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Small Nucleotide Variant Analysis Using RNA Fusion Panel (SMURF): Making the Most of RNAseq Data in Solid Tumours

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ABSTRACT

Background: RNA-based fusion panels using targeted next-generation sequencing of formalin-fixed paraffin-embedded tumour tissue specimens are used for various tumour types to detect rearrangements/fusions. Using bioinformatic approaches, the data obtained from RNA sequencing (RNA-Seq) can also be used for small nucleotide variants analysis (single nucleotide variants and indels).

Methods: The performance characteristics of applying Small nucleotide variant analysis Using RNA Fusion panel (SMURF) analysis to RNA-Seq (RNA sequencing) data obtained from an RNA-based fusion panel was evaluated and compared to results obtained from a dedicated DNA-based panel.

Results: This demonstrated that an RNA fusion panel can be enhanced to identify small nucleotide variants to maximise the utility of this panel. Although coverage depth across various genes, based on gene expression, was found to be quite variable, which is expected, there were certain genes found to consistently display coverage depth suitable for variant identification, such as *GNAS, GNAQ, NRAS*, and *CTNNB1*.

Conclusion: This analysis suggests that the variants in these genes can be confidently reported from RNA-Seq data obtained from an RNA-based fusion panel.

1 | Introduction

Over the past several years, RNA-based fusion panels leveraging targeted next-generation sequencing (NGS) have been increasingly employed to analyse formalin-fixed paraffin-embedded (FFPE) tumour tissue specimens from patients with a wide range of malignancies. These panels enable the sensitive and specific detection of gene rearrangements and fusion events, which are critical for diagnostic, prognostic, and therapeutic decision-making. In addition to identifying structural alterations, this approach facilitates the characterisation of allele-specific expression patterns, providing deeper insights into tumour biology and potential mechanisms of oncogenesis [1–6]. Although sequencing DNA from cells obtained from FFPE or fine needle aspiration (FNA) is a well-established method for detecting small nucleotide variants (single nucleotide variants (SNVs) and indels), recent studies have explored the use of RNA as an alternative template for pathogenic variants detection [7]. Currently, most laboratories manage both DNA and RNA workflows separately to ensure proper detection of pathogenic variants and fusions [8].

Using bioinformatic approaches, data obtained from RNA sequencing (RNA-Seq) can also be used for analysis of small

nucleotide variants [9–12]. This approach is based on the principle that DNA regions encoding actively expressed genes are transcribed into mRNA, allowing pathogenic variants within coding regions to be identified [13]. Maximising information obtained from one test may enhance its diagnostic yield.

Performance characteristics of applying the SMURF (Small nucleotide variant analysis Using RNA Fusion panel) analysis to the RNA-Seq data obtained from an RNA-based fusion panel was determined by comparing results to a dedicated DNA-based panel.

2 | Materials and Methods

2.1 | Study Population

Cases with solid tumours tested on both a 1375-gene pan-cancer RNA fusion panel and 70-gene DNA panel were included in this study. A wide spectrum of tumour types was tested, including bone and soft tissue tumours, melanomas, lung adenocarcinomas, and glial tumours. In all cases, the same tissue block was used for both panels. A total of 26 samples with 42 variants were included in the analysis. Splice site variants and variants in the promoter region were excluded from analysis, as these would not be adequately sequenced by RNA-Seq. The study is consistent with the NHMRC Ethical Considerations in Quality Assurance and Evaluation Activities (2014) guidelines (QA/115179/MonH-2024-461258(v1)).

2.2 | Test Principle and Method

2.2.1 | DNA-Based 70-Gene Panel

Targeted NGS was performed on DNA extracted from patient tissue. FFPE tissue specimens were macrodissected prior to extraction when the tumour purity was low and required enrichment. Generation of DNA libraries by hybridisation capture was achieved using a custom 70-gene panel, followed by sequencing on an Illumina MiniSeq (Illumina Inc., San Diego, CA, USA). Data processing, including sequencing data alignment to the reference genome GRCh37 (hg19), and small variant calling was performed with the DRAGEN Enrichment application via Illumina BaseSpace Sequence Hub. Variants were annotated and analysed using Velsera Clinical Genomics Workspace (CGW v6.14). The limit of detection for this assay has been validated in this laboratory to a variant allele frequency (VAF) of 5%. Results were interpreted and reported in accordance with AMP/ASCO/CAP guidelines using HGVS nomenclature [14].

