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Original Research

RNA-Based and DNA-Based Next-Generation Sequencing of *KIT* and *PDGFRA* Mutations in Gastrointestinal Stromal Tumors: Analytical Performance and Epidemiologic Insights

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ABSTRACT

Mutations in the *KIT* and *PDGFRA* proto-oncogenes are key drivers in gastrointestinal stromal tumors (GIST) and guide targeted therapy. While DNA-based next-generation sequencing (NGS) is standard for mutation detection, RNA sequencing (RNA-Seq) may offer complementary advantages. This study evaluates RNA-Seq performance and presents molecular epidemiologic data from a large DNA-sequenced GIST cohort. RNA-Seq was performed on 24 GIST cases previously analyzed by DNA-based NGS (16 *KIT* mutants, 5 *PDGFRA* mutants, 3 wild-type) using the Agilent SureSelectXT RNA Direct Library Prep Kit and an in-house analysis pipeline. RNA-Seq was successful in 21 cases, identifying 13 of 14 *KIT* mutations and all *PDGFRA* mutations, including single nucleotide variants (SNV), insertions, and in-frame deletions (3–27 bp). One complex 45 bp deletion-insertion was not detected, though low-level evidence (<10% of reads) was present. Separately, DNA-based NGS results from 579 GIST cases were reviewed to assess mutation prevalence and clinicopathologic associations. Mutations were found in 83.2% of cases (403 *KIT*, 79 *PDGFRA*), with secondary *KIT* mutations in 6.0%. Mutation site correlated with tumor location and patient age; secondary mutations were more frequent in non-gastric tumors. RNA-Seq demonstrates high accuracy for detecting clinically relevant *KIT* and *PDGFRA* mutations and may complement DNA-based profiling. The DNA cohort provides broader context for mutation prevalence and patterns in clinical practice.

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumor of the gastrointestinal tract[1]. These tumors originate from interstitial cells of Cajal (ICC) which participate in the regulation of gastrointestinal motility. Mutations in the *KIT* and *PDGFRA* proto-oncogenes are present in approximately 85% and 10% of GISTs, respectively[1]. In fewer than 5% of cases, GISTs harbor alterations in other genes such as *SDH, NF1, BRAF, PIK3CA*, or *RAS*[2-4]. The specific mutations identified in *KIT* and *PDGFRA* are clinically significant, as they can predict sensitivity or resistance to targeted therapies[5,6].

DNA sequencing—either Sanger or next-generation sequencing (NGS)—is routinely used to detect mutations in *KIT, PDGFRA*, and other relevant genes in GIST. A limited number of studies have explored the utility of RNA-based next-generation sequencing (RNA-Seq) for mutation detection in GIST[7]. RNA-Seq offers potential advantages over DNA NGS, including the ability to: (1) quantify expression levels of mutated genes, and (2) confirm the impact of suspected splice site mutations identified by DNA NGS[8]. While DNA NGS remains the preferred method for mutation profiling, there are clinical scenarios where tissue availability restricts testing to either DNA or RNA. In such cases, it is important to determine whether RNA-Seq can reliably detect clinically relevant mutations.

In this study, we compare the accuracy of RNA-Seq with DNA NGS for detecting *KIT* and *PDGFRA* mutations in GIST, assess gene expression levels in mutated tumors, and evaluate the impact of splice site

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mutations, in order to assess technical concordance between RNA-Seq and DNA NGS. Additionally, we present molecular epidemiologic data from a large cohort of 560 GIST patients.

Materials and Methods

IRB Approval

This study was approved by Mayo Clinic Institutional Review Board (IRB Study ID: 18-01163).

DNA isolation, Library Preparation, and NGS

DNA was extracted from tumor-rich areas of unstained formalin-fixed paraffin-embedded (FFPE) slides using the QIAamp DSP DNA FFPE Tissue *KIT* (Qiagen Inc, Valencia, CA). Selected tissue areas contained ≥20% tumor cells. As previously described, DNA NGS utilized the Ion Ampliseq Hotspot Panel V2 and the Ion AmpliSeq Library Kit 2.0 (Life Technologies) to assess hotspot mutations in the *KIT* and *PDGFRA* genes . Sequencing was performed on an Illumina HiSeq instrument. This assay only detects mutations in the *KIT* and *PDGFRA* genes and did not include other genes associated with GIST.

RNA isolation, Library Preparation, and NGS

Total RNA was isolated using the miRNA Easy *KIT* (Qiagen). mRNA isolation, complementary DNA (cDNA) synthesis, and NGS library preparation were performed using the Illumina TruSeq Library Preparation *KIT*, v2. RNA quantity and concentration of RNA were measured with a Qubit instrument (Qiagen) and the RNA quality was assessed using an Agilent Bioanalyzer to determine RNA Integrity Number (RIN). RNA-Seq was performed using the Agilent SureSelectXT RNA Direct Library Preparation kit with v7 baits and sequencing was performed on an Illumina HiSeq 2500 instrument [9,10].

DNA NGS Bioinformatics

As previously described, sequencing data was processed with a CLC Bio Genomics Server and a custom-built bioinformatic pipeline to align the reads to the GRch37/hg19 reference genome[11]. Variant calling was performed using the CLC server, and variants with 5% or more mutant allele frequency were reviewed to determine pathogenicity[11].

