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Comprehensive molecular screening by next generation sequencing reveals a distinctive mutational profile of *KIT/PDGFRA* genes and novel genomic alterations: results from a 20-year cohort of patients with GIST from north-western Greece

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ABSTRACT

Introduction Gastrointestinal stromal tumours (GIST) are mesenchymal neoplasms that usually carry an activating mutation in *KIT* or platelet-derived growth factor receptor alpha (*PDGFRA*) genes with predictive and prognostic significance. We investigated the extended mutational status of GIST in a patient population of north-western Greece in order to look at geopraphic/genotypic distinctive traits.

Patient and methods Clinicopathological and molecular data of 38 patients diagnosed from 1996 to 2016 with GIST in the region of Epirus in Greece were retrospectively assessed. Formalin-fixed paraffin-embedded tumours were successfully analysed for mutations in 54 genes with oncogenic potential. Next generation sequencing was conducted by using the Ion AmpliSeqCancer Hotspot Panel V.2 for DNA analysis (Thermofisher Scientific).

Results Among 38 tumours, 24 (63.16%) and seven (18.42%) of the tumours harboured mutations in the *KIT* and *PDGFRA* genes, respectively, while seven (18.42%) tumours were negative for either *KIT* or *PDGFRA* mutation. No mutations were detected in five (13.16%) cases. Concomitant mutations of *BRAF* and fibroblast growth factor receptor 3 (*FGFR3*) genes were observed in two patients with *KIT* gene mutation. Two patients with *KIT*/*PDGFRA* wild-type GIST had mutations in either *KRAS* or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) genes. There was no significant survival difference regarding the exonic site of mutation in either *KIT* or *PDGFRA* gene. The presence of a mutation in pathway effectors downstream of *KIT* or *PDGFRA*, such as *BRAF*, *KRAS* or *PIK3CA*, was associated with poor prognosis. Adverse prognosticators were also high mitotic index and the advanced disease status at diagnosis.

Conclusions We report comparable incidence of *KIT* and *PDGFRA* mutation in patients with GIST from north-western Greece as compared with cohorts from other regions.

Key questions

What is already known about this subject?

- Prognostic factors such as the tumour site and size, the mitotic count and the type of certain *KIT* or *PDGFRA* mutations on gastrointestinal stromal tumours (GIST) have been already described.
- Data beyond *KIT* and *PDGFRA* mutation are scarce and lacks a population-based study from a region of south-east Europe.

What does this study add?

- This is a population-based study of patients with GIST from north-western Greece.
- We examined the extended mutational profile of patients with GIST by using next generation sequencing.
- We report concomitant mutations of *BRAF* and *FGFR3* genes in two patients with *KIT* mutation. We also detected rare mutations on *PIK3CA* and *KRAS* genes in two patients with *KIT*/*PDGFRA* wild-type GIST.
- We show that the patients with a mutation in a downstream effector of KIT and PDGFRA signalling such as *BRAF*, *KRAS*, *PIK3CA* genes had poor prognosis.

How might this impact on clinical practice?

- In the near future, methods to investigate the comprehensive molecular profile of patients with GIST may be implemented in the clinical practice to uncover mutations with prognostic and predictive significance.

Interestingly, we identified rare mutations on *RAS*, *BRAF* and *PIK3CA* genes in patients with poor prognosis.

INTRODUCTION

Gastrointestinal stromal tumours (GISTs) are mesenchymal neoplasms originating at any segment of the gastrointestinal tract.[1] The term was coined by Mazur *et al* in 1983 to describe a morphologically broad spectrum of tumours of the gastric wall.[2] GISTs are rare tumours and comprise about 1% of all gastrointestinal malignancies. They most frequently arise in the stomach (60%) and less frequently in the small intestine (30%). They can also occur in other parts of the gut (10%) and rarely in the mesentery and omentum. Thirty per cent of GISTs have a malignant clinical phenotype with increased frequency of intra-abdominal and liver metastasis.''

