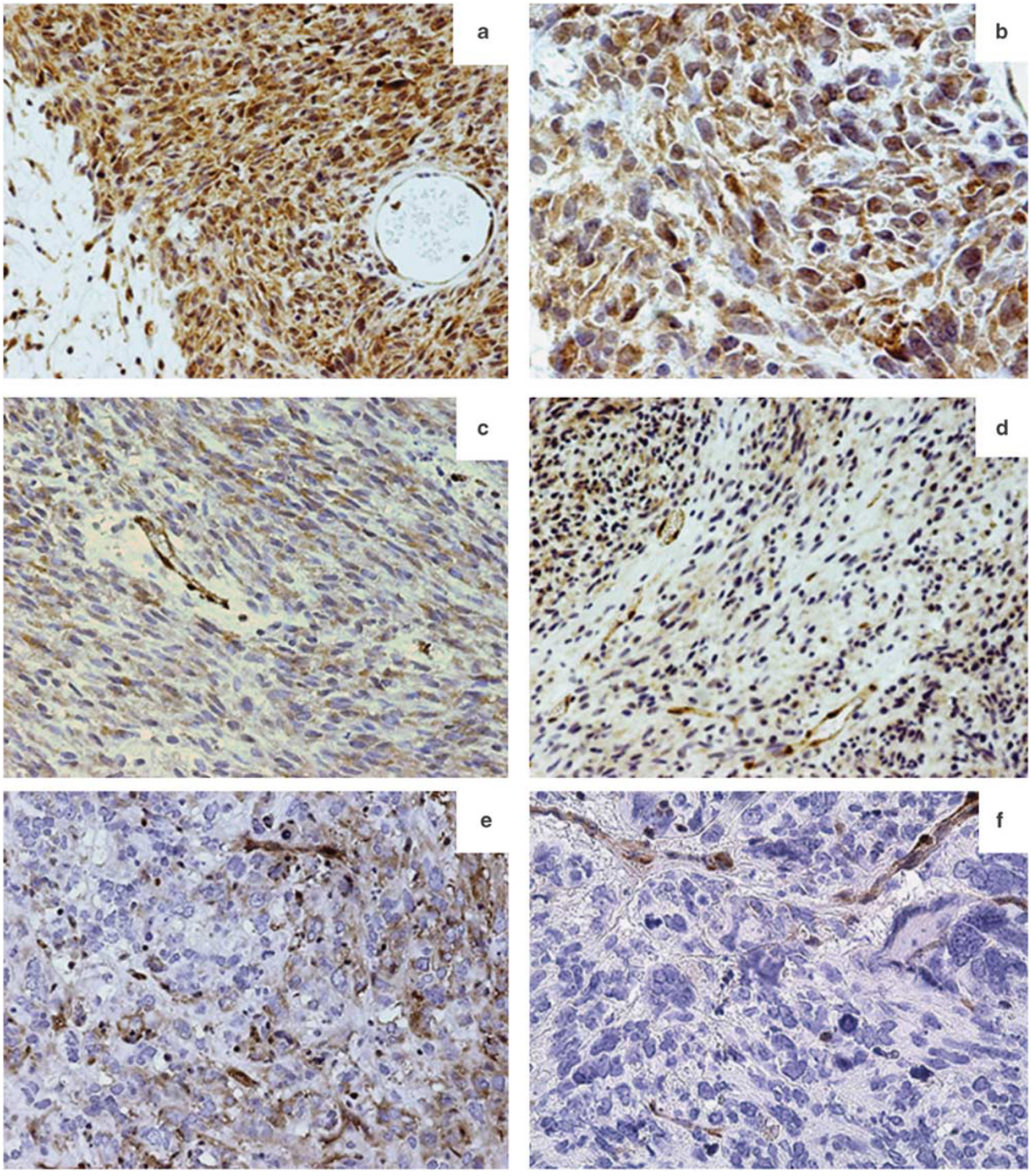

Figure 1 Examples of the PTEN immunostaining in GIST. Normal staining was defined as intense, cytoplasmic, and nuclear PTEN immunoreactivity in majority of tumor cells, with the staining intensity equal to that of the vascular endothelium (which served as internal positive control); original magnifications $\times 100$ (**a**) and $\times 400$ (**b**). The weaker reactivity of neoplastic cells in comparison with the vascular endothelium in $>50\%$ of the neoplastic cells; original magnification $\times 200$ (**c**) and $\times 100$ (**d**). Reduced (**e**) or absent (**f**) PTEN expression in tumor specimens; original magnification $\times 200$. Immunostains counterstained with hematoxylin.

