Next-generation sequencing demonstrates the rarity of short kinase variants specific to quadruple wild-type gastrointestinal stromal tumours

Newton A C S Wong ,[1,2] Olivier T Giger,[3] Rogier ten Hoopen,[4] Ruth T Casey,[5] Kirsty Russell,[2] Claire Faulkner[2]

ABSTRACT

Aim There is no known specific biomarker or genetic signal for quadruple wild-type (qWT) gastrointestinal stromal tumours (GISTs). By next-generation sequencing (NGS) of different GIST subgroups, this study aimed to characterise such a biomarker especially as a potential therapeutic target.

Methods and results An NGS panel of 672 kinase genes was applied to DNA extracted from 11 wild-type GISTs (including three qWT GISTs) and 5 *KIT/PDGFRA* mutated GISTs. Short variants which were present in qWT GISTs but no other GIST subgroup were shortlisted. After removing common population variants, in silico-classified deleterious variants were found in *CSNK2A1*, *MERTK*, *RHEB*, *ROCK1*, *PIKFYVE* and *TRRAP*. None of these variants were demonstrated in a separate cohort of four qWT GISTs.

Conclusions Short kinase variants which are specific to qWT GISTs are rare and are not universally demonstrated by this whole subgroup. It is therefore possible that the current definition of qWT GIST still covers a heterogenous population.

INTRODUCTION

The traditional definition for a wild-type gastrointestinal stromal tumour (GIST) is a lack of activating mutations in *KIT* exons 9, 11, 13 and 17, and *PDGFRA* exons 12, 14 and 18.[1] Based on this definition, wild-type GISTs comprise approximately 10% of all GISTs.[1] Wild-type GISTs may be subdivided on molecular grounds into those which (1) show succinate dehydrogenase (SDH) deficiency (through germline SDHx mutations or somatic SDHC epimutations), (2) show *BRAF* or *KRAS* mutation, (3) are associated with neurofibromatosis 1 (NF1) or (4) show none of the above features (often referred to as quadruple negative or wild-type GISTs).[2] However, a common feature to all these subgroups of wild-type GISTs is poorer response to the tyrosine kinase inhibitor imatinib.[3] This drug remains the licensed first-line therapy for all GISTs, at least in the United Kingdom, and while wild-type GISTs may show a better response to second line therapies such as sunitinib,[4] there remains a clinical need for more predictably effective therapies for wild-type GIST patients. A more directed and biologically robust method of identifying these therapies is to characterise key molecular signatures of these tumours and to then consider therapies which target these molecules.

Kinases can be targeted by specific antibody (eg, anti-EGFR) or inhibitor (eg, anti-BRAF) therapies such as those currently used for melanoma and certain carcinomas.[5–7] Because wild-type GISTs lack the oncogenic drive provided by *KIT* or *PDGFRA* mutation, it is biologically plausible that alternative kinases are activated instead. Indeed, this has already been demonstrated for some subgroups of wild-type GISTs, e.g. succinate dehydrogenase (SDH) deficient wild-type GISTs usually demonstrate activation of the kinase IGF1R.[8]

By virtue of their definition, quadruple wild-type (qWT) GISTs currently lack a distinct molecular signature and therefore, specific molecular targets for therapy. A small proportion of qWT GISTs demonstrate *FGF1R* or *NTRK3* variants,[9–11] but there has not yet been any demonstration of a kinase variant specific to this group of GISTs. The following study therefore aimed to characterise such a variant by screening different subgroups of wild-type GISTs, and especially qWT GISTs, using next-generation sequencing (NGS) with a panel covering more than 600 kinases. Any potentially deleterious kinase variants were then validated by targeted assays and finally tested for among a second group of qWT GISTs.

