V600E \textit{BRAF} mutations are alternative early molecular events in a subset of \textit{KIT/PDGFRA} wild-type gastrointestinal stromal tumours

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\textbf{ABSTRACT}

\textbf{Background:} A small subset (10–15%) of gastrointestinal stromal tumours (GISTs) lack mutations in \textit{KIT} and \textit{PDGFRA} (wild-type GIST). Recently, a novel \textit{BRAF} exon 15 mutation (V600E) was detected in imatinib-naive wild-type high-risk intestinal GISTs (4%). However, the frequency and distribution of \textit{BRAF} mutations within the spectrum of GISTs, and whether they might represent secondary events acquired during tumour progression, remain unknown.

\textbf{Methods:} 69 GISTs (39 \textit{KIT} mutants, 2 \textit{PDGFRA} mutants and 28 wild-type) were analysed for mutations in \textit{BRAF} exon 15 and \textit{KRAS} exon 2. To assess the stage at which these mutations might occur in GIST, a considerable number of incidental gastric (n = 23) and intestinal (n = 2) tumours were included.

\textbf{Results:} \textit{BRAF} mutations (V600E) were detected in 2 of 28 wild-type GISTs (7%), but in none of the 41 \textit{KIT/PDGFRA} mutants. No \textit{KRAS} mutation was detected. The two \textit{BRAF}-mutated GISTs measured 4 mm in diameter and originated in the gastric body and the jejunalum in two men (mean age, 76 years). Both tumours were mitotically inactive \textit{KIT}-positive spindle-cell GISTs that were indistinguishable histologically from their more common \textit{KIT}-mutated counterparts.

\textbf{Conclusion:} \textit{BRAF} mutations represent an alternative molecular pathway in the early tumorigenesis of a subset of \textit{KIT/PDGFRA} wild-type GISTs and are per se not associated with a high risk of malignancy. Mutations in \textit{KIT}, \textit{PDGFRA} and \textit{BRAF} were mutually exclusive in this study. Results from this and a previous study indicate that \textit{BRAF}-mutated GISTs show a predilection for the small bowel (four of five tumours), but this needs further evaluation in larger studies.

Gastrointestinal stromal tumours (GISTs), the most common primary gastrointestinal (GI) mesenchymal tumours, are \textit{KIT} positive and \textit{KIT}-signalling driven neoplasms. The stomach represents the most common site involved (60–70%), followed by the small bowel (20–30%).\textsuperscript{1} GISTs harbour activating \textit{KIT} mutations in a majority of cases (≥80%).\textsuperscript{2} Less commonly (10%), mutations in the homologue oncocogene platelet-derived growth factor receptor \textalpha{} (PDGFRA) are detected in GISTs that often display gastric location and epithelioid morphology.\textsuperscript{3} Activating mutations of \textit{KIT} or PDGFRA represent early initiating molecular events and are mutually exclusive. On the other hand, a minority of the cases (10–15%), particularly GISTs in paediatric patients,\textsuperscript{4} those affected by the Carney triad,\textsuperscript{7} neurofibromatosis 1 (NF1)-associated GISTs,\textsuperscript{8} and a small subset of sporadic adult GISTs, are wild-type for \textit{KIT} and \textit{PDGFRA}. The pathogenetic mechanisms underlying these wild-type GISTs are poorly understood. \textit{BRAF} is a member of the RAS–RAF–MEK–ERK pathway that is involved in cell cycle regulation and oncogenic modulation of cellular responses to growth signals by activating the mitogen-activated protein kinase (MAPK) pathway.\textsuperscript{9} \textit{BRAF} mutations are commonly detected in diverse benign and malignant tumours, including benign congenital and acquired melanocytic nevi (61%),\textsuperscript{7,9} malignant melanoma (50–70%),\textsuperscript{7,10–12} papillary thyroid carcinoma (35–60%),\textsuperscript{7,11} colorectal adenoma/carcinoma (5–20%)\textsuperscript{13} and others.\textsuperscript{7} Most \textit{BRAF} mutations cluster in a hot-spot at nucleotide 1799 in exon 15 leading to substitution of valine by glutamic acid at codon 600 (V600E).\textsuperscript{7} It has been demonstrated that \textit{BRAF} and \textit{KIT} mutations represent mutually exclusive alternative oncogenic events in malignant melanoma.\textsuperscript{10,12} This led Agaram et al to investigate a series of \textit{KIT/PDGFRA} wild-type GISTs for \textit{BRAF} mutations in a recent study.\textsuperscript{16} They detected V600E \textit{BRAF} mutations in 4% of wild-type GISTs, and in one patient with acquired imatinib resistance who lacked secondary \textit{KIT/PDGFRA} mutations or other apparent cause of resistance. However, no \textit{KIT/PDGFRA}-mutated imatinib-naive GISTs were analysed for \textit{BRAF} mutations. Accordingly, it remains unclear whether \textit{KIT/PDGFRA} mutations are mutually exclusive with \textit{BRAF} mutations. Furthermore, there is evidence that \textit{BRAF} mutations may act in a synergistic manner with other secondary concomitant genetic events, including \textit{KRAS} mutations and other genetic alterations, to enhance tumour progression and aggressiveness in melanoma, colorectal cancer and thyroid carcinoma.\textsuperscript{11,12,17–19} Taking the above observations into consideration, we analysed a series of \textit{KIT/PDGFRA} mutants and sporadic adult wild-type GISTs for \textit{BRAF} and \textit{KRAS} mutations to further explore the frequency of \textit{BRAF} mutations in GISTs, and to assess whether coexisting \textit{KRAS} mutation might be responsible for the reportedly aggressive clinical course of \textit{BRAF}-mutated GISTs.