which still redundant AKT signaling existed, as proven by incomplete downstream p-S6 inhibition (sixfold higher in *PTEN*si *vs* non-*PTEN*si cells). This effect disappeared under the combined treatment regimen.

Interestingly, NVP-BEZ235 treatment gave rise to MAPK phosphorylation in non-*PTENsi* cells and at higher extent in *PTEN*si cells (2- and 2.8-fold, respectively). The MAPK activation was substantially and equally abolished by imatinib alone or

Table 3 Correlation of PTEN loss by FISH/aCGH and PTEN protein expression by immunohistochemistry with clinicopathological data in GIST under study

Categories	*PTEN by FISH/aCGH*					*PTEN protein by immunohistochemistry*					
	n	*With loss*	*% Of total*	*Statistical analysis*	P-*value*	n	*Absent*	*Reduced*	*Abnormal % of total*	*Statistical analysis*	P-*value*
Total	108	26	24			112	10	26	32		
Gender											
Male	63	14	22	Male *vs* female	0.59	64	7	18	39	Male *vs* female	0.07
Female	45	12	27			48	3	8	22		
Primary tumor site											
Gastric	52	9	17	Gastric *vs* non-gastric	0.11	54	4	6	18	Gastric *vs* non-gastric	0.002
Non-gastric	56	17	30			58	6	20	45		
GIST category											
Imatinib naïve[a]	55	5	9	Imatinib naïve *vs* imatinib treated	<0.001	58	0	9	16	Imatinib naïve *vs* imatinib treated	<0.001
V. low/Low risk	24	1	4	High risk/meta *vs* imatinib treated	0.11	25	0	0	0.0	High/meta *vs* imatinib treated	0.7
Inter. risk	11	0	0	High risk/meta *vs* low/Inter. risk	0.03	13	0	0	0.0	High/meta *vs* V. low/low/Inter. risk	<0.001
High risk	13	2	15	High risk *vs* meta	0.48	13	0	5	38	High/Inter. *vs* low risk	0.02
Meta	7	2	28			7	0	4	57	High *vs* Inter./low risk	<0.001
Imatinib treated	53	21	40			54	10	17	50		
Mutation status										WT *vs* PDGFRA	0.2
KIT mutants	83	22	26	*KIT* 9 *vs KIT* 11 mutants	0.2	58	9	23	37.2	*KIT* 9 *vs KIT* 11	0.5
PDGFRA mutants	18	1	6	*KIT vs PDGFRA* mutants	0.054	19	0	2	10.5	*KIT vs PDGFRA*	0.02
KIT/*PDGFRA*-WT	7	3	43	*KIT* mutants *vs* WT	0.3	7	1	1	28.5	*KIT* mutants *vs* WT	0.6
With secondary *KIT* mutation	21	9	43	With secondary *KIT* mutation *vs* without	0.6	21	6	6	57.1	With secondary *KIT* mutation *vs* without	0.4

Abbreviations: aCGH, array comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; Inter., intermediate; Meta, metastasis; V. low, very low; WT, wild type.
[a]Risk of recurrence for primary imatinib naive GIST was assessed according to the Armed Forces Institute of Pathology criteria.[23]