2.2.2 | RNA-Based 1375-Gene Fusion Panel

The hybrid capture method used by this laboratory utilises the Illumina TruSight RNA Pan-Cancer Panel, targeting 1375 cancer genes and detecting fusions by spanning all exons of all those genes. This qualitative assay can identify multiple fusion partners on a single sequencing platform, even without prior knowledge of gene rearrangements. At least 20 ng of extracted RNA was required to proceed to library preparation. After RNA extraction, RNA-Seq libraries were generated. This process included fragmentation, cDNA synthesis, adapter ligation and library amplification. Unique oligonucleotide indexes were then added for downstream multiplex sequencing. Libraries were then hybridised to biotin-labelled probes specific for target RNA regions. Streptavidin beads were used to capture biotinylated probes, with magnet-based removal of any unbound fragments. Post-capture libraries were quantified using the Promega QuantiFluor RNA System (Promega Corporation, Madison, WI, USA) to ensure optimal cluster density on the flow cell. Sequencing was performed using the Illumina NextSeq 550 sequencing system. Illumina DRAGEN RNA application was used for secondary analysis. The gene fusion module uses DRAGEN RNA spliced aligner to perform split-read analysis on chimeric alignments to detect potential breakpoints. Additionally, small variant calling was performed along with variant annotation via Nirvana (BaseSpace). QC coverage depth metrics were generated on an input BED file. The hard-filtered.vcf.gz was used for further downstream analysis. This file was uploaded to navify Mutation Profiler, an ARTG listed analysis software (Roche Diagnostics, North Ryde NSW, Australia) to perform tertiary analysis. A virtual analysis panel was used to evaluate variants in genes that were common across both panels.

3 | Results

3.1 | Overlap of Genes Across the Two Panels

Out of the 70 genes in the DNA panel, 62 were available in the RNA-based panel. Only the genes common across both panels were evaluated.

3.2 | Mean Coverage Depth of Genes in RNA-Based Panel

QC coverage depth metrics were generated using an input BED file. For the purposes of this study, only the mean coverage depth of genes also found in the DNA panel were evaluated. Mean coverage depth was quite variable, with some genes displaying consistently high coverage depth (more than 200×), including *AKT1, ARID1A, CDH1, CDK4, CTNNB1, EPCAM, FGFR1, GNAS, IDH1, IDH2, PDGFRA, PDGFRB, PPP2R1A, PTEN*, and *SF3B1*. Other genes with mean coverage depth between 100× and 200× included *APC, ATM, BAP1, BRAF, CDK6, EGFR, ERBB2, ERBB3, FBXW7, FGFR2, GNA11, GNAQ, KRAS, MAP2K1, MET, MLH1, MSH6, MYC, NF1, NF2, NRAS, PIK3CA, RB1, SMAD4, TP53*, and *VHL*. Genes with poor mean coverage depth (less than 50×) included *ALK, AR, BRCA1, BRCA2, CD274, CDKN2A, ESR1, HRAS, MYCN, PALB2, POLD1, RET*, and *ROS1*.

Figure 1 (box plot) shows the mean coverage depth and range across genes in the three major tissues examined, i.e., soft tissue, lung and skin. The figure also demonstrates the mean coverage depth on the DNA panel.