RNA NGS Bioinformatics

RNA-seq paired-end reads for the samples were processed using the internally developed Mayo Analysis Pipeline for RNA-Seq (MAP-Rseq) [9]. MAP-RSeq employs STAR [12] to align reads to the reference human genome build 38. Aligned reads were processed through multiple parallel modules. Variant calling was performed using the RVBoost module. Gene and exon expression quantification was conducted using the Subread[13] package to obtain raw and normalized fragments per kilobase per million mapped reads (FPKM). Quality control was assessed using FASTQC, MultiQC [14] and RSEQC [15]. Results from alignment, gene expression quantification and quality control were consolidated in a HTML report. Integrative Genomics Viewer (IGV)[16] was used to visualize RNA-Seq alignments for case 5 which harbored a deletion within *KIT* exon 11. Human genome variation society (HGVS) nomenclature is used for variants. RNA-seq data will be made available upon request.

Statistical analysis

Categorial variables were compared using the chi-squared test, and continuous variables were analyzed using the Independent Student's t-test. All analyses were univariate and descriptive. P-values are considered significant if they are less than 0.05.

Results

Molecular epidemiologic findings

We identified 607 individuals who underwent DNA-based NGS for *KIT* and *PDGFRA* mutations in the Mayo Clinic Molecular Genetics Lab between August 2015 and December 2018. Of these, 579 cases were included in the study based on a strong clinical or pathologic impression of GIST. Among these, 463 (80.0%) were Mayo Clinic Laboratory reference specimens and 116 (20.0%) were Mayo Clinic patients. The cohort included 336 male (58.0%) and 243 (42.0%) females (p=0.007) with a median age of 62 years (mean 60.8, range 16-92) (**Figure S1**).

Tumor origin was most commonly gastric (N=264, 45.6%), followed by the small bowel (N=129, 22.3%) and colorectum (N=39, 6.7%). In 137 cases (23.7%), the site was unspecified due to extensive intra-abdominal involvement or metastatic presentation. Rare sites included the cervix (N=1), esophagus (N=1), pancreas (N=2), retroperitoneum (N=5), and spleen (N=1) (**Table S1).** DNA NGS was successful on 560 of the 579 (96.7%) patients. Mutations in *KIT* or *PDGFRA* were identified in 482 cases (83.2%) with *KIT* mutations 403 (83.6%) and *PDGFRA* mutations in 79 (16.4%). Twenty-nine cases harbored two *KIT* mutations. Mutation distribution by exon is shown in Figure 1A.

Table 1 summarizes gender, age, and tumor location across *KIT*-mutant, *PDGFRA*-mutant, and wild type (WT) groups. Patients with *KIT* and *PDGFRA* mutations were significantly older than patients with WT tumors (p<0.0001). While male predominance was observed across all groups, sex distribution did not differ significantly (p=0.61). Tumor location varied significantly, with wild-type GISTs more frequently arising in non-gastric sites, while *PDGFRA*-mutant tumors predominantly occurred in the stomach (p < 0.0001).

Among *KIT*-mutant cases, mutation site was significantly associated with tumor location (colorectal, gastric, and small bowel) (p=0.0003, **Table S2).** Exon 11 mutations were more common in gastric tumors. No significant difference was observed between colorectal and small bowel tumors (p=0.65). For *PDGFRA* mutant cases, mutation site was not associated with tumor location (p=0.24**).**

Secondary mutations

Among the 482 cases harboring *KIT* or *PDGFRA* mutation, 29 (6.0%) exhibited two *KIT* mutations, whereas no tumors demonstrated more than one *PDGFRA* mutation. When VAF's differed by <10% the exon containing the classically observed exon (i.e. exon 11 or 9) for the primary mutation was designated as the location of the primary mutation (**Table S3)**[17]. However, in one case (Case 15), the exon 13 mutation exhibited a markedly higher VAF (34.5%) than the exon 11 mutation, making the distinction between primary and secondary events uncertain. Secondary mutations were identified in exons 11, 13, 14 and 17, of *KIT* with exon 11 being the most frequent site. Figures 1B and 1C show the distribution of primary and secondary mutations by exon. Consistent with previous reports, recurrent secondary mutations such as V654, and N822K were observed[18]. No significant differences in age or sex were noted between patients with one versus two *KIT* mutations (p=0.054 and 0.590, respectively). However, cases with two mutations were significantly more likely to arise in non-gastric locations (81.8% vs. 33.7%, p<0.0001, **Table S4**). As previously described primary mutations in exons 9 and 11 of *KIT* were most often classified as the primary event, whereas alterations in exons 13/14 and 17 were more commonly observed as secondary mutations[18–20].

The frequency of mutation types in primary and putative secondary mutations is summarized in **Table S6** and **Table S7**, respectively.