The annual incidence of GIST is estimated at 10–15 per million per year.[3] The median age at diagnosis is 65 and the prevalence is equal between men and women. Most of the cases are sporadic (95%), but they can also be associated with genetic syndromes such as familial GIST, neurofibromatosis type 1, Carney's triad and Carney-Stratakis triad.

The majority of GISTs carry an activating mutation of *KIT* proto-oncogene or the plateled-derived growth factor receptor alpha (*PDGFRA*) gene. Both genes encode proteins that belong to the receptor tyrosine kinase class III family. *KIT* and *PDGFRA* orchestrate signalling transduction pathways that promote proliferation and inhibit apoptosis when activated by their respective ligand stem cell factor or platelet-derived growth factor. Gain-of-function mutations lead to constitutively active signalling and result in neoplasia.[4 5] GISTs are suggested to originate from the stem cell precursors of the interstitial Cajal cell (ICC).[6] Both GISTs and ICC express the receptor tyrosine kinase KIT and their development relies on KIT receptor signalling.[4]

Macroscopically, GISTs are well circumscribed and vary in size ranging from less than 1 cm to more than 40 cm.[7] Tumour sections reveal grey to pink colour and areas of cystic degeneration, haemorrhage and infarction. Microscopically, GISTs have either spindle cell or epithelioid cell morphology and contain eosinophilic collagen structures stained with periodic acid-Schiff stain. Positive staining for CD117 (KIT) (>95% GISTs) and/or discovered on GIST 1 markers is the hallmark of diagnosis.[8] However, negative staining does not rule out the diagnosis, and mutational analysis of *KIT* and *PDGFRA* can be helpful in these cases.

The mutational profile of GISTs is of great biological significance.[9] Approximately, 85% of GISTs have an active mutation in the *KIT* and 5% in the *PDGFRA* gene. The most common mutation of *KIT* is located in exon 11 (70%) which is the region that encodes the juxtamembrane domain of the receptor. The second most common mutation is in exon 9 (10%) encoding the extracellular domain of *KIT*. Exon 9 mutants show less sensitivity to imatinib.[10] Mutations of exons 13 and 17 encoding the ATP-binding pocket and the activation loop, respectively, are rare (1%–2%). Mutations in exon 18 account for the majority (>80%) of the *PDGFRA* mutations and may be associated with imatinib resistance. Rare mutations in exons 12 and 14 of the *PDGFRA* gene have also been reported. Finally, 5%–15% of GIST are characterised as wild type. The latter do not harbour mutations of either *KIT* or *PDGFRA* gene and are potentially resistant to imatinib.

Prognostic factors such as the tumour site and size, the mitotic count and the type of certain *KIT* mutations have been well evaluated.[11] Although GIST is considered to be responsive to tyrosine kinase inhibitors, primary and acquired resistance to such treatment is frequently reported and it is often attributed to secondary mutations.[12] Moreover, ethnic and geographical variations in the mutational profile and genetic determinants of resistance have not been extensively studied. Nevertheless, data from comprehensive analysis of genomic alterations are scarce. In this study, we present data from a retrospective analysis of patients diagnosed with GIST within a 20-year period in the region of north-western Greece. We examined clinicopathological features of this rare tumour and we investigated the prognostic significance of the mutational status of *KIT* and *PDGFRA* as well as of 52 additional oncogenes and tumour suppressor genes by the use of next generation sequencing (NGS) technology.

PATIENTS AND METHODS

Patient characteristics and tumour samples

Clinical and molecular data of 38 patients diagnosed with GIST in north-western Greece within a period of 20 years (1996–2016) were retrieved. GIST diagnosis was made by distinctive histopathology and the presence of *KIT* expression in biopsies obtained from the primary tumour in treatment-naive patients. Demographic and clinical factors were also evaluated. Informed consent was obtained from all patients before testing.

Tissue selection and DNA extraction

H&E stained tissue sections of formalin-fixed paraffin-embedded (FFPE) tumour samples were used as a guide for the localisation of neoplastic areas. Only specimens with a minimum of 75% tumour cell content were selected for this study. The selected neoplastic areas were manually microdissected and DNA was extracted from unstained 10 µm thick FFPE sections using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The Qubit DNA HS assay kit (Life Technologies) was used to quantify purified DNA.