3.3 | Variant Comparison Between Analyses

A total of 42 variants across 26 samples were compared on both panels (Table 1). All variants were identified in both data sets,

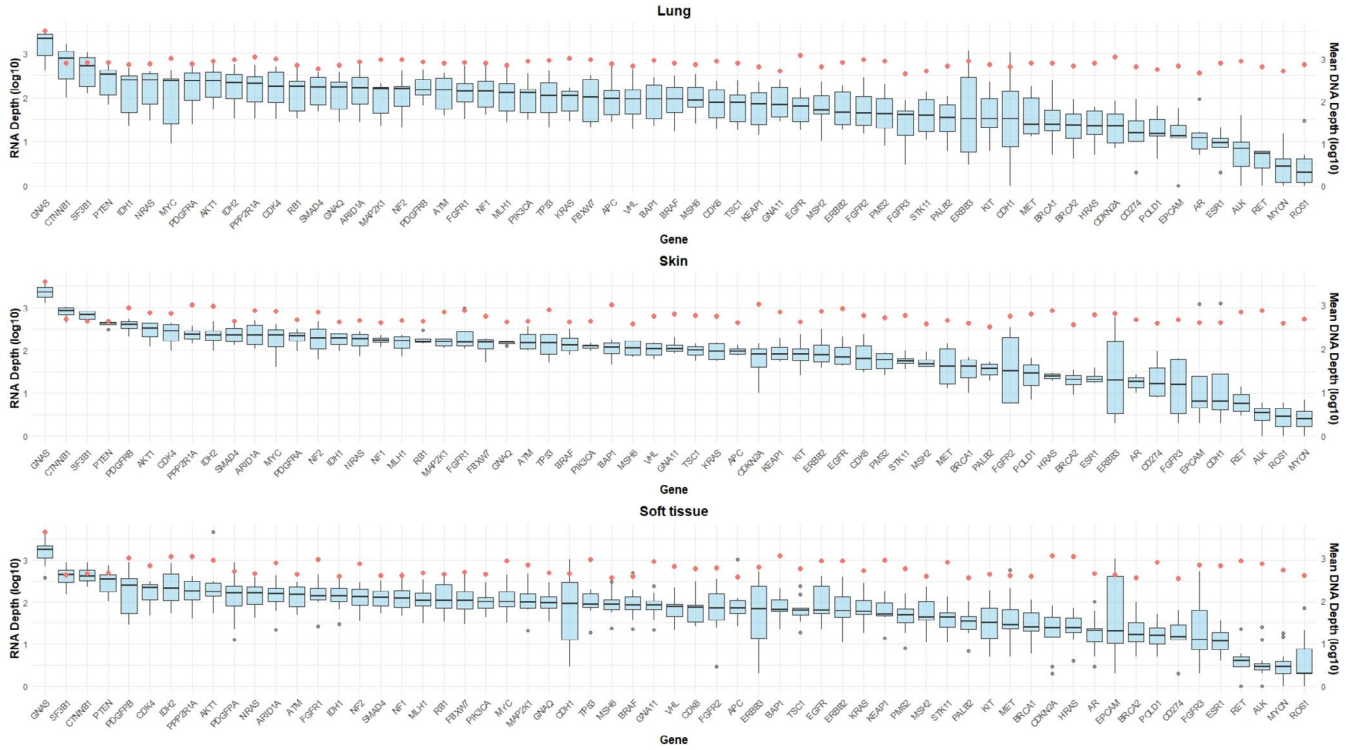

FIGURE 1 | Box plots displaying mean coverage depth across various genes present in RNA-Seq data in three subgroups of tissues: Lung, skin and soft tissue. The corresponding mean DNA-seq depth is displayed by the red dot.

except two (ATM Y1442Ifs in sample 9 and NF1 L844F in sample 12) (Figure 2). No false positive variants were identified by RNA-Seq that were missed by DNA-Seq, resulting in a Positive Percent Agreement (PPA) of 95%. Since no false positives were observed, the Negative Percent Agreement (NPA) could not be calculated.

Initially not called by the SMURF analysis, these two variants were evaluated further and able to be visualised on IGV using BAM files. Further examination indicated that both variants were called as candidates but filtered out as "weak evidence". The DRAGEN RNA pipeline uses a threshold where only variants with SQ $\geq$11.8 are filtered in as PASS, with both these variants slightly below the cutoff (10.63 and 10.79, respectively). To remedy this, the threshold was lowered to 10. On repeated analysis following this modification, both variants were identified. Throughout these analyses, there was no evidence that RNA-Seq did not identify variants due to nonsense mediated decay (NMD).