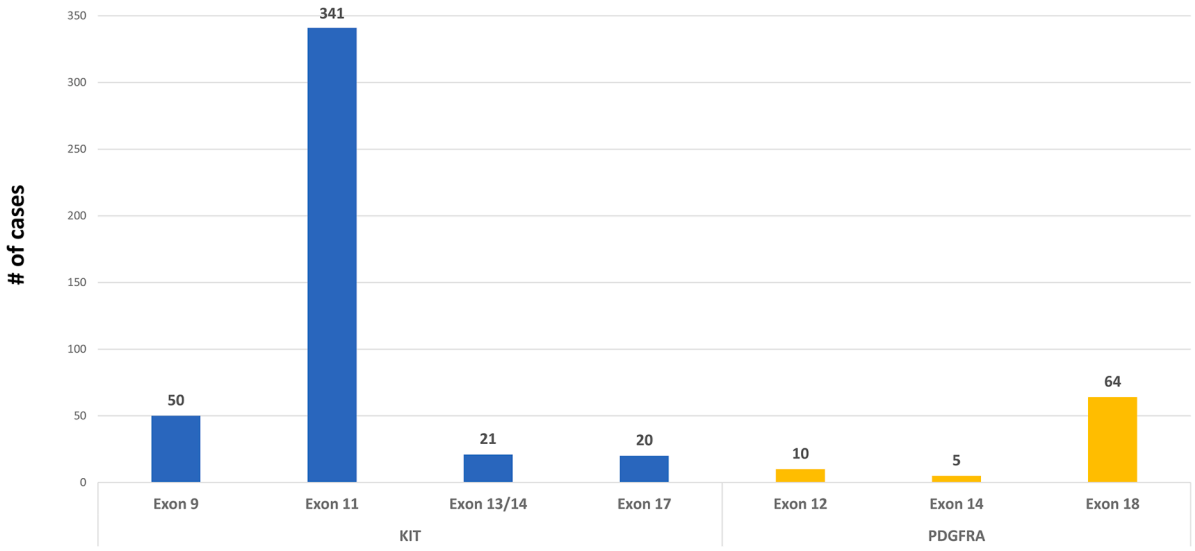

Figure 1A. Frequency of *KIT* and *PDGFRA* mutations by exon.

Table 1
GIST demographics.

Demographic	*KIT* mutant (N=403)	*PDGFRA* mutant (N=79)	WT (N=78)	P-value* (Three way)	P-value* *KIT* vs. *PDGFRA*	P-value* *KIT* vs. *WT*	P-value* *PDGFRA* vs. *WT*
Age (Mean, SD)	62.0 (12.6)	62.9 (12.4)	51.9 (16.8)	<0.0001	0.10	<0.0001	<0.0001
Age (N, %)							
≤30 years	5 (1.2)	1 (1.3)	12 (15.4)	<0.0001	0.53	<0.0001	<0.0001
>30 years	398 (98.8)	78 (98.7)	66 (84.6)				
Sex							
Male	231 (57.3)	50 (63.3)	45 (57.7)	0.61	0.38	1.00	0.51
Female	172 (42.7)	29 (36.7)	33 (42.3)				
Tumor site**				<0.0001	<0.0001	0.0036	<0.0001
Gastric	173 (56.8)	61 (95.3)	22 (35.4)				
Non-gastric	132 (43.2)	3 (4.7)	40 (64.6)				
Tumor site***				0.0005	<0.0001	0.0087	<0.0001
Gastric	173 (58.2)	61 (95.3)	22 (36.7)				
Small bowel	95 (32.0)	1 (1.5)	30 (50.0)				
Colorectal	29 (9.8)	2 (3.2)	8 (13.3)				

Abbreviations: WT= wild type.
* P-values are calculated using the chi-squared test and are considered significant if they are less than 0.05.
** Cases with unidentified tumor sites (N= 85) or metastases (N=44) were not included in this comparison.
*** Tumors with small numbers of cases including cervix (N=1), esophagus (N=1), pancreas (N=2), retroperitoneum (N=5), and spleen (N=1) were not included in this analysis but were included in the 'Non-gastric' group in the above comparison.

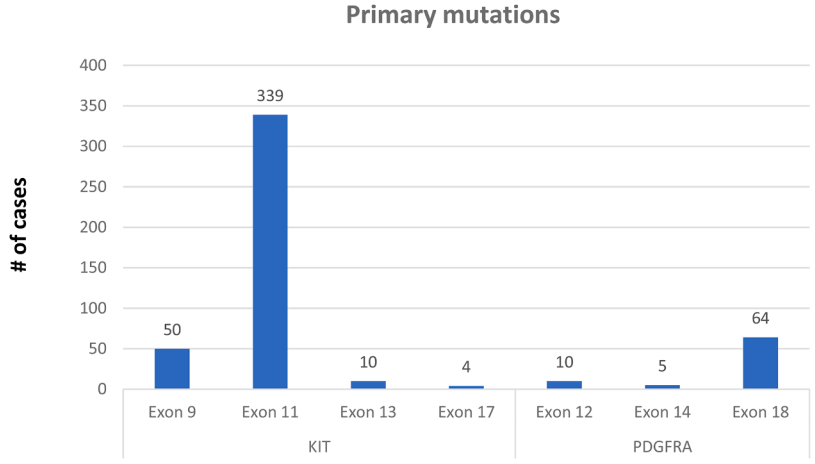

Figure 1B. Frequency of primary mutations in *KIT* and *PDGFRA* by exon. Primary mutations (except for case 15) were identified as having a higher VAF than other mutations in that gene.

