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ORIGINAL ARTICLE

Evaluating gene fusions in solid tumors – Clinical experience using an RNA based 53 gene next-generation sequencing panel

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Abstract

Given the known association of gene fusions with solid tumor morbidity and the need to clarify the role of fusions in therapeutic, prognostic and diagnostic outcomes, we reviewed the positive yield rate for fusions in solid tumors using cases that were referred to our laboratory for clinical testing. We retrospectively evaluated results from 183 solid tumor samples that were received during a 24 month period for testing using the FusionSeq™ assay, an RNA-based Next Generation Sequencing (NGS) panel of 53 genes known to form fusions in solid tumors. Positive yield rate (actionable fusions) was evaluated for all samples tested, as a correlate for clinical utility. Twenty five fusions (actionable, variants of uncertain significance – VUS, and benign) were identified, of which 7 were classified as actionable gene fusions, resulting in an overall positive yield rate of ~3.8% (7/183). Sixteen mostly novel fusions were classified and reported as VUSs. Five fusion events were classified as false positives, occurring due to mispriming or wild-type read through while 2 were classified as likely benign. Additionally 68% of fusions (17 of 25) detected in our study were present in prostate, colorectal, and gynecological cancers, suggesting that the frequency of fusions identified is dependent on specific tumor type. The high number of novel fusions identified highlights the potential for fusions in precision medicine.

Keywords Fusions, Solid tumors, Next-generation sequencing, Clinical utility.

Introduction

Fusion variants are hybrid genes that occur due to genomic rearrangements such as chromosomal inversions, deletions and/or translocations and have been identified in both hematological neoplasms and solid tumors. Gene fusions as they occur can be associated with oncogenic properties, and may often drive tumorigenesis in a wide array of cancer types [1,2]. Some fusions are known to occur in a single type of tumor, like the *PAX8-PPARγ* fusion in Thyroid Cancer [3], while others such as the *ETV6-NTRK3* fusion have been found across several cancer types including infantile fibrosarcoma, secretory breast carcinoma and acute myeloid leukemia [4].

Tumorigenic properties of gene fusions depend upon their ability to either upregulate the activity of a proto-oncogene (e.g. by fusing a strong promoter to a proto-oncogene), forming a fusion protein with oncogenic functionality (e.g. by causing a constitutive activation of a tyrosine kinase) or inducing a loss of function (e.g. by truncating a tumor suppressor gene). About 20% of human cancer morbidity is estimated to be caused by gene fusions [5], with the highest frequency of gene fusions being seen in hematological malignancies (~90%). The Cancer Genome Atlas (TCGA) Fusion gene data portal (www.tumorfusions.org) lists 20,731 gene fusions across 33 different solid tumor types, with breast invasive carcinoma having the highest number of fusions (20%) and uveal melanoma having the least number of fusions (0.2%) [2], suggesting that frequency of fusions is dependent on specific tumor type [6].

The technological revolution of massively parallel or next-generation sequencing in the recent years has allowed for the simultaneous detection of multiple fusions either at the DNA or RNA level with a higher confidence, and in a shorter period

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of time. Evaluation of solid tumors for fusions is currently common practice and standard of care. Focus on fusions for therapeutic indications, began in 2001 with the development of the first tyrosine kinase inhibitor (TKI) targeting BCR-ABL, imatinib mesylate (Glivec; Novartis), which was approved by the Food and Drug Administration (FDA) as a cancer treatment [7]. This TKI was one of the first targeted therapies used for cancer treatment and led to a major improvement of CML prognosis, with a remission observed in 80% of cases. In the past 20 years, though a significant number of fusions have been identified across adult and pediatric tumors [2,8], only a small number have been functionally characterized, with even fewer of those gene partners (*ABL1, ALK, ROS1, RET, NTRK, FGFR, BRAF, ETS, EWS*) being evaluated as therapeutic targets [9]. Evaluation of solid tumors for fusions may result in the identification of novel, uncharacterized fusions, contributing to future studies of potential clinical utility. This ultimately benefits the patients, with greater options for therapeutic intervention. In this study, we outline our clinical experience using a 53 gene next-generation sequencing panel for the evaluation of fusions across 183 cases. We present the actionable fusions and the variants of uncertain significance (VUS) identified, and correlate them to cancer type.