3.4 | VAF and Coverage Depth Comparison

VAF and coverage depth of variants identified in both analyses were evaluated. Overall, VAFs for most variants were similar. There were two cases (case 8 and case 22) where there was a significant difference in the VAF of the variants by DNAseq and SMURF analysis, likely due to allele-specific expression (ASE). On excluding these two variants from the analysis, regression analysis produced an R^2 value of 0.72, indicating good concordance between the two methods (Figure 3). Variants with higher coverage depths on RNA-Seq included hotspot variants in *GNAS, GNAQ, NRAS*, and *CTNNB1* genes, with similar VAFs for these hotspot variants across both analyses (Table 1).

4 | Discussion

4.1 | Challenges Associated With RNA-Seq for Small Variant Detection

The use of RNA for detecting gene mutations is inherently limited to regions of genes that are actively expressed and relies on both the stability and abundance of mRNA transcribed from the gene of interest in specific cell types [9]. In addition, RNA requires careful monitoring of input quality (e.g., integrity, fragment size), especially in FFPE samples, where RNA degradation is common [15]. Also, such analyses may not be able to detect all variants that involve splice sites [16, 17]. The application of RNA-based sequencing for small nucleotide variants is not common practice, due to the intrinsic complexity of splicing and RNA editing [18]. There is also the possibility of errors being introduced by reverse transcription (RT) during library preparation and increased alignment errors near splicing junctions [19]. It is important to note that nonsense variants might be missed due to nonsense-mediated decay; thus, it is important to have strict quality control measures in place where the coverage depth across the hotspots being reported is evaluated and visualised [18]. Another disadvantage of using RNA-based SMURF analysis is that some significant variants in the promoter region, especially the *TERT* promoter, will not be detected if they are not transcribed [9]. This limits utility in testing of brain tumours, where *TERT* promoter variants are important for the diagnosis of glioblastomas [20].

TABLE 1 | Comparison of variants identified in both DNA sequencing and SMURF analysis, with read depths and VAFs (*after modifying DRAGEN RNA pipeline SQ threshold).

			DNA panel		RNA panel: SMURF		Concordance
Sample	**Gene**	**Variant**	**VAF**	**Depth**	**VAF**	**Depth**	
1	GNAS	R201H	36	307	33	425	Yes
2	CTNNB1	T41A	14	274	25	262	Yes
3	GNAS	R201H	33	949	37	1215	Yes
4	PIK3CA	H1047R	17	421	23	179	Yes
	TP53	C242F	14	640	24	185	Yes
	TP53	R280S	14	289	20	123	Yes
5	GNAQ	Q209L	10	121	10	220	Yes
6	TP53	G266E	61	553	75	252	Yes
7	CTNNB1	T41A	20	296	28	707	Yes
8	CDKN2A	D108Y	49	356	100	52	Yes
	TP53	T155P	37	287	51	74	Yes
9	ATM	Y1442Ifs	36	294	24	148	No/Yes*
	TP53	R158G	10	1046	16	44	Yes
10	CTNNB1	S45F	52	147	36	586	Yes
11	TP53	G266E	34	384	51	218	Yes
12	NRAS	Q61L	45	239	65	353	Yes
	NF1	L844F	24	225	26	228	No/Yes*
13	TP53	P301Qfs*44	24	408	57	207	Yes
	PTEN	Y16D	22	705	38	610	Yes
14	NRAS	Q61R	18	157	44	248	Yes
	NF2	K253fs	40	810	42	64	Yes
15	EGFR	E746_A750del	25	676	13	15	Yes
	TP53	V272M	81	427	73	26	Yes
16	APC	K714Sfs*4	15	120	25	36	Yes
17	KRAS	G12D	18	430	33	39	Yes
	BRAF	G464V	36	548	40	73	Yes
	TP53	R273L	16	630	12	50	Yes
18	GNAS	R201H	21	234	18	590	Yes
19	TP53	V173L	17	308	13	23	Yes
20	GNAQ	Q209H	8.3	84	13	150	Yes
21	TP53	M246T	79	957	88	368	Yes
22	EGFR	L858R	43	1504	59	578	Yes
	TP53	R273C	50	524	94	231	Yes
23	TP53	R283P	12	775	32	139	Yes
24	KRAS	G12D	33	556	40	125	Yes
	ARID1A	Q614*	62	492	57	42	Yes