Ion Ampliseq NGS

NGS was conducted by using the Ion AmpliSeq Cancer Hotspot Panel v2 for DNA analysis (Thermofisher

Scientific). The Ion AmpliSeq Cancer Hotspot Panel v2 (Ampliseq, Thermofisher Scientific) is designed to amplify 207 amplicons covering approximately 2800 COSMIC mutations from 50 oncogenes and tumour suppressor genes and four fusion-gene transcripts in FFPE tumour samples (ComPlit DX assay: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAQ*, *GNAS*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *NTRK1*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *ROS1*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, *VHL* genes).

Library preparation

DNA concentrations were measured using the Qubit 2 fluorometer in combination with the Qubit2 dsDNA HS assay kit. For DNA library construction, 10 ng of DNA from each of the 38 FFPE samples were used. An amplicon library was thus generated from total DNA using the Ion AmpliSeq Library Kit V.2.0 according to the manufacturer's instructions. Briefly, amplicon amplification was performed using the Ion Ampliseq HiFi Master Mix. The amplicons were then digested with FUPA reagent and barcoded with the IonCode Barcode Adapters. Subsequently, the amplified products were purified from the other reaction components using AgencourtAMPure XP PCR purification system (Beckman Coulter, Brea, California, USA).

For libraries originated from genomic DNA, the Ion Library Equaliser Kit method was used for normalising library concentration at ~100 pM without the need of extra instrumentation. Finally, equal volumes of each normalised DNA library were combined and clonally amplified on Ion Sphere particles (ISP) by emulsion PCR performed on the Ion One Touch 2 instrument with the Ion PI HiQ OT2 200 Kit (Thermofisher Scientific) according to the manufacturer's instructions.

Quality control was performed using the Ion Sphere Quality Control kit (Thermofisher Scientific) to ensure that 10%–30% of template positive ISP were generated in the emulsion PCR. Finally, the template-positive Ion PI ISP were enriched in the Ion OneTouch ES instrument, loaded on an Ion PI Chip v3 and sequenced on an Ion Proton Sequencer with the Ion PI HiQ Sequencing 200 Kit according to the manufacturer's instructions.

Data analysis

NGS data analysis was performed with the Ion Reporter Software within Torrent Suite Software (Thermofisher Scientific). Statistical analysis was made with SPSS V.17.

RESULTS

Patient demographics and tumour characteristics

Thirty-eight patients (26 males, 12 females) originating from north-western Greece were diagnosed with GIST and managed in the Department of Medical Oncology at Ioannina University Hospital, between 1996 and 2016. Twenty tumours were larger than 5 cm and 15 harboured necrosis. More than one and more than five mitoses per 10 high-power fields were observed in 17 and 15 tumours, respectively. Ki67 immunostaining was as follows: <2% of malignant cells in nine cases, 2%–20% in 22 and >20% in seven. The tumour relapsed in eight patients later during the course of the disease, while nine patients died at a median follow-up of 78 months (online Supplementary table 1). The median overall survival of the entire GIST cohort was 171 months (95% CI 135 to 199).

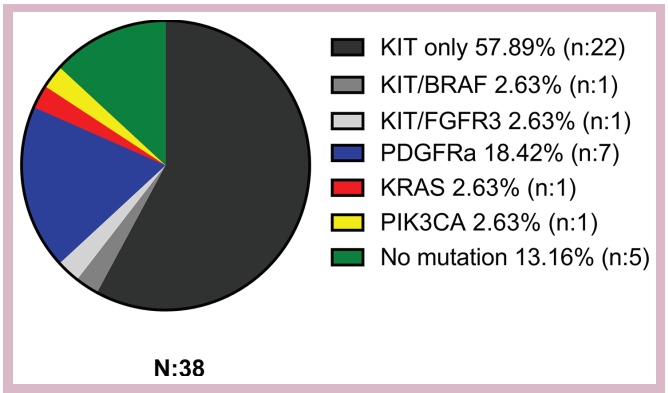

Figure 1 Percentage of mutations detected in 38 gastrointestinal stromal tumours analysed.