Materials and methods

Case cohort

To identify genetic associations for therapeutic, prognostic and diagnostic purposes, a total of 305 cases were referred by oncologists to The Jackson Laboratory (JAX) Clinical Genomics Laboratory to be processed using a clinically validated (CLIA/CAP) assay called the FusionSeq™. This assay incorporates an RNA-based panel for 53 genes (Supplemental Table 1) known to form fusions in solid tumors, during the time period of April 2016–May 2018. Formalin Fixed Paraffin Embedded (FFPE) samples, which were shipped to the JAX CLIA lab by the ordering physicians, were sectioned in-house, (5 μM, 10 unstained slides and 1 stained with Hematoxylin and Eosin), reviewed for neoplastic content, and areas of tumor were marked off by a pathologist to enable macro-dissection for nucleic acid extraction. Samples which did not meet the established neoplastic content cut-offs ($>$50%), were either processed under deviations, ($\geq$30% up to 50%) or failed for not meeting specimen requirements ($\leq$30%).

Fusion detection

Total nucleic acid (TNA) was extracted from macro dissection-enriched FFPE tissue sections, followed by cDNA synthesis and sequencing, using a modified and validated Archer FusionPlex Solid Tumor Panel assay (Archer Dx, Boulder, CO). Post extraction included additional quality control (QC) metrics to establish cut-offs for RNA concentration and quality. A sample was failed for RNA quality if it did not pass the extraction QC of yielding 200 ng of nucleic acid. In addition, RNA purity was evaluated using a nanodrop spectrophotometer (Thermo Fisher Scientific) for OD 260/280 ratios, and a Bioanalyzer (Agilent Genomics) to measure the DV_{200} score by calculating the areas under the curve for all fragments greater than 200 bp. Samples with an OD260/280 ratio $>$1.6 and with DV_{200} scores greater than 30% were acceptable for downstream processing. A qPCR QC step was also executed to determine synthesized cDNA quality, which requires a cycle threshold (Ct) value of $\leq$30. Illumina MiSeq sequencer generated 150 bp paired-end sequence reads with a minimum of 1.5 million reads per sample. The fusion detection limit for all fusions was 5% of wild-type with a minimum of $\geq$5 supporting reads and $\geq$3 unique start sites (fusion supporting reads).

Sequence analysis, variant interpretation and clinical reporting

Fusion analysis was performed using the Archer™ Analysis Software, which aligns against the hg19 human genome build and enables protein translation predictions for all reported fusions in our cohort (Archer analysis software, Manual Section 3.2.7.3). In addition, these predictions were cross-checked manually using the chromosomal breakpoints in the fusion partners for the given protein-coding transcripts. We also evaluated canonical splice sites across fusions and those fusion genes with intronic breakpoints that preserved the canonical splice donor/acceptor sites. This minimized the chance of exon skipping and protein truncation during transcription and translation, and was therefore thought to be in-frame. Fusions determined to be actionable were confirmed either by re-running the sample or using Taqman Reverse Transcription PCR (RT-PCR), before being included in the clinical report. Supplemental Table 2 lists the primer/probe sets that were used to confirm fusions.

Evidence of association between genomic variants and potential response to therapy or availability of clinical trials is curated from the peer-reviewed literature, publicly available databases, the JAX clinical knowledgebase (JAX-CKB), clinicaltrials.gov, FDA.gov and clinical practice guidelines. Variants were classified into four tiers based on the 2017 joint consensus guidelines by AMP/ASCO/CAP on interpretation of sequence variants in cancer [10]. The four tiers include strong clinical significance (Tier I), potential clinical significance (Tier II), unknown clinical significance (Tier III), and benign or likely benign variants (Tier IV).

Those variants referred to as ‘actionable’ met the criteria for Tier I or Tier II clinical significance and were further categorized as therapeutic, prognostic, or diagnostic. Variants were classified as Tier I (strong clinical significance) if they were indicated on-label for an FDA approved drug in the patient’s tumor type or if clinical practice guidelines recommended treatment decisions based on the presence of the variant in the patient’s tumor type. Variants were classified as Tier II (potential clinical significance) if clinical trial evidence supported off-label use of an FDA-approved drug in targeting the mutation or if the variant served as a marker for inclusion within an ongoing trial. A variant was classified as Tier III if evidence was conflicting or insufficient to satisfy conditions for classification as a Tier I, II or IV mutation [10]. Tier IV variants were those classified as benign or likely benign based on the recommendations [10] and were not reported for routine testing. Benign or likely benign variants are those that are observed at high allelic frequency in the general or specific subpopulation databases and have no existing published evidence of cancer association [10].