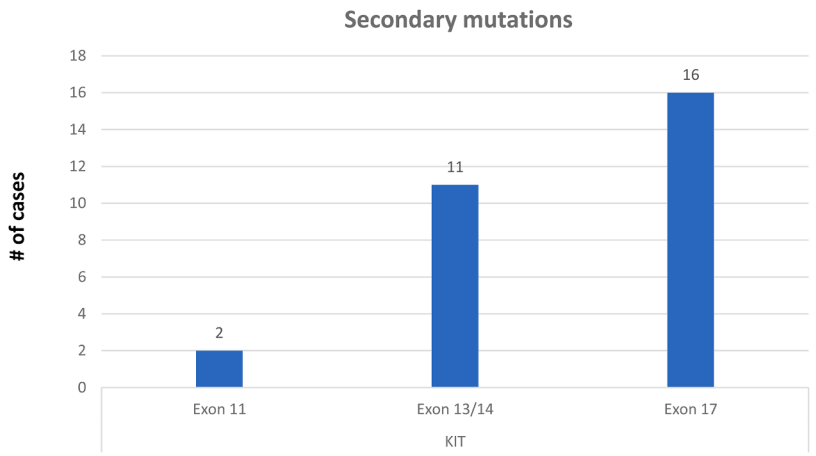

Figure 1C. Frequency of secondary mutations in *KIT* by exon. Secondary mutations (except for case 15) were identified as having a lower VAF than the primary mutation.

RNA-Seq Cohort

Twenty-four GIST cases previously analyzed by DNA NGS were selected for RNA-Seq: 16 with a *KIT* mutation (one with two *KIT* mutations), 5 with *PDGFRA* mutations, and 3 WT cases. RNA-seq was successful in 21 cases: 14 *KIT*-mutant (including one with two mutations), 5 *PDGFRA*-mutant, and 2 WT cases. RNA-Seq detected 13 out of 14 *KIT* mutations and all 5 *PDGFRA* mutations and confirmed WT status in the two WT cases (Table 2). Detected mutations included 8 deletions, 5 insertions, and 5 single nucleotide variants. All deletion or- insertion mutations were in-frame and varied in length from 3 to 27 base pairs. One 45bp deletion (case 15) was not detected by RNA-Seq, though low-level evidence was present in the raw data, suggesting a bioinformatic rather than a chemistry error. In case 5 case, RNA-Seq confirmed splice acceptor site alteration in codon 550, corresponding to a 1648-1674 deletion identified by DNA NGS (Figure 2A-C).

Expression and Variant Allele Frequencies

Expression levels of the *KIT* and *PDGFRA* mutations were high across all *KIT* and *PDGFRA* mutant cases (Table 2), consistent with their activating nature. The mean number of supporting reads for the seventeen *KIT* mutations was 4117.1 (SD=2724.4, range= 212-9836), and for *PDGFRA* was 3655.8 (SD=2430.9, range=1783-7656). RNA VAF's ranged from 17.2% to 87.8%. No significant correlation was found between DNA and RNA VAF's for KIT (r = -0.296, p=0.304) or *PDGFRA* (r=0.824, p=0.086) (**Figures S2** and **S3**). Interestingly, the IHC results showed diffuse positivity of Kit in WT cases 22 and 24 despite the absence of *KIT* or *PDGFRA* mutations. IHC results were not available for case 23.

Discussion

In this study, we demonstrate that RNA-Seq has high analytical performance for detecting clinically actionable *KIT* and *PDGFRA* mutations in GIST, identifying 18/19 mutations successfully detected by DNA NGS (95% sensitivity). This finding is clinically relevant because RNA-based approaches are increasingly needed in scenarios where tissue quantity is limited or when confirmation of splice-altering variants is required. In parallel, our analysis of 579 GISTs provides one of the larger real-world, laboratory-based molecular epidemiology datasets, illustrating current mutation frequencies, associations with anatomic site, and the prevalence of secondary mutations in routine clinical practice. Together, these results highlight complementary utilities of DNA and RNA sequencing in GIST and underscore the diagnostic and therapeutic implications of accurate mutation detection.

The demographic and anatomic patterns in our cohort align closely with established GIST epidemiology, with most patients diagnosed between the sixth and seventh decades of life[21]. A modest male predominance was also observed, although the basis for this sex difference remains unclear[22,27]. Our cohort included a higher proportion of wild-type tumors (14%) than the ~5% reported in population-based studies, a discrepancy likely reflecting referral bias, as *KIT/PDGFRA*--positive tumors may have been diagnosed by immunohistochemistry and therefore not submitted for sequencing, whereas IHC-negative cases were more often sent for molecular testing. The younger age of WT cases in our series further supports enrichment for *SDH*- and *NF1*-associated GIST, consistent with prior reports. Collectively, these patterns illustrate how test utilization and referral practices shape the molecular landscape observed in large clinical sequencing cohorts[7,28,29]. However, the number of WT tumors subjected to RNA-Seq was relatively small, limiting our ability to comprehensively evaluate RNA-based detection performance or expression characteristics in this heterogeneous subgroup.