\textbf{METHODS}

\textbf{Clinicopathological features and \textit{KIT/PDGFRA} mutation status of the cohort}

The study included 69 tumours from 49 patients. Seventeen patients had 2–3 distinct tumours. Some of the latter group of patients who had multiple
primary sporadic gastric tumours have been included in a previous study.\textsuperscript{20} In all, 41 tumours had a known tyrosine kinase mutation (35 had KIT exon 11 mutations, 4 had KIT exon 9 mutations, and 2 had PDGFR\textsuperscript{A} exon 18 mutations) and 28 tumours were wild-type for KIT exons 9, 11, 13, 17 and PDGFR\textsuperscript{A} exons 12, 14 and 18. KIT/PDGFR\textsuperscript{A} mutant tumours were of gastric (n = 29), small bowel (n = 9) and metastatic or unsure location (n = 3). KIT/PDGFR\textsuperscript{A} wild-type tumours originated in the stomach (n = 11), small bowel (n = 10), colon (n = 1), oesophagus (n = 1), and uncertain or metastatic sites (n = 5). According to the National Institutes of Health (NIH) consensus criteria,\textsuperscript{21} KIT/PDGFR\textsuperscript{A}-mutated tumours fitted the very low risk (n = 13), low risk (n = 10), intermediate risk (n = 1) and high risk (n = 9) categories. On the other hand, wild-type tumours with a known risk stratification were at very low risk (n = 12), low risk (n = 3), intermediate risk (n = 2) and high risk (n = 6) for aggressive behaviour. None of the patients had evidence of NF1, Carney triad or paediatric GISTs, and none had imatinib therapy prior to surgery.

**Molecular analysis for KIT, PDGFR\textsuperscript{A}, BRAF and KRAS mutations**

Extraction of tumour DNA from formalin-fixed paraffin-embedded material, PCR amplification, and direct sequencing of KIT exons 9, 11, 13, 17 and PDGFR\textsuperscript{A} exons 12, 14, 18 were performed using the methods previously described.\textsuperscript{22} For BRAF and KRAS mutation analysis, the sequences of the primers used for the PCR are listed below.