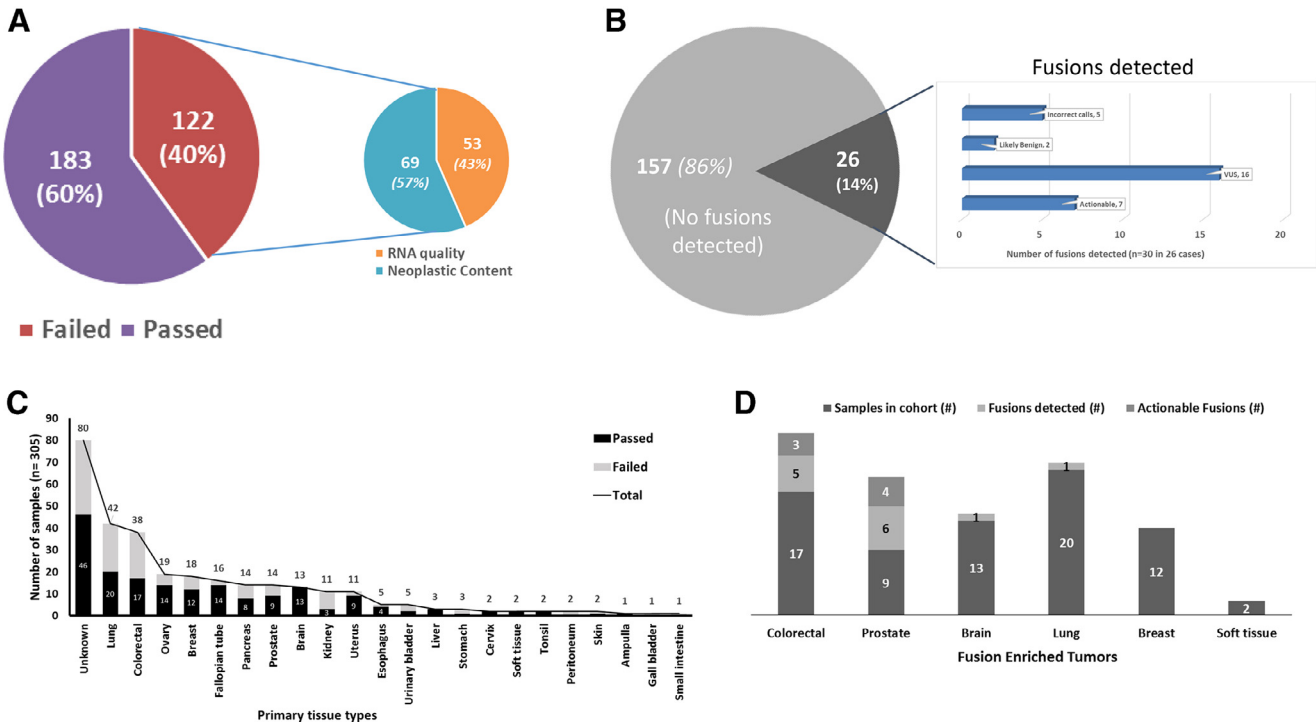

Fig. 1 Evaluation of the cohort as demonstrated by the number of samples that passed or failed initial assessment of neoplastic content/RNA quality (A), those that had fusions present (B), the primary tumor types that were obtained for testing (C), and the number of fusions identified in tumor types that are known to be fusion enriched (D).

Statistical analysis

The positive predictive value and the 95% confidence interval (CI) for the FusionSeq™ assay was determined using the following formulas: positive predictive value (PPV) = True positive (TP)/True positive + False positive (FP) and, 95% CI of PPV= PPV +/− 1.96* Standard error (SE) (PPV) [11,12]. Significance tests were performed using two-tailed Fisher's Exact Test to generate *p* values [13].

Results

Positive yield rate of fusion evaluation in solid tumors

From a total of 305 cases that were referred for testing of the fusion gene panel, 122 cases (40%) failed for two primary reasons, RNA quality (43%, 53 of 122) and insufficient neoplastic content (57%, 69 of 121). The remaining 183 cases (60%) were successfully processed to generate data on the FusionSeq™ assay (Fig. 1(A)). Of the 183 cases, 157 (86%) had no detectable fusions, 26 cases (14%) had 30 fusions detected by the Archer software (Fig. 1(B)), of which 7 were deemed actionable, 16 were classified as VUSs, 2 were evaluated to be likely benign, and 5 were false positive calls due to mispriming and wild-type read through events. Fusion genes classified as clinically actionable or a VUS are listed in Table 1. Detection of 7 actionable fusions resulted in a positive yield rate of 3.8% (7 of 183), with the PPV of fusion detection for the FusionSeq™ assay in our cohort being 76.6% (66.7–86.5%, 95% CI).