In our RNA-Seq cohort, 21 of 24 cases yielded analyzable data. All *PDGFRA* mutations and 13 of 14 *KIT* mutations were identified, including single nucleotide variants, insertions, and in-frame deletions (). No *KIT, PDGFRA, BRAF, SDH* (*SDHA, SDHB, SDHC, SDHD*), or *NF1* mutations were detected in WT cases. All mutation-positive cases by DNA NGS demonstrated high *KIT* or *PDGFRA* mRNA expression, consistent with the activating nature of these mutations. However, DNA variant allele frequencies (VAFs) did not correlate significantly with mutant transcript expression levels, which may reflect tumor heterogeneity, transcriptional regulation, or RNA stability.

RNA-Seq also confirmed the functional impact of splice site mutations. In Case 5, a deletion spanning positions 1648-2 to 1674 detected by DNA NGS predicted exon 11 skipping, which was validated by RNA-Seq (Figure 2B and C). Although histopathology and immunohistochemistry (IHC) are often sufficient for diagnosing GIST, molecular testing remains essential for guiding targeted therapy because specific *KIT* and *PDGFRA* mutations correlate with treatment response[1]. For instance, mutations in *KIT* exons 13/14 or 17, and the *PDGFRA* D842V variant, predict poor response to imatinib[23], whereas D842V is associated with sensitivity to second-generation inhibitors like avapritinib [24].

The high concordance between RNA-Seq and DNA NGS supports RNA-Seq as a viable alternative when DNA is limited or transcript-level analysis is needed. Additionally, RNA-Seq provides functional insights—such as confirmation of splice site effects—that may enhance personalized treatment strategies.

Other RNA-based methods, such as RT-PCR, have also been used to detect mutations in GIST[25]. Funasaka et al. reported a 96.9%

Table 2
DNA NGS and RNA-Seq results for the RNA-Seq Cohort.

Case #	Tumor percentage	Gene	Mutation Type	Variant nomenclature	Exon	Length of Insertion or Deletion (bp)	Genomic Coordinates	RNA/DNA NGS Concordance	DNA NGS VAF	RNA NGS Wild type Counts	RNA NGS Variant Counts	RNA NGS VAF*
1	70	*KIT*	SNV	c.1727T>C p.L576P	11	NA	chr4: g.55593661	Concordant	46.1%	4166	4061	49.4%
2	90	*KIT*	DEL	c.1671_1676del p. W557_V559delinsC	11	6	chr4: g.55593604	NA**	42.9%	NA	NA	NA
3	80	*KIT*	INS	c.1504_1509dup p.A502_Y503dup	9	6	chr4: g.55592180	Concordant	42.6%	27113	7228	21.0%
4	70	*KIT*	DEL	c.1671_1676del p. W557_V559delinsC	11	9	chr4: g.55593605	Concordant	52.9%	1185	1482	55.6%
5	90	*KIT*	DEL	c.1648_1674del p.K550_K558del	11	27	chr4: g.55593582	Concordant	20.0%	3513	9836	73.7%
6A	90	*KIT*	DEL	c.1671_1676del p. W557_V559delinsC	11	6	chr4: g.55593605	Concordant	47.3%	5421	4480	45.2%
6B	90	*KIT*	DEL	c.2466T>G p.N822K	17	NA	chr4: g.55599340	Discordant	48.0%	NA	NA	NA
7	80	*KIT*	DEL	c.1653_1669delinsTC p. M552_W557delinsR	11	15	chr4: g.55593587	Concordant	70.0%	1020	212	17.2%
8	80	*KIT*	INS	c.1504_1509dup p.A502_Y503dup	9	6	chr4: g.55592180	Concordant	34.1%	5480	1543	22.0%
9	90	*KIT*	DEL	c.1651_1662del p.P551_E554del	11	12	chr4: g.55593585	Concordant	32.0%	6559	4066	38.3%
10	90	*KIT*	INS	c.1504_1509dup p.A502_Y503dup	9	6	chr4: g.55592180	Concordant	52.4%	17845	4722	20.9%
11	70	*KIT*	INS	c.1504_1509dup p.A502_Y503dup	9	6	chr4: g.55592180	Concordant	73.2%	14982	5165	25.6%
12	80	*KIT*	INS	c.1504_1509dup p.A502_Y503dup	9	6	chr4: g.55592180	Concordant	49.6%	12593	3425	21.4%
13	60	*KIT*	DEL	c.1656_1676del p.M552_V559delinsI	11	21	chr4: g.55593589	NA**	20.0%	NA	NA	NA
14	80	*KIT*	DEL	c.1679_1681del p.V560del	11	3	chr4: g.55593608	Concordant	56.5%	632	4551	87.8%
15	60	*KIT*	DEL	c.1676_1725del50insCACAG p. V559_T574delinsA	11	45	chr4: g.55593610	Discordant	10.0%	NA	NA	NA
16	80	*KIT*	DEL	c.1735_1737del p.D579del	11	3	chr4: g.55593669	Concordant	40.5%	9442	8004	45.9%
17A	80	*KIT*	SNV	c.1669T>A p.W557R	Failed to detect	NA	chr4: g.55593603	NA**	NA	4735	2369	33.3%
17B	80	*PDGFRA*	SNV	c.1682T>A p.V561D	12	NA	chr4: g.55141036	Concordant	39.0%	8203	7656	48.3%
18	80	*PDGFRA*	DEL	c.2526_2537del p.I843_D846del	18	12	chr4:g 55152094	Concordant	42.4%	1782	1783	50.0%
19	60	*PDGFRA*	SNV	c.2525A>T p.D842V	18	NA	chr4:g 55152093	Concordant	42.5%	2617	2196	45.6%
20	80	*PDGFRA*	SNV	c.2525A>T p.D842V	18	NA	chr4:g 55152093	Concordant	35.5%	3695	2380	39.2%
21	70	*PDGFRA*	SNV	c.2525A>T p.D842V	18	NA	chr4:g 55152093	Concordant	45.7%	4159	4264	50.6%
22	NA	WT	None	NA	NA	NA	NA	Concordant	NA	NA	NA	NA
23	NA	WT	None	NA	NA	NA	NA	Concordant	NA	NA	NA	NA
24	NA	WT	None	NA	NA	NA	NA	NA**	NA	NA	NA	NA