Mutation analysis

Mutation analysis revealed the presence of mutations in 33 of 38 GISTS (86.84%), while no mutations were detected in five cases (13.16%). Of the 54 oncogenes and tumour suppressor genes sequenced, alterations were detected in *BRAF*, *PIK3CA*, *KRAS*, *FGFR3*, *PDGFRA* and *KIT* genes. The most common site of mutation was in the *KIT* gene (24 cases, 63.16%) followed by the *PDGFRA* gene (seven cases, 18.42%) (figure 1). Notably, seven patients (18.42%) had tumours negative for *KIT*/*PDGFRA* gene mutation. All mutations are summarised in table 1.

KIT mutations

Of the 24 *KIT* mutated GIST, 19 (79.17%) harboured mutations in exon 11, three (12.5%) in exon 9 and two (8.33%) in exon 13. No mutations were detected in the remaining exons (2, 10, 14, 15 and 17) of the *KIT* gene. More specifically, most of the activating exon 11 mutations were clustered in the proximal part between codons 550 and 560 and consisted of small in-frame deletions and point mutations. In the distal part of exon 11, one point mutation (p.L576P) was found in two samples. In the remaining two mutated exons of the *KIT* gene, exons 9 and 13, a 6-nucleotide insertion GCCTAT between 1509 and 1510 nucleotides causing an insertion of two amino acids (p.Y503_F504insAY) and an amino acid substitution at position 642 (p.K642E) were detected, respectively. The median overall survival of patients with *KIT*-mutated tumours was 171 months (95% CI 125 to 199), with no significant survival differences observed between various exonic mutations.

Table 1 The mutational profile and the allelic frequencies of 38 gastrointestinal stromal tumour cases

Gene	Case no.	Exon	Nucleotide	Codon	Allelic frequency (%)/ case no.
KIT	2, 24, 38	9	c.1509_1510insGCCTAT	p.Y503_F504insAY	32%, 43%, 35%
KIT	26, 36	11	c.1651_1662del12	p.P551_E554del	52%, 65%
KIT	9, 23	11	c.1652_1654delCCA	p.P551_M552>L	51%, 49%
KIT	13	11	c.1665_1679del15	p.V555_V560>V	47%
KIT	20	11	c.1663_1716del53	p.V555_D572del	94%
KIT	8	11	c.1666_1671delCAGTGG	p.Q556_W557del	47%
KIT	22	11	c.1669T>G	p.W557G	41%
KIT	25, 34	11	c.1669_1674delTGGAAG	p.W557_K558del	52%, 43%
KIT	16*	11	c.1669_1680del12	p.W557_V560del	67%
KIT	3, 35	11	c.1669_1683del15	p.W557_E561del	86%, 90%
KIT	6	11	c.1676T>C	p.V559A	49%
KIT	10	11	c.1679T>A	p.V560D	60%
KIT	31	11	c.1676T>A	p.V559D	49%
KIT	14	11	c.1682T>A	p.V561D	9%
KIT	7, 29	11	c.1727T>C	p.L576P	66%, 50%
KIT	12, 15†	13	c.1924A>G	p.K642E	60%, 48%
PDGFRA	5, 18, 27, 30	18	c.2525A>T	p.D842V	39%, 55%, 40%, 51%
PDGFRA	37	18	c.2525_2534delinsC	p.D842_H845delinsA	57%
PDGFRA	19, 28	18	c.2527_2538del12	p.I843_D846delIMHD	39%, 54%
BRAF	16*	11	c.1405G>A	p.G469R	6%
FGFR3	15†	9	c.1150T>C	p.F384L	49%
KRAS	21	2	c.35G>A	p.G12D	70%
PIK3CA	17	9	c.1633G>A	p.E545K	37%

*Concomitant *KIT*/*BRAF* mutation (case 16).
†Concomitant *KIT*/*FGFR3* mutation (case 15).