MATERIALS AND METHODS

Formalin-fixed paraffin-embedded (FFPE) tissue blocks for an anonymised cohort of 11 wild-type GISTs (comprising 2 paediatric and 2 adult SDH deficient GISTs, 2 NF1-related GISTs, 2 *BRAF* mutated GISTs and 3 qWT GISTs), 3 *KIT* mutated GISTs and 2 *PDGFRA* mutated GISTs had been identified and retrieved from the files of the Department of Histopathology at the Bristol Royal Infirmary, Bristol, for a previous study.[12] While all these tissue blocks showed at least 50% neoplastic cell content (NCC), the latter varied between the blocks, and none were completely devoid of non-neoplastic cells (eg, inflammatory cells and blood vessels). Mutant allele frequency would thus vary artefactually across all cases due to varying NCCs alone and therefore was not reported in the following results.

DNA was extracted from each block and sequencing libraries prepared using an Agilent SureSelect panel, which was customised to include all potentially relevant targets and covered 672 kinase genes (online supplementary file 1). DNA sequencing was performed using the Illumina NextSeq, which has sufficient capacity to enable

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[1]Department of Cellular Pathology, Southmead Hospital, Bristol, UK
[2]South West Genomic Laboratory Hub, Southmead Hospital, Bristol, UK
[3]Department of Pathology, University of Cambridge, Cambridge, UK
[4]Department of Oncology, University of Cambridge, Cambridge, UK
[5]Department of Medical Genetics, University of Cambridge, Cambridge, UK

Correspondence to
Dr Newton A C S Wong, Department of Cellular Pathology, Southmead Hospital, Bristol BS10 5NB, UK; Nacs.Wong@bristol.ac.uk

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Table 1 Clinicopathological features of the gastrointestinal stromal tumour (GIST) subgroups

Feature	Quadruple wild type (n=7)	Paediatric SDH deficient (n=2)	Adult SDH deficient (n=2)	NF1 related (n=2)	*BRAF mutated* (n=2)	*KIT/PDGFRA mutated* (n=5)
Sex						
Male	3	0	1	1	2	2
Female	4	2	1	1	0	3
Age (years)						
Mean (range)	54 (24–61)	17 (16–18)	44 (27–61)	49 (45–53)	66 (54–78)	65 (50–77)
Tumour location						
Stomach	1	2	2	0	0	4
Small bowel	5	0	0	2	2	1
Fallopian tube	1	0	0	0	0	0
Morphology						
Spindle	2	0	0	2	2	2
Mixed cell type	2	0	2	0	0	1
Epithelioid	3	2	0	0	0	2

NF1, neurofibromatosis1; SDH, succinate dehydrogenase.

multiplexing of up to 16 samples per run, generating sufficient depth of coverage (300×) to enable the detection of short variants. To exclude FFPE-related artefacts, samples were tested in duplicate.

The resulting data were analysed bioinformatically (using the software packages BWA V.0.7.9a, Picard V.1.67, Bedtools V.2.26.0, Samtools V.1.2, Vardict git(0752bad985cdb1b499f87a5ce32c7a486e0fcc63) and fastQC V.0.11.4 and deconstructSigs) and with annotation software (Cancer Genome Interpreter and Alamut V.2.11) to remove common population variants and to assess any variants for predicted effect on gene/protein function. Specific Sanger sequencing assays were designed for any predicted deleterious variants which were specific to qWT GISTs. These targeted gene assays were applied to the original three qWT GISTs and then also to a further anonymised cohort of four qWT GISTs from the University of Cambridge.

RESULTS

Table 1 presents a summary of available clinicopathological features of the GIST subgroups studied.

There was a shortlist of 26 gene variants that were present among any of the three qWT GISTs but were not seen among any of the other wild-type GISTs or *KIT*/*PDGFRA* mutated GISTs. Following the exclusion of likely common population variants, in silico analysis classified only six remaining variants to be deleterious (table 2). None of these six variants was present in two or more qWT GISTs. Targeted sequencing assays for each deleterious variant validated three variants (table 2). These targeted assays did not demonstrate any variants among a further cohort of four qWT GISTs (table 2).