(Continues)

TABLE 1 | (Continued)

Sample	Gene	Variant	DNA panel		RNA panel: SMURF		Concordance
			VAF	Depth	VAF	Depth	
	PIK3CA	E542K	33	311	38	74	Yes
	ATM	Q401*	62	492	68	37	Yes
25	CTNNB1	T41A	17	250	27	629	Yes
	BRCA2	Q940*	51	207	50	20	Yes
26	NF1	C582Lfs*6	72	236	70	40	Yes
	PTEN	G127*	69	271	49	328	Yes

It's also important to note that gene expression can vary depending on the tissue being analysed. For instance, the Androgen Receptor (AR) is highly expressed in gonadal tissues, but shows lower expression in skin and soft tissues, as observed in our dataset [21]. We are already aware that prioritizing high specificity through stringent filtering strategies during secondary analysis often comes at the cost of sensitivity [22]. This trade-off is well recognized in genomics research. In our case, this was further demonstrated by the two variants that were initially missed, only being detected after we lowered the filtering threshold.

4.2 | Advantages of Using the SMURF Approach

The advantage of pairing small nucleotide variant detection and fusion analysis in one protocol is that it reduces processing time and conserves tissue sample [23]. Genes that are uniformly expressed at a higher level can be evaluated for identifying small exonic variants with confidence.

In addition to SNVs, this approach was also able to identify small indels. Hagiwara et al. have previously demonstrated that RNA-Seq data can be used to identify indels using RNAIndel software, robustly predicting 88%–100% of somatic indels [24]. Another advantage of RNA-Seq is that the highly expressed genes have more reads, which increases the chances of identifying a small nucleotide variant occurring at a low VAF [19]. Alignment errors at splicing junctions can be mitigated by using two different aligners that employ distinct clipping strategies [19]. It is also recommended to filter out soft-clipped regions in RNA-Seq reads [19].

Coudray et al. demonstrated that variants identified by RNA-Seq displayed better representation of glioblastoma-related pathogenic variants, as catalogued by COSMIC [25]. In the same study, it was observed that RNA-Seq showed fewer uniquely mapped reads than whole exome sequencing.

This study, like others, demonstrated a good correlation between VAFs obtained by the SMURF analysis and the DNA-based estimate [13]. However, some samples displayed larger differences, which may be attributable to differences in gene expression. This study also identified some genes with better read depth from RNA-Seq than DNA-based sequencing, including *GNAS, GNAQ, NRAS*, and *CTNNB1*. This analysis suggests that hotspot variants in these genes can be confidently reported from the SMURF analysis.

Currently, most laboratories apply sequential DNA- and RNA-based NGS protocols to capture all possible alterations; however, this approach may lead to tissue sample exhaustion and increased laboratory workload [13]. In soft tissue tumour cases, where most of the NGS-based testing is performed to look for diagnostic fusion transcripts, SMURF analysis could be particularly useful in cases where there is also a need to examine certain hotspot pathogenic variants (e.g., *CTNNB1* for desmoid fibromatosis and *GNAS* for fibrous dysplasia). In this analysis, both of those genes have consistently displayed high read depth levels and high levels of concordance in VAFs for variants detected by both the DNA- and RNA-based panels.

Other studies have reported variable success rates with the identification of pathogenic variants by RNA-Seq compared to DNA sequencing. Most have reported a variant identified in 40%–50% of cases [9, 18, 26]. The success rate for this study identified 94% of variants in its initial form and 100% in this small cohort, after slightly decreasing the SQ threshold. It should be noted that many of the variants in RNA-Seq data for this study were present at a read depth of less than 100×. If a threshold of 100× or 50× read depth is chosen, then RNA-Seq would identify 68% and 88% of the variants, respectively. Exclusion of splice site variants has also led to higher concordance rates in this analysis.