PDGFRA mutations

Regarding *PDGFRA*, all mutations were localised in exon 18. In three samples (42.86%), two deletions and one deletion/insertion mutation were detected, whereas the drug resistance-associated p.D842V mutation was detected in four (57.14%) samples. The median overall survival of patients with *PDGFRA*-mutated tumours was 159 months (95% CI 115 to 180), with no significant survival differences observed between various exonic *PDGFRA* mutations or patients with *PDGFRA* D842V mutation (median overall survival of 125 months).

Concomitant mutations

Considering the scarcity of comprehensive genomic data in GIST literature, we investigated the incidence of mutations in additional oncogenes that may be implicated in GIST development and progression. As shown in table 1, two cases with concomitant *KIT* and *BRAF* (case 16) and *KIT* and *FGFR3* (case 15) mutations were detected. The patient with concurrent *KIT*/*BRAF* mutations was placed on adjuvant treatment with imatinib but he developed progressive disease with liver metastases 12 months later. He was then switched to sunitinib and he had an overall survival of 55 months. The patient with simultaneous *KIT*/*FGFR3* mutations received adjuvant treatment with imatinib with no tumour relapse so far (17 months). In addition to the concomitant mutations above, we found two rare mutations in *KIT*/*PDGFRA* wild-type GIST. In particular, we report a patient with a *KRAS* c.35G>A (G12D) and a patient with a *PIK3CA* c.1633G>A (E545K) mutation, respectively. The patient with the *KRAS* mutation had a gastric GIST, he was managed with subtotal gastrectomy but he relapsed quickly locoregionally and despite imatinib therapy, he succumbed at an overall survival of 15 months. The patient with the *PIK3CA* mutation had locally advanced GIST at diagnosis which relapsed quickly and she was then placed on imatinib but with a limited survival of 4 months.

Correlation of histopathological and molecular features

In our study, 25 of the 38 cases were composed of spindle cells (65.79%), five were composed of epithelioid cells (13.16%), while eight were of mixed subtype (21.05%). From the 24 *KIT* mutated cases, seven showed epithelioid or mixed histology (two epithelioid, five mixed), whereas the rest showed spindle histology. From the seven *PDGFRA*

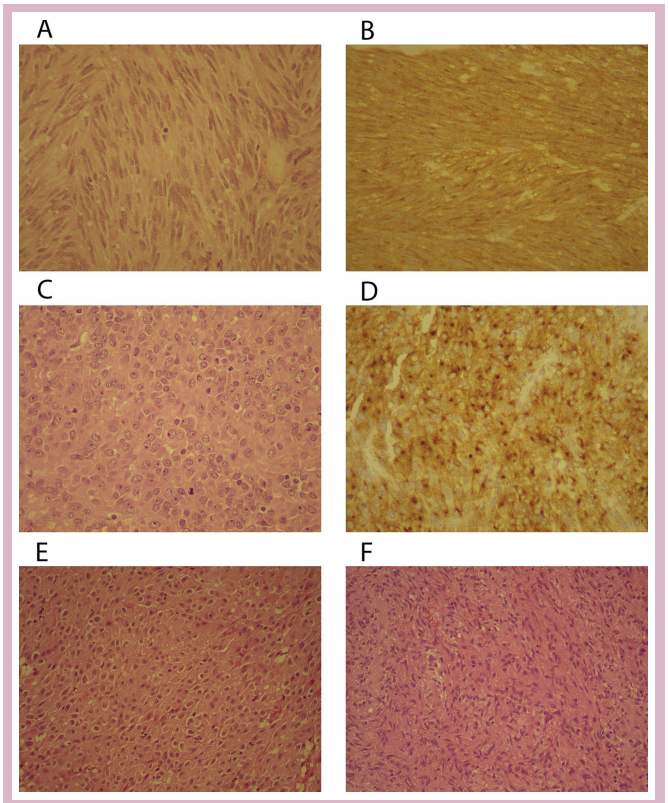

Figure 2 Histological–molecular correlation in gastrointestinal stromal tumour (GIST). (A and B) Spindle cell GIST with *KIT* mutation ((A) H&E ×400 and (B) KIT immunohistochemistry, DAB ×400). (C and D) Epithelioid GIST with concominant *KIT* and *BRAF* mutation ((C) H&E ×400 and (D) KIT immunohistochemistry, DAB ×400). (E) Epithelioid GIST with *PDGFRA* mutation (H&E ×200). (F) Spindle cell GIST with undetected mutation (H&E ×200).