* RNA NGS VAF = RNA NGS Variant Counts/ (RNA NGS Variant Counts + RNA NGS Wild type Counts)

** Cannot compare due to failed RNA testing:

Case 2 (DV200 = 28): RNA library preparation failed due to insufficient library concentration. This DV200 value falls within the intermediate range in which RNA-seq success is variable, consistent with reduced RNA integrity.

Case 13 (DV200 = 37): RNA library preparation failed due to low library concentration, despite DV200 >30. This highlights that while higher DV200 values are generally associated with success, other factors such as RNA input quantity and specimen quality may still affect library yield, particularly in archival FFPE tissue.

Case 17a (DV200 not available): RNA-seq was attempted; however, NGS library preparation failed due to technical library issues, and a pathogenic *PDGFRA* mutation was instead detected by Sanger sequencing.

SNV= Single nucleotide variants, DEL=Deletion, INS=Insertion, NA=Not applicable, Discordant= Mutation detected by DNA NGS was not detected by RNA-Seq

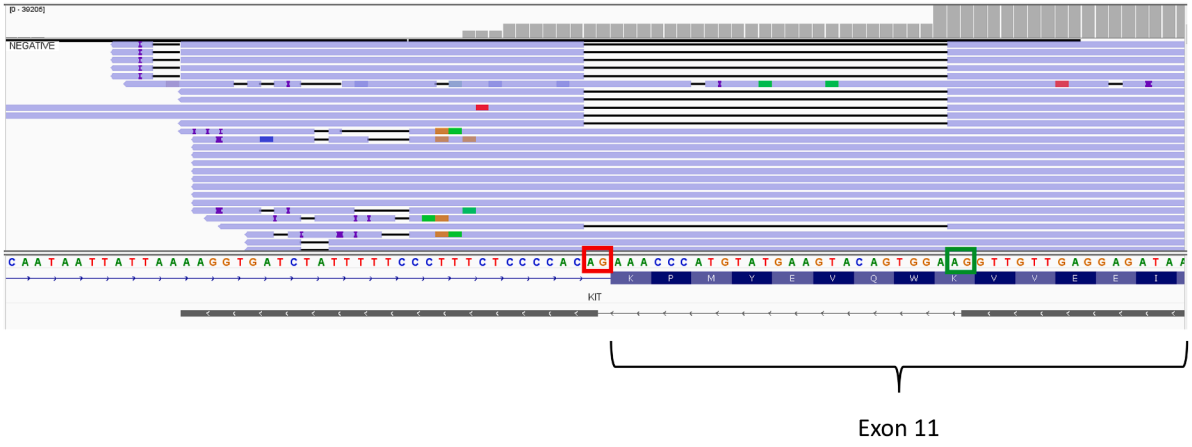

Figure 2A. DNA NGS shows in IGV a c.1648-2_1672del, a 27bp deletion for case 5 of the RNA-seq cohort. Note that this is designated as c.1648_1674del using standard nomenclature. The predicted splice acceptor sites in the reference sequence are annotated with green and red boxes.