In some of the cases in this study there was a marked difference in VAFs of variants identified by DNAseq and RNA-Seq-SMURF e.g., the *CDKN2A* D108Y in case 8 was identified at 49% VAF in DNAseq but at 100% by SMURF analysis. Similarly, *TP53* R273C in case 22 was present at 50% VAF in DNAseq but at 94% VAF in SMURF analysis. The reason for this variation could be that the RNA-Seq data may have allele-specific differential expression of pathogenic variants [27, 28]. This may lead to some of the variants being falsely implicated to be of germline origin. On the other hand, the over-representation of the normal allele is well documented in cases of heterozygous pathogenic variants in RNA-Seq, when there is an expression imbalance between the mutant and wild-type alleles [7, 29]. Some previous studies have attributed these differences to technical variations in RNA-Seq methodologies [30]. This study, however, did not find marked overexpression of normal alleles by RNA-Seq, which may be due to mutant alleles being hyperactive or being more stable than the wild-type allele [31, 32].

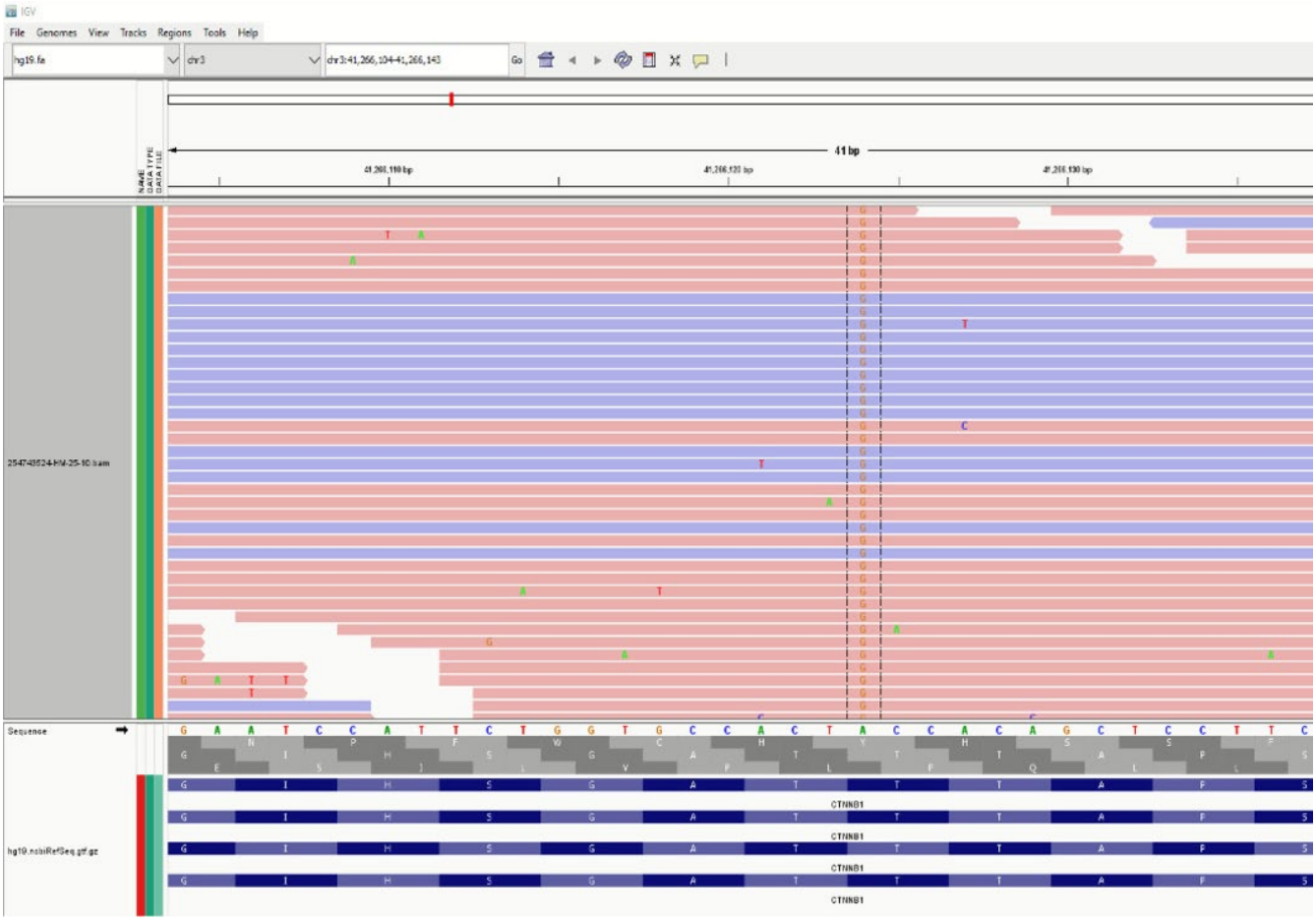

FIGURE 2 | IGV visualisation of a *CTNNB1* variant in RNA-Seq data.

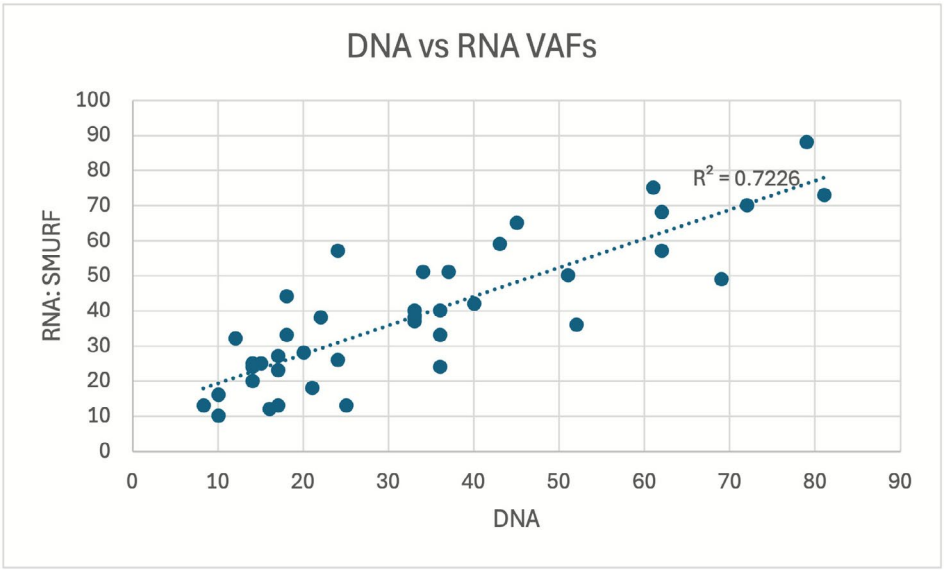

FIGURE 3 | Regression analysis comparing VAFs of variants identified by both DNA and RNA sequencing.

5 | Conclusion

While RNA fusion panels are designed principally for rearrangement detection, the capacity to call expressed SNVs and indels offers valuable insights. When carefully implemented, RNA-based panels can serve as a powerful adjunct in solid tumour workflows that require both fusion and small nucleotide variant detection.

This study demonstrated a proof of concept that the utility of an RNA fusion panel can be enhanced by also identifying small nucleotide variants from only minor modifications to the

bioinformatic workflow. Although coverage depth across various genes was found to be quite variable, certain genes (e.g., *GNAS, GNAQ, NRAS*, and *CTNNB1*) were found to display coverage depth consistently good enough for this type of analysis. We propose therefore that hotspot variants in these genes may be confidently reported from RNA-Seq data obtained from an RNA-based fusion panel.

Thus, SMURF analysis using RNA-Seq data can be considered a "rule in" type of test with high specificity but lower sensitivity, due to lower read depths across some genes. Therefore, whilst this approach may be able to markedly increase the utility of data obtained from RNA-based tests, it will still remain important to employ orthogonal tests (DNA sequencing) where unexpected negative results are obtained.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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