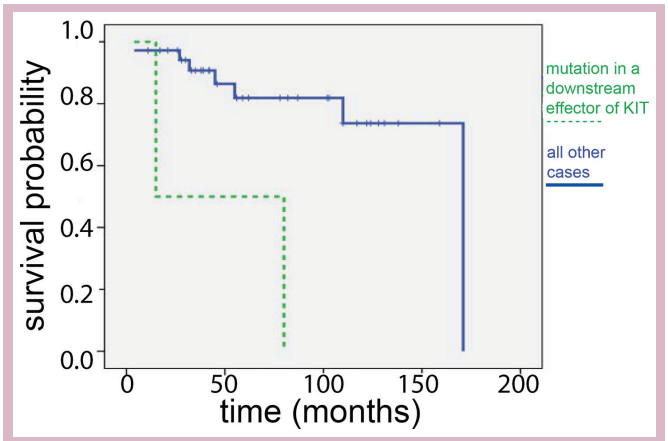

Figure 3 Patient survival by *KRAS, BRAF, PIK3CA* mutation status. Comparison between patients with a mutation in a downstream effector of *KIT* (*KRAS, BRAF, PIK3CA*) and all other cases.

mutated cases, five were of epithelioid or mixed cell morphology (two epithelioid, three mixed; 71.43%) and two were of spindle cell morphology (28.57%). From the seven GIST that were negative for *KIT/PDGFRA* mutation, one was of epithelioid histology subtype and the rest were of spindle histology subtype. Overall, *PDGFRA*-mutated GISTs were more often epithelioid than the *KIT* mutated and the *KIT/PDGFRA* wild-type GIST. Interestingly, the *KIT*-mutated case with concomitant *BRAF* mutation was also of epithelioid histology (figure 2). Regarding KIT immunohistochemistry, all *KIT*-mutated GIST expressed KIT protein in tissue sections. KIT immunohistochemical staining, even focally, was also detected in five out of seven PDGFRa-mutated GISTs and in three out of seven *KIT/PDGFRA* wild-type GIST.

Prognostic parameters

Among the clinical and molecular variables analysed, those that retained prognostic significance were the Ki67 immunostaining, the disease extent and the presence of an activating mutation downstream of *KIT* and *PDGFRa* genes such as *RAS*, *BRAF* or *PIK3CA*.

The median overall survival of patients with low Ki67 staining (<2%) was not reached versus 170 months (95% CI 67 to 215) in patients with intermediate Ki67 expression (2%–20%) versus only 42 months (95% CI 25 to 58) in patients with high Ki67 staining (>20%) (log-rank, two-sided p=0.01). The median overall survival of patients with localised tumours at diagnosis was 171 months (95% CI 110 to 197) versus 55 months (95% CI 13 to 79) in patients with metastatic dissemination at diagnosis (log-rank, two-sided p=0.011). The presence of an activating mutation in the RAS/RAF axis (*KRAS* or *BRAF* genes) or the PI3K/AKT axis (*PIK3CA* gene), downstream of KIT/PDGFRA signalling pathway, was associated with a median overall survival of 15 months (95% CI 15 to 68) versus a median overall survival of 171 months (95% CI 125 to 197) in the absence of *KRAS*, *BRAF* and *PIK3CA* mutations (log-rank, two-sided p=0.003) (figure 3).

DISCUSSION

In the present study, we investigated the mutational profile in 38 Greek patients with GIST managed in an academic department in north-western Greece. Although other Greek groups have published clinicopathological and molecular data,[13 14] this is the first population-based study on Greek patients with GIST. To the best of our knowledge, it also consists the first report from a region of south-east Europe.