The entire cohort of 305 cases included 69 types of pathological diagnosis (Table 2) across 23 tissue types (Fig. 1(C)). We evaluated tumor primaries known to be enriched for fusion genes (colorectal, brain, breast, lung, prostate and soft tissue) from the 183 successfully processed cases and identified 5 fusions (29%, 5 of 17) in colorectal cancer, 6 fusions in prostate cancer (67%, 6 of 9), 1 in brain (8%, 1 of 13), 1 in lung (5%, 1 of 20) and none in the 12 breast or 2 soft tissue tumors (Fig. 1(D)). Metastatic cancer of unknown primary (CUP) along with adenocarcinoma of unknown primary were the biggest cohort (26%, 80 of 305 cases) with 46 of the 80 (57.5%) successfully passing the FusionSeq™ assay (Table 2). Other prevalent diagnoses in our tested sample cohort included lung adenocarcinoma (20 cases) and colorectal cancer (17 cases) (Table 2).

Fusions detected (actionable, variants of uncertain significance and likely benign)

Seven clinically actionable fusions were identified in our cohort (Table 1); *TMPRSS2-ERG* was the most common fusion, being identified in 4 cases of prostate adenocarcinoma. Colorectal adenocarcinoma had the 3 other actionable fusions, 2 known, *EIF3E-RSPO2* and *PTPRK-RSPO3,* and 1 novel fusion, *QKI-BRAF* (Table 1). In our cohort, prostate (44%, 4 of 9) and colorectal cancer (18%, 3 of 17) were the only two fusion enriched tumor types [2] found to have actionable fusions (Table 1 and Fig. 1(D)).

Table 1 Fusion genes, actionable and variants of uncertain significance (VUS) detected in the cohort.[a]

Cases	Fusion Gene	Class	type	Effect	5 prime partner	5 prime partner -breakpoint exon	5 prime partner Transcript	3 prime partner	3 prime partner -breakpoint exon	3 prime partner Transcript	Primary tissue	Pathological diagnosis
1	TMPRSS2-ERG	Tier II	Actionable	GOF	TMPRSS2	exon 1	NM_005656	ERG	exon 4	NM_001243428	Prostate	Prostate adenocarcinoma
2	TMPRSS2-ERG	Tier II	Actionable	GOF	TMPRSS2	exon 1	NM_005656	ERG	exon 4	NM_001243428		
3	TMPRSS2-ERG	Tier II	Actionable	GOF	TMPRSS2	exon 1	NM_005656	ERG	exon 4	NM_001243428		
4	TMPRSS2-ERG	Tier II	Actionable	GOF	TMPRSS2	intron 1	NM_005656	ERG	exon 4	NM_001243428		
5	**ABCC4-ETV4**	Tier III	VUS	Unknown	ABCC4	exon 1	NM_005845	ETV4	exon 4	NM_001986		
6	MSMB-NCOA4	Tier III	VUS	Unknown	MSMB	exon 3	NM_002443	NCOA4	exon 2	NM_005437		
7	**EWSR1-PKM**	Tier III	VUS	Unknown	EWSR1	exon 12	NM_013986	PKM	exon 6	NM_002654	Unknown	Cancer of Unknown primary
8	**GPR107-MUSK**	Tier III	VUS	Unknown	GPR107	exon 1	NM_001136557	MUSK	exon 15	NM_005592		Adenocarcinoma of Unknown primary
9	**PHIP-AKT3**	Tier III	VUS	Unknown	PHIP	exon 1	NM_017934	AKT3	exon 1	NM_005465		Squamous cell carcinoma of Unknown primary
10	TMPRSS2-ERG	Tier III	VUS	GOF	TMPRSS2	exon 1	NM_005656	ERG	exon 5	NM_001243428		Epithelioid malignancy of Unknown primary
11	EIF3E-RSPO2	Tier II	Actionable	GOF	EIF3E	exon 1	NM_001568	RSPO2	exon 2	NM_178565	Colorectal	Colorectal adenocarcinoma
12	PTPRK-RSPO3	Tier II	Actionable	GOF	PTPRK	exon 7	NM_002844	RSPO3	exon 2	NM_032784		
13	**QKI-BRAF**	Tier II	Actionable	GOF (inferred)	QKI	intron 3	NM_206854	BRAF	intron 9	NM_004333		
14	**TMEM66-NRG1**	Tier III	VUS	Unknown	TMEM66	exon 1	NM_016127	NRG1	exon 6	NM_001160004		
15	**FGFR3-SPDYE4**	Tier III	VUS	Unknown	FGFR3	exon 8	NM_001163213	SPDYE4	exon 2	NM_001128076		
16	**ZNF84-BRAF**	Tier III	VUS	Unknown	ZNF84	exon 5	NM_003428	BRAF	Intron 9	NM_004333	Brain	Glioblastoma multiforme
17	ESR1-MTHFD1L	Tier III	VUS	Unknown	ESR1	exon 1	NM_000125	MTHFD1L	exon 16	NM_001242768	Fallopian tube	Fallopian tube carcinoma
18	**DNASE2-MAST1**	Tier III	VUS	Unknown	DNASE2	exon 5	NM_001375	MAST1	exon 21	NM_014975		Fallopian tube serous carcinoma
19	**TAF6L-AKT3**	Tier III	VUS	Unknown	TAF6L	exon 1	NM_006473	AKT3	exon 1	NM_005465		Fallopian tube serous carcinoma
20	ESR1-AKAP12	Tier III	VUS	Unknown	ESR1	exon 2	NM_001122740	AKAP12	exon 5	NM_005100	Ovary	Ovarian serous carcinoma
21	ESR1-MTHFD1L	Tier III	VUS	Unknown	ESR1	exon 2	NM_000125	MTHFD1L	exon 17	NM_001242768		Ovarian serous carcinoma
22	EWSR1-WT1	Tier III	VUS	GOF	EWSR1	exon 8	NM_013986	WT1	exon 8	NM_024426		Ovarian carcinoma
23	**EWSR1-GORASP2**	Tier III	VUS	Unknown	EWSR1	exon 13	NM_013986	GORASP2	exon 10	NM_015530	Lung	Adeno-squamous carcinoma of lung