**BRAF** exon 15 forward:
5'-CTCCATGAAAGGTTCTACTCCGAGTAAATATTAG-3'  
BRAF exon 15 reverse:
5'-AGCCTCAATTTCTCTCAGGCTGGTGAAATATGG-3'  
KRAS exon 2 forward:
5'-TTTCTCTATGATGCTGAAATGTGCCG-3'  
KRAS exon 2 forward nested:
5'-TTTCTCTATGATGCTGAAATGTGCCG-3'
   
**KRAS** exon 2 reverse nested:
5'-GCTTTTTTGTAGCTGATTACATGCTGATA-3'.

BRAF and KRAS amplification required one step and two steps of amplification, respectively. For the first and the nested PCR, a 50 cycle amplification step was performed using an AmpliTaq Gold (Applied Biosystems, Foster City, California, USA) under the following conditions: 20 s at 95°C for denaturation, 10 s at 55°C for annealing, and 40 s at 72°C for elongation. Sequencing PCR and sequence analysis were performed as described previously.\textsuperscript{22} To investigate for a possible coexistence of different mutations in the same tumour, all tumours were examined for mutations in each of the above four genes, even if a mutation was detected in one gene.

**Verification PCR**

Each sample in which a mutation could be detected by PCR was reconsidered for amplification using the respective exon primers exclusively. Four independent amplifications were done, and all confirmed or excluded the mutations that were initially suspected.

**RESULTS**

**BRAF and KRAS mutation status**

Two of the 28 KIT/PDGFR\textsuperscript{A} wild-type tumours (7%) revealed a heterozygous BRAF V600\textsuperscript{E} mutation in exon 15 of the gene. On the other hand, none of the 41 tumours with known KIT or PDGFR\textsuperscript{A} mutations had a BRAF mutation. None of the 69 tumours had a mutation in exon 2 of the KRAS gene. Normal mucosa was analysed for BRAF mutation in both mutated cases and demonstrated a wild-type sequence, thus excluding a germline BRAF mutation.

**Pathological features of the BRAF mutant GISTs**

The two patients were males with a mean age of 76 years (table 1). Patient no. 1 had two tumours (3 and 4 mm in diameter) originating in the gastric body; both tumours were incidental autopsy findings (fig. 1A). One tumour harboured a BRAF mutation and the other was wild-type for KIT/PDGFR\textsuperscript{A}/BRAF/KRAS. Patient no. 2 had a 4 mm jejunal GIST found during surgery for gastric carcinoma. All tumours were mitotically inactive spindle-cell GISTs that were indistinguishable histologically from their KIT-mutated counterparts detected at comparable early stage. The gastric tumour from patient no. 1 showed variable stromal hyalinisation (fig. 1B). The small bowel tumour from patient no. 2 was moderately cellular with numerous skeinoid fibres (fig. 1C). All tumours strongly expressed CD117 (fig. 1D). CD34 was expressed by the gastric tumours, but not by the small bowel tumour. The latter expressed focally smooth muscle actin. P16 immunostaining showed weak, but variable, nuclear staining in scattered tumour cells in both cases (<20%, data not shown). Both tumours qualified as very low risk for aggressive behaviour according to the NIH consensus criteria,\textsuperscript{21} corresponding to prognostic group 1 in the Miettinen’s classification.\textsuperscript{2} The molecular findings from BRAF-mutated gastric GIST from patient no. 1 are illustrated in fig 2.
DISCUSSION

This study represents the second report of *BRAF* mutations in GISTs, and the first one to investigate *KIT/PDGFRA*-mutated GISTs for *BRAF* and *KRAS* mutations. In a recent study, Agaram et al detected the novel V600E *BRAF* mutation in 3 of 61 *KIT/PDGFRA* wild-type GISTs (4%) and in 1 of 25 patients with secondary imatinib resistance without detectable secondary mutations in *KIT/PDGFRA* (4%).16 All three patients in that study were middle-aged women with high-risk spindled small bowel GISTs, with occasional sclerosis and calcification in one case (table 1). The patient with secondary resistance had a *PDGFRA*-mutated primary epithelioid gastric GIST. The resistant clone harboured the primary *PDGFRA* mutation and the secondary V600E *BRAF* mutation. No mutations in the *NRAS* gene were detected in that study.16