A few, in silico-classified deleterious variants were found among the other wild-type GIST subgroups as follows: *COL1A1* (c.613C>G p.(Pro205Ala)) in one of two paediatric GISTs; *CHEK2* (c.1420C>T p.(Arg474Cys)) in one of two adult SDH deficient GISTs and *MTOR* (c.495A>C p.(Arg165Ser)) in the other adult SDH deficient GIST; *ERBB4* (c.2440A>G p.(Asn814Asp)), *COL1A1* (c.613C>G p.(Pro205Ala)) and *MAPKAPK5* (c.139C>T p.(Arg47Trp)) in one of two NF1-related GISTs; *MLH1* (c.779T>G p.(Leu260Arg)) and *CHEK2* (c.1427C>T p.(Thr476Met)) in one of two *BRAF* mutated GISTs.

Table 2 Six, in silico-classified deleterious variants which were demonstrated among qWT gastrointestinal stromal tumours (GISTs)

Gene variant genotype and predicted protein change	Present in NGS-tested qWT GISTs? (*validated?)			*Present in further cohort of qWT GISTs?			
	Case Bq1	Case Bq2	Case Bq3	Case Cq1	Case Cq2	Case Cq3	Case Cq4
CSNK2A1 c.859A>C p.(Ser287Arg)	N (n/a)	Y (UM†)	N (n/a)	UM	UM	N	UM
MERTK c.275C>T p.(Pro92Leu)	Y (Y)	N (n/a)	N (n/a)	N	N	N	N
RHEB c.487A>G p.(Ile163Val)	Y (UM)	N (n/a)	N (n/a)	UM	UM	UM	UM
ROCK1 c.2279_2280delinsCT p.(Gly790Ala)	Y (Y)	N (n/a)	N (n/a)	UM	N	N	N
PIKFYVE c.1496C>T p.(Ser499Leu)	N (n/a)	Y (Y)	N (n/a)	UM	N	N	N
TRRAP c.6929C>T p.(Ala2310Val)‡	Y (UM)	N (n/a)	N (n/a)	UM	UM	UM	N

*As tested for by targeted Sanger sequencing assays.
†Unmatched (ie, no polymerase chain reaction (PCR) product or a non-specific PCR product which did not align with reference sequence). These failures were attributed primarily to suboptimal DNA template quality.
‡Variant reported by NGS as c.6875C>T p.(Ala2292Val).
n/a, not applicable; NGS, next-generation sequencing; qWT, quadruple wild type.

DISCUSSION

The principal aim of our study was to screen for and characterise kinase variants which are specific to qWT GISTs and which might therefore serve as biomarkers and/or therapeutic targets for this subset of tumours. There have been a handful of previous analyses of qWT GISTs using large panel technologies, though with different study aims and methodologies.[9–11 13] Two particular kinase genes with variants reported by some of these studies are *FGFR1* and *NTRK3*. Shi and colleagues applied the FoundationOne assay (covering the coding regions of more than 182 cancer-related genes) to 24 GISTs lacking alterations in the KIT/PDGFRA/RAS/NF1 pathways, of which 12 did not show SDH alterations.[9] Three of these tumours (two qWT and one with unknown SDH status) showed *FGFR1* variants (two fusions and one missense mutation—all classified as deleterious), but five non-wild-type GISTs also showed *FGFR1* variants (one amplification classified as likely deleterious and the remaining as of uncertain signficance).[9] Pantaleo and colleagues performed whole-exome sequencing on nine qWT GISTs, of which one case harboured an *FGFR1* missense mutation.[10] Shi and colleagues reported an *ETV6-NTRK3* fusion in two GISTs; while one tumour was described as wild-type with SDHB expression, complete molecular phenotypes of both tumours were not given and it is unclear whether this fusion was assessed for and/or found among other GIST subgroups.[9] Finally, using RNA-based NGS and then fluorescence in situ hybridization, Brenca and colleagues described an *ETV6-NTRK3* fusion in one of five qWT GISTs and not among 26 GISTs which were mainly *KIT*/*PDGFRA* mutated.[11]

The kinase-orientated panel used in our study did cover *FGFR1* and *NTRK3* (online supplementary file 1), but no short variants of either kinase were found among three qWT tumours. Our NGS assay was not set up to assess for large structural variants such as fusions, but the above literature data already indicate that neither *FGFR1* nor *NTRK3* variants are specific to and/or universally demonstrated by all qWT GISTs. Large structural variant analysis is less accurately performed on FFPE tissue-derived DNA by NGS technology, and we did not have access to good quality RNA from our cases to permit such optimal analysis.