The overall extended mutational rate (54 gene panel) in our registry was 86.84%, approximating the frequencies observed in previous studies. However, the mutation rate for *KIT* was 63.16% and for *PDGFRA* 18.42%, whereas the estimated percentages from clinical trials were 80%–85% and 5%–10%, respectively.[10 15] This disparity could be attributed to different patient characteristics, as most of these case series were phase III studies that recruited patients with advanced GIST, whereas our report is a population-based study of patients with GIST of various stages. Similarly, a prospective epidemiological study of patients with GIST in the Rhone-Alpes region in France demonstrated comparable incidence of *KIT* (67%) and

PDGFRA mutations (16%).[16] Presumably, the favourable prognosis of patients with *PDGFRA* mutation accounts for its decreased incidence in patients with advanced diseased in phase III clinical trials.[17]

Population studies have shown variable rates of *KIT* and *PDGFRA* mutation as well. Steigen *et al* demonstrated mutation rates of 75% for *KIT* and 10% for *PDGFRA* in northern Norway,[18] while Braconi *et al* found *KIT* and *PDGFRA* mutations in 79% and 12% of tumour samples, respectively, in Ancona, Italy. Additionally, Mazzola *et al* showed mutation rates of 65% for *KIT* and 10% for *PDGFRA* in the south of Switzerland,[19] while Wozniak *et al* demonstrated mutation rates of 69.3% and 12.9% for *KIT* and *PDGFRA,* respectively.[20] Regarding data beyond Europe, Braggio *et al* reported mutation frequencies of 74.5% for *KIT* and 7.3% for *PDGFRA* mutation in Brazilian patients,[21] while Du *et al* found *KIT* mutation rates of 76.6% and *PDGFRA* rates of 2.8% in China.[22] These findings indicate ethnic/genetic variations. Environmental as well as genetic risk factors related to polymorphisms in DNA repair mechanisms or xenobiotic metabolism may contribute to the generation of a distinctive population.[23] Interestingly, the north-west Hellenic region is a mountainous territory, prone to isolation and development of distinct genetic 'niches' with conserved, proprietary genetic traits.

Regarding the exonic site of mutation, exon 11 of the *KIT* gene was the most common site (79.17%) and exon 9 the second most frequent location (12.5%) followed by mutations in exon 13 (8.33%). Notwithstanding some fluctuations in the frequencies, our results are in line with other European cohorts.[16 20] Several lines of evidence indicate that exon 9 mutations are associated with an increased risk for tumour progression.[24] Furthermore, certain types of mutations such as exon 11 duplications may be correlated with favourable prognosis.[17] Conversely, deletions of the codon 557–558 may be associated with poor clinical outcome.[25 26] We did not observe significant differences in the overall survival and progression-free survival regarding the type of *KIT* mutation. With respect to *PDGFRA*, all mutations were located in exon 18 and the patients had benign clinical course. *PDGFRA* mutation is associated with a favourable disease outcome based on the reports of Wozniak *et al* from a multicenter analysis of a European registry[24] and Joensuu *et al* from a pooled analysis of population-based studies and individual data.[17] Moreover, we did not find significant difference in the overall survival between patients with *PDGFRA* D842V mutation and other *PDGFRA* mutations. However, the lack of difference in the overall survival between groups with different types of mutations in our cohort may be attributed to the small sample size, different tumour stage at diagnosis, varying follow-up times (76.31% of patients being alive at the time of the analysis) and distinct 'all comers' clinicopathological characteristics.

In addition to *KIT* and *PDGFRA*, we investigated the mutational status of several other oncogenes that may drive GIST development.[27] *BRAF* mutation affects a small subset of patients with *KIT/PDGFRA* wild-type GIST, and the V600E mutation in exon 15 is the most commonly found one.[28–31] *BRAF* mutation is of great therapeutic relevance given the fact that BRAF kinase is a downstream effector of KIT/PDGFRA signalling, while imatinib targets exclusively the upstream tyrosine kinases.[32 33] In the current study, we found a case with concomitant mutations of *KIT* and *BRAF*. Rosi *et al* showed that concomitant mutations of *BRAF* and *KIT* are an extremely rare event.[34] However, such cases may be underrepresented because investigations for genomic alterations of *BRAF* are usually reserved for GIST negative for *KIT* and *PDGFRA* mutations. Furthermore, we demonstrated that the patient with this dual mutation had poor prognosis. This is in accordance with the study of Miranda *et al* who showed that *BRAF* mutation conferred resistance to imatinib.[35]