In the current study, we demonstrated that the V600E *BRAF* mutation may occur in early-stage incidental GISTs at very low risk for malignant behaviour, thus suggesting gate-keeper alternative molecular events in a subset of *KIT/PDGFRA* wild-type GISTs. The two *BRAF* mutant tumours in our study were ≤5 mm in greatest diameter, mitotically inactive, *KIT*-positive and *KIT/PDGFRA* wild-type with spindle morphology. Their histology was indistinguishable from their *KIT*-mutated incidental counterparts reported in more detail previously.23 Analysis of normal tissue in both cases revealed a wild-type *BRAF* sequence, thus ruling out a germline mutation. None of our 41 *KIT/PDGFRA*-mutated GISTs revealed a *BRAF* mutation indicating that these mutations are mutually exclusive, similar to the observation for *KIT* and *BRAF* mutations in malignant melanoma.10 15

The oncogenic transforming properties of the *BRAF* V600E mutation have been demonstrated in diverse cancer types including melanocytes, colorectal cancer and thyroid cancer.11 12 17–19 24 In particular, it has been shown that mutant *BRAF* (V600E) cooperates functionally with Rac1b,25 Akt311 and other genetic pathways to sustain cell viability and proliferation in tumours and cancer cell lines. However, the prevalence of V600E *BRAF* mutations in congenital and acquired melanocytic naevi89 suggests that the mutation per se is not sufficient for tumour progression, and that additional genetic alterations and activation of other cooperating oncogenic pathways are needed for a tumour to gain a malignant potential.681 11 9 Our observations were in line with the previous report that considered loss of nuclear expression of the tumour suppressor oncoprotein p16 as a possible cofactor in the progression of *BRAF*-mutated GISTs30 and melanocytic lesions.24

Unfortunately, no frozen tissue was available to evaluate the oncogenic activity of the mutant *BRAF* in our cases. However, future studies might shed light on the mechanisms by which...
BRAF mutations represent early molecular events in the pathogenesis of a subset of wild-type gastrointestinal stromal tumours (GISTs). BRAF mutations are per se not indicative of malignancy, and other molecular events are needed for tumour progression. BRAF mutated GISTs are not distinguishable from KIT-mutated GISTs on morphological grounds. BRAF and KIT/PDGFRα mutations seem to be mutually exclusive events in GIST.

constitutively activated BRAF might influence KIT activation in this subset of GISTs.

Our observation, that BRAF mutations may occur in minute clinically benign GISTs of small bowel and stomach location without a significant predilection for certain age groups or gender, contrasts with the observations of Agaram et al. It is likely that this represents selection bias in both studies and that BRAF mutations probably occur in a wider range from benign incidental to clinically malignant GISTs in the spectrum of the disease. It is remarkable from the previous study and the current study that 4 of 5 GISTs with a primary BRAF mutation were of small bowel origin, suggesting a strong predilection for tumours arising in the small bowel. Whether BRAF-mutated GISTs would be more prone to genetic instability, and hence would follow a malignant course in later stages of the disease, remains unclear. However, there is sound evidence that BRAF mutations may facilitate the acquisition of secondary genetic events through induction of a higher genetic instability, thereby resulting in a more aggressive clinical course.17–18 Lack of NRAS mutations in the previous study16 and of KRAS mutations in our study suggests that mutations in the SAS family of oncogenes probably play no or little role in GIST pathogenesis, but this needs further validation.

In summary, we demonstrated that V600E BRAF mutations represent alternative early molecular events in a subset of KIT/PDGFRα wild-type GISTs and that they are mutually exclusive to KIT/PDGFRα mutations. The clinicopathological profiles of BRAF-mutated GISTs remain to be further delineated in larger future studies.

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