Apart from *FGFR1*, Shi and colleagues listed six other genes with variants more commonly found in their 24 wild-type GIST cohort compared with non-wild-type GISTs: *ARID1B*, *ATR*, *LTK*, *PARK2*, *SUFU* and *ZNF217*.[9] Only the last two did not show variants in non-wild-type GISTs, and for each of *SUFU* and *ZNF217*, variants were only found in 2 out of the 24 wild-type GISTs.[9] While Pantaleo and colleagues described variants of some novel genes (ie, *MAX*, *CHD4* and *CTNND2*) among their nine qWT GISTs, each variant was only seen in one of the nine tumours, and it is unknown whether these variants are harboured by non-wild-type GISTs.[10] Most recently, Urbini and colleagues reported *FGF4* copy number gain to be specific to qWT GISTs, though this gain was not shown by two out of eight such tumours analysed.[13] Our data are therefore similar to those of all these studies in recording a few variants which appeared to be specific to qWT GISTs but were not universally demonstrated by all these tumours studied. While our finding is in some ways disappointing, it may still be important in indicating the current criteria for qWT GIST define a heterogenous population. It was difficult to prove this hypothesis with our study, which was limited by the relatively small number of qWT GISTs studied. However, one way of exploring the hypothesis and potentially teasing out further subgroups is to pool, where possible, raw data from existing and future studies of these tumours to bioinformatically interrogate a much larger population in which trends may then be more obvious. Interestingly, there have been increasing numbers of independent reports of *NF1* variants in qWT GISTs,[13–15] therefore suggesting a proportion of such neoplasms may actually represent unrecognised NF1 syndrome.[15]

Of the six deleterious variants that we demonstrated only among qWT GISTs, at least three have some link with tumorigenesis. *ROCK1* regulates cell mobility, and its overexpression may increase tumour cell invasion and angiogenesis.[16] *RHEB* is recognised as a member of the RAS superfamily, its unregulated activity may therefore drive tumour cell proliferation, and its overexpression has been demonstrated in a variety of neoplasms including liver, bladder and lung cancers.[17] How *PIKFYVE* variants may contribute to neoplasia is less clear, but inhibitors of its protein are toxic to glioblastoma and melanoma cell lines.[18 19]

Our NGS study did also demonstrate a few, in silico-classified deleterious variants among the four other wild-type GIST subgroups. These other variants were not validated by single-gene assays because our study was primarily focused on qWT GISTs. However, it is worth mentioning two in particular as each was present in more than one wild-type GIST subgroup. *CHEK2* variants (two different genotypes) were present in one adult SDH deficient GIST and one *BRAF* mutated GIST. *CHEK2* is a tumour suppressor gene and regulates cell division at a DNA damage checkpoint.[20] *CHEK2* is recognised as a breast cancer susceptibility gene and its variants have also been associated with prostate cancer.[21] The same *COL1A1* variant was present in one paediatric GIST and one NF1-related GIST. However, while germline *COL1A1* variants underlie some forms of osteogenesis imperfecta,[22] a role for this collagen-related gene in tumorigenesis is currently unclear.

In conclusion, our study has shown that short kinase variants which are specific to qWT GISTs are rare and are not universally demonstrated by this whole subgroup. It is therefore possible that the current definition of qWT GIST still covers a heterogenous population.

Take home messages

- Short kinase variants which are specific to quadruple wild-type GISTs are rare and are not universally demonstrated by this whole subgroup.
- It is therefore possible that the current definition of qWT GIST still covers a heterogenous population.

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Twitter Kirsty Russell @krussyo

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ORCID iD

Newton A C S Wong http://orcid.org/0000-0002-9537-4106

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