Fibroblast growth factor receptor (FGFR) axis is another tyrosine kinase receptor signalling pathway that is implicated in oncogenesis by inducing proliferation and inhibiting apoptosis through the RAS/RAF/MEK/ERK and PI3K/AKT pathway. Mutations, amplifications and fusions that lead to constitutively active conformation of the FGFR have been reported in diverse tumours.[36] Shi *et al* reported actionable alterations of *FGFR1* gene that consisted of kinase fusions in *KIT/PDGFRA* wild-type GIST.[37] Here, we report a case of GIST with concomitant mutation of *FGFR3* in exon 9 and KIT in exon 13. FGFR may drive oncogenesis in *KIT/PDGFRA* wild-type GIST, but its role as an auxiliary mutation is unknown. Preclinical studies have shown that activation of the FGFR pathway restored c-KIT phosphorylation during imatinib treatment possibly through crosstalk with the MAPK pathway.[38] However, the patient of our cohort has been placed on imatinib treatment without having tumour relapse so far (17 months).

KRAS mutations are frequently detected in various tumours but little is known in GIST where it seems to be an extremely rare event. Lasota *et al* failed to detect such mutations by sanger sequencing and pyrosequencing in a large cohort of 514 GIST.[39] Other studies failed to detect *KRAS* mutation as well.[29–31 40] Nevertheless, Miranda *et al* demonstrated the rare occurrence of *KRAS* mutation concurrently with exon 11 mutations in *KIT* gene (two cases) and exon 18 mutation in *PDGFRA* gene (one case) in GIST samples retrieved from the Ticino Registry.[35] In the same study, it was also experimentally shown that the expression of *KRAS* mutants mediated resistance to imatinib treatment in a cell culture line cotransfected with a constitutively active *KIT* gene. Here, we found the presence of *KRAS* mutation in a patient with an overtly malignant GIST by next generation sequencing. Likewise, Hechtman *et al* found a *KRAS* G12V (c.35G>T) mutation in a patient with a clinically aggressive GIST by next generation sequencing.[41] Similarly, Gao *et al* also reported the presence of *KRAS* mutation in a small subset of patients that have been previously characterised as wild-type GIST by sanger sequencing.[42] We speculate that the adoption of NGS technology in population-based studies on GIST

would unveil uncommon genomic alterations, providing us the opportunity to study their prognostic relevance.

PI3K pathway is another major cell signalling pathway that drives carcinogenesis by inducing cell survival and proliferation.[43] Daniels *et al* and Lasota *et al* reported concurrent *PIK3CA* mutations with exon 11 KIT mutations in imatinib naive GIST that may represent secondary oncogenic events related to GIST progression. Such mutations are rare and associated with clinically aggressive phenotype.[30 44] Furthermore, Falchook *et al* demonstrated the presence of *PI3KC* mutation in *BRAF* mutated GIST that was detected after treatment with BRAF inhibitor, thus indicating acquired resistance to this investigational compound.[33] Here, we demonstrate the presence of a *PIK3CA* mutation in a patient with *KIT*/*PDGFRA* wild-type GIST who did not respond to imatinib and had short survival. This is the first report of a *PIK3CA* mutation in wild-type GIST. We believe that the lack of comprehensive genomic screening in clinical studies may underestimate such rare mutations.

In conclusion, we report comparable *KIT* and *PDGFRA* mutation rate in patients with GIST from north-western Greece compared with registries from other European regions. Although the retrospective, small nature of our cohort precluded us from studying the prognostic impact of *KIT* and *PDGFRA* mutation status, we report the presence of rare mutations in downstream effectors of KIT such as *BRAF*, *KRAS* or *PIK3CA* in patients with GIST with poor prognosis. Our data highlight the oddities between different populations with GIST and underscore the significance of the comprehensive molecular profiling in population-based studies. In the era of personalised medicine, the latter is of particular importance in view of the emergence of primary and acquired resistance to tyrosine kinase inhibitors.

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