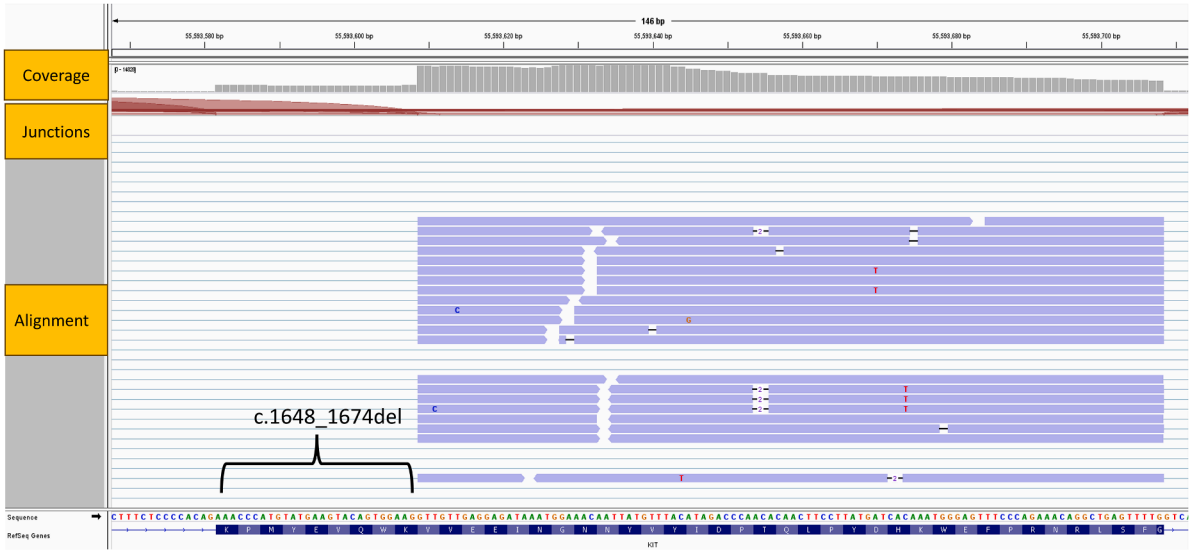

Figure 2B. RNA-seq analysis of the c.1648_1674del splice site mutation. The IGV visualization displays, from top to bottom: 1) Coverage track (gray bars): represents read depth at each nucleotide position, 2) Splice junction track (red): shows canonical splicing from exon 10 to exon 11, as well as an alternative splice junction from exon 10 to a site 27 base pairs into exon 11, also illustrated in Figure 2C, 3) Read alignment track (blue): displays sequenced reads spanning exon 11, 4) Reference track: Shows the nucleotide and corresponding amino acid sequences of exon 11 from the KIT gene.

detection rate for *KIT* and *PDGFRA* mutations in fine-needle aspirate samples using RT-PCR[25]. However, RNA-Seq offers a broader transcriptomic view, enabling simultaneous assessment of diverse variants, splice alterations, and gene expression patterns in a single assay. This is particularly valuable in GIST, where secondary mutations—especially in exons 13 and 17—can influence therapeutic sensitivity[26].

[29]

WT cases in our cohort were younger (mean age 51.9 years) compared to *KIT* (62.0 years) and *PDGFRA* (62.9 years) mutant cases (Table 1), consistent with established association of *SDH* and *NF1* related GIST with early onset. As previously reported, *KIT* exon 11 mutations—particularly deletions in codons 550–579—were most common, with frequently observed variants including c.1669_1674del (p.W557_K558del), c.1504_1509dup (p.A502_Y503dup), c.1676T>A (p.V559D), and c.1679T>A (p.V560D), found in 10.3%, 9.9%, 5.9%, and 3.6% of mutant cases, respectively[30] .Most *PDGFRA* mutations arose in gastric tumors, with c.2525A>T (p.D842V) being the most prevalent (9.9%) consistent with previous cohorts and large external datasets such as cBioPortal [17,31,32].

Putative secondary *KIT* mutations were identified in 6.0% of *KIT*-mutant cases. These mutations were more frequently located in exons 13, 14, or 17, whereas primary mutations were typically found in exons 9 and 11[33]. Several variants appeared exclusively as secondary mutations, suggesting acquisition under selective therapeutic pressure. The most common secondary mutation was c.1961T>C (p.V654A), observed in seven cases. Although treatment history was unavailable, these alterations are well recognized mechanisms of TKI resistance. Early detection of resistance-associated mutations is critical for optimizing therapy[34,35]. For example, *KIT* exon 9 mutations are may respond better to higher-dose imatinib or TKIs such as sunitinib or regorafenib [36]. Similarly, mutations in exons 13/14 or 17 may respond better to second- or third-generation tyrosine kinase inhibitors such as bezuclastinib or sunitinib [33].

Vanden Bempt et al. previously reported challenges in detecting *KIT* exon 11 deletions using targeted RNA-Seq[7]. In our RNA-Seq assay failed to detect a 45 bp deletion, indicating a limitation in identifying larger insertion or deletion events. The largest deletion successfully detected in our study was 27 bp. Although our sample size is limited, these findings highlight the need for further evaluation of assay performance for large structural variants.