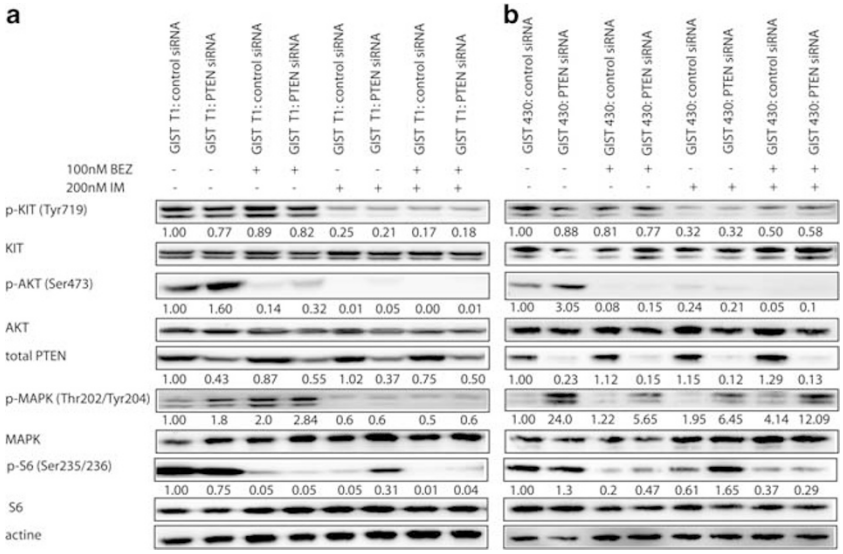

Figure 2 Short interfering (siRNA) knockdown of *PTEN* in imatinib-sensitive GIST-T1 (**a**) and imatinib-resistant GIST430 (**b**) cell lines. The effect of PTEN silencing on KIT downstream signaling was evaluated by western blotting under DMSO, imatinib, or NVP-BEZ235 alone, or combined treatment. For densitometry analysis, bands were normalized to actin expression and compared with control (diluting medium) as previously described.[12]

under the combined treatment in both, non-*PTEN*si and *PTEN*si cells.

In imatinib-resistant GIST430 cells, knockdown of *PTEN* induced the increase of p-AKT (3-fold) and even more remarkable of p-MAPK (24-fold). The AKT phosphorylation was significantly inhibited under NVP-BEZ235 or imatinib treatment alone or combination in *PTEN*si and in non-*PTEN*si cells. In contrast, MAPK hyperactivation in *PTEN*si cells was still substantially higher compared with control cells under both NVP-BEZ235 (5.6-fold) and imatinib (6.4-fold). Noteworthy, combined treatment of NVP-BEZ235 with imatinib led still to a 4- and 12-fold overactivation of MAPK in non-*PTEN*si and *PTEN*si cells, respectively. The S6 protein, downstream intermediate of the mTOR pathway, was overactivated by 30% in *PTEN*si cells in comparison with control. The NVP-BEZ235 treatment resulted in reduction of S6 phosphorylation in non-*PTEN*si and, to a lesser extent, in *PTEN*si cells (80 and 50%, respectively). Imatinib treatment induced 40% reduction of p-S6 in control cells; in contrast, a 60% increase in the level of p-S6 was observed in *PTEN*si cells. Markedly, this overactivation of S6 protein in *PTEN*si *vs* non-*PTEN*si cells was only partially reverted by combined treatment.

Discussion

First, we carried out FISH and/or aCGH analysis of *PTEN* in a heterogeneous cohort of GIST. Mono-allelic *PTEN* loss occurred in 24% of cases, whereas bi-allelic *PTEN* loss was encountered only in one tumor. In the imatinib-naïve GIST, a positive correlation between mono-allelic loss of *PTEN* and high-risk/metastatic tumors were found ($P=0.03$). These findings are in agreement with aCGH analysis reported by Ylipää *et al*,[17] as they observed the loss of chromosome 10q (on which *PTEN* maps) in the specific GIST subgroup, in patients with poor clinical outcome. As such, this event is likely to occur in the late stage of GIST evolution. As revealed by RT-qPCR analysis, *PTEN* loss was associated with substantially lower or absent *PTEN* transcript levels ($P=0.002$). In contrast to Yang *et al.*,[18] we do not have evidence that receptor tyrosine kinase inhibitor treatment can cause loss of PTEN expression by epigenetic silencing, as we did not identify PTEN promoter methylation in any of the analyzed imatinib-resistant GIST. This finding together with the lack of evidence for inactivation of the *PTEN* by inactivating mutations suggests that the aberrantly low *PTEN* expression in GIST might be mainly due to mono-allelic loss.