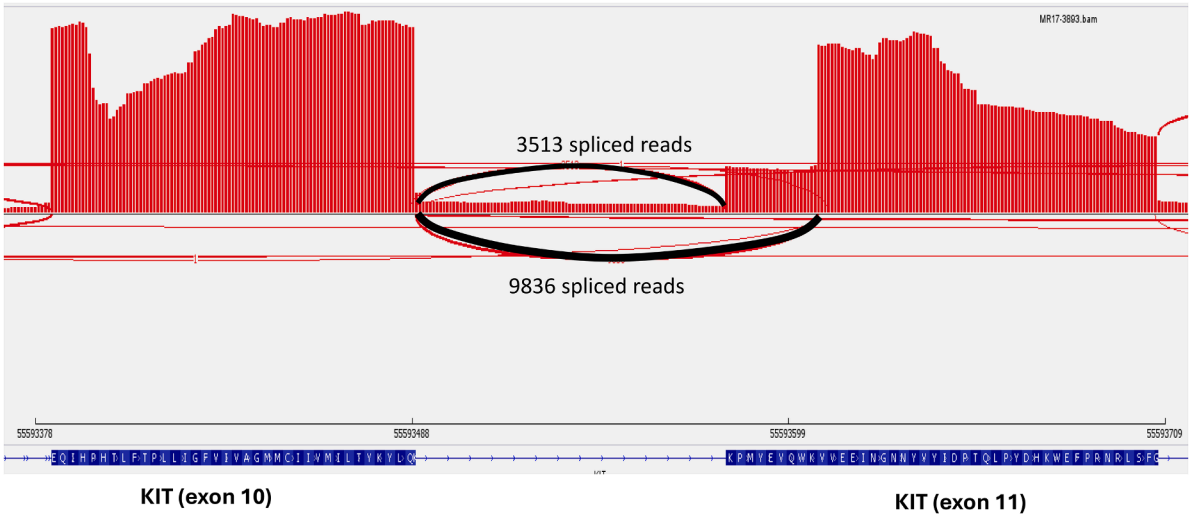

Figure 2C. The bar plot (red) represents read coverage along the exon for case 5. The Sashimi plot shows (as black curves) reads that are spliced from the end of exon 10 to the normal splice acceptor site (wild type-3513 spliced reads) and new acceptor splice site (mutant-9836 spliced reads).

In summary, our study demonstrates that RNA-Seq shows high concordance with DNA NGS for detecting clinically relevant *KIT* and *PDGFRA* mutations and provides additional functional insights, including reliable detection of splice-site alterations and most insertion or deletion variants. The poor correlation observed between DNA and RNA VAFs highlights underlying transcriptional or cellular heterogeneity that warrants further investigation. Our large reference-laboratory cohort also revealed several epidemiologic features not well described in prior literature, including a substantial proportion of tumors with unspecified primary sites and multiple rare anatomic locations. Within this cohort, we quantified and characterized dual *KIT* mutations at an unprecedented scale and identified a novel association between dual mutations and non-gastric tumor location, suggesting potential biological or therapeutic implications. The paired DNA/RNA/IHC evaluation further confirmed that strong *KIT* immunoreactivity can occur in genetically wild-type tumors, underscoring the need for molecular testing even when IHC suggests *KIT* pathway activation. Together, these findings emphasize the complementary strengths of DNA- and RNA-based sequencing for comprehensive GIST characterization and highlight opportunities to expand RNA-Seq for broader detection of rare drivers, transcriptional signatures, and clinically informative biomarkers that may refine personalized management strategies.

Our study's main limitations include: 1) the relatively small RNA-seq cohort, which was designed for comparison with DNA-based NGS rather than independent epidemiologic analysis, 2) the use of archival FFPE specimens, where progressive RNA degradation over time led to occasional library failures and reduced ability to call variants, 3) challenges in detecting complex variants, exemplified by a missed 45-bp *KIT* deletion due to short-read limitations, 4) RNA-seq–based mutation detection may have reduced sensitivity for variants present at very low allele fractions or with low transcriptional expression, including subclonal alterations, and 5) the absence of clinical outcome or survival data, which prevented correlation with treatment response. Future studies incorporating matched clinical outcomes may clarify whether mutant transcript abundance or expression patterns correlate with therapeutic response or resistance evolution.

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Portions of this manuscript, including the abstract and cover letter, were prepared with the assistance of AI-based tools for language refinement, organization, and formatting. These tools were used to improve clarity and consistency. All scientific content, interpretations, and conclusions were developed and verified by the authors without reliance on AI-generated analysis. The final manuscript was thoroughly reviewed and approved by all authors.

Author Contributions

Developed methods: BAP, JSV, RM, NF, JD; provided analysis: SJ, SA, BAP, RM, NF, MAA, JD, KCH; interpretation of data: SJ, SA, BAP, JSV, RM, NF, MAA, JD, KCH; wrote the original draft: SJ, SA, KCH; validated data: BAP; conceptualized the study: KCH; curated data: SJ, SA, BAP, MAA, KCH; supervised the study: KCH. All the authors reviewed and edited the final version of the manuscript.

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Soumya Jaladi: Writing – review & editing, Writing – original draft,

Formal analysis, Data curation, Conceptualization. **Saba Alvand:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Beth A. Pitel:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Jesse S. Voss:** Writing – review & editing, Validation, Methodology. **Rohini Mopuri:** Writing – review & editing, Methodology, Data curation. **Numrah Fadra:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Sham Sunder:** Writing – review & editing, Formal analysis. **Mazen A. Atiq:** Writing – review & editing, Data curation. **Jaime Davila:** Writing – review & editing, Methodology, Data curation. **Sounak Gupta:** Writing – review & editing. **Benjamin R. Kipp:** Writing – review & editing. **Kevin C. Halling:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2026.102812.

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