Our findings were corroborated at the protein level by immunohistochemical analysis. In the imatinib-naïve cohort, reduced PTEN expression was mainly associated with the high-risk/metastatic tumors ($P<0.001$). An inverse correlation between PTEN immunoreactivity and disease progression in primary GIST has been previously reported.[19,20] In our imatinib-treated GIST, the frequency of tumors with reduced or absent PTEN expression reached 50%. This result is notable because to the best of our knowledge data about the incidence of PTEN deficiency in imatinib-treated GIST has not been reported as of yet. Importantly, a subset of imatinib-progressive GIST with mono-allelic *PTEN* loss showed complete lack of PTEN reactivity by

immunohistochemistry, suggesting a bi-allelic inactivation. In these cases, we cannot exclude the presence of microdeletions/rearrangements of the second *PTEN* allele that were under the detection limit of the techniques used in the current study. Alternatively, other inactivation mechanisms might have a role, as PTEN expression could be positively and negatively regulated by transcription factors or microRNAs, as well as posttranslationally regulated by phosphorylation, oxidation, and acetylation.[1,2] Downregulation of PTEN results in hyperactivation of the PI3K/AKT/mTOR pathway, leading to proliferative advantage of neoplastic cells. A similar role was attributed to PTEN in other types of cancers.[9]

Subsequently, we explored the effect of siRNA PTEN silencing on KIT downstream signaling *in vitro* using GIST cell lines. Noteworthy, in both imatinib-sensitive and imatinib-resistant GIST cells, *PTEN* silencing resulted in overactivation of both, AKT and MAPK; the latter being exceptionally hyper-phosphorylated in imatinib-resistant cells. Overactivation of AKT was expected, as PTEN is a negative regulator of the PI3K-AKT pathway, whereas the latter might be explained by the extensive mTOR-negative feedback loops and cross-talks between the PI3K and the RAS-mediated MAPK pathways that have been well documented among the signaling networks driving tumor progression.[21,22]

Finally, we have investigated the effect of PI3K/mTOR inhibition, alone or in combination with imatinib, on *PTEN*si GIST cell lines.

In imatinib-sensitive GIST-T1, NVP-BEZ235 treatment resulted in only partial inactivation of AKT in *PTEN*si cells, whereas enhancing the activation of MAPK. Nevertheless, the effect of PTEN silencing on AKT and MAPK activation in these cells was counteracted by imatinib alone and even more substantially by the combined treatment of NVP-BEZ235 and imatinib.

In imatinib-resistant GIST-430 cells, NVP-BEZ235 treatment led to less efficient AKT and MAPK inhibition in *PTEN*si in comparison to non-*PTEN*si cells. Likewise, imatinib did not inhibit sufficiently the overactivated MAPK and downstream S6 proteins in *PTEN*si cells. Combined treatment resulted in still substantial MAPK hyper-phosphorylation, and only partially counteracted S6 activation.

In summary, our data strongly support an important role for PTEN downregulation in GIST progression. Partial or total PTEN depletion occurs frequently in imatinib-resistant GIST. *In vitro* studies suggest that PTEN insufficiency leads to upregulation of the PI3K/AKT and MAPK pathways. Depending on the molecular context of the individual tumors, the MAPK activity might be even paradoxically further enhanced under certain dual-specific PI3K/mTOR inhibitors. Our results highlight the importance of molecularly sub-classifying GIST before exposing patients to innovative targeted treatments.

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Disclosure/conflict of interest

P Schöffski received research funding and honoraria for advisory functions from Novartis. P Rutkowski received honoraria from Novartis, Pfizer, and he served as a member of Advisory Board for Novartis and Bayer. All remaining authors have declared no conflict of interest.

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