*False positives and likely benign fusions are not listed in this table. Novel fusion genes are **bolded.** GOF – Gain of function. Only fusions deemed actionable were confirmed by RT-PCR (see methods, supplemental table 2)

Table 2 Pathological diagnosis of all cases received for testing ($n = 305$).

Primary tumor site and diagnosis	No. of samples	No. passed	Primary tumor site and diagnosis	No. of samples	No. passed	Primary tumor site and diagnosis	No. of samples	No. passed
Ampulla	**1**	**1**	**Kidney**	**11**	**3**	**Skin**	**2**	**1**
Ampullary adenocarcinoma	1		Clear cell renal cell carcinoma	3		Metastatic melanoma	2	
Brain	**13**	**13**	Renal cell carcinoma	7		**Small intestine**	**1**	**0**
Anaplastic astrocytoma	1		Round cell sarcoma of Kidney	1		Small intestine adenocarcinoma	1	
Anaplastic oligodendroglioma	1		**Liver**	**3**	**3**	**Soft tissue**	**2**	**2**
Glioblastoma multiforme	8		Hepatocellular Carcinoma	3		Desmoid cancer	1	
Gliosarcoma	1		**Lung**	**42**	**20**	Liposarcoma	1	
Meningioma	2		Lung Non-Small cell carcinoma	2		**Stomach**	**3**	**1**
Breast	**18**	**12**	Lung Small cell carcinoma	2		Gastric Adenocarcinoma	2	
Breast adenocarcinoma	8		Lung adenocarcinoma	24		Gastric carcinoma	1	
Breast carcinoma	9		Lung carcinoid	1		**Tonsil**	**2**	**2**
Inflammatory breast cancer	1		Lung carcinoma	1		Tonsillar carcinoma	1	
Cervix	**2**	**2**	Lung squamous cell carcinoma	7		Tonsillar squamous cell carcinoma	1	
Cervical adenocarcinoma	2		Adeno-squamous carcinoma of lung	1		**Unknown**	**80**	**46**
Colorectal	**38**	**17**	Mesothelioma	3		Adenocarcinoma of Unknown Primary	17	
Colorectal Adenocarcinoma	20		**Ovary**	**19**	**14**	Extramammary Paget disease	1	
Colorectal carcinoma	17		Ovarian carcinoma	6		Epithelioid malignancy	1	
Rectal squamous cell carcinoma	1		Ovarian granulosa cell tumor	1		Metastatic Cancer of Unknown Primary	58	
Esophagus	**5**	**4**	Ovarian carcinosarcoma	1		Neuroendocrine neoplasm	2	
Esophageal adenocarcinoma	1		Dysgerminoma and Immature teratoma of ovary (1 each)	2		Squamous cell carcinoma	1	
Esophageal carcinoma	3		Ovarian serous carcinoma	9		**Urinary bladder**	**5**	**2**
Squamous cell carcinoma of esophagus	1		**Pancreas**	**14**	**8**	Large cell neuroendocrine carcinoma	1	
Fallopian tube	**16**	**14**	Pancreatic adenocarcinoma	6		Urothelial carcinoma of bladder	2	
Fallopian tube carcinoma	9		Pancreatic carcinoma	8		Urothelial papillary carcinoma of bladder	1	
Serous (5) and Transitional type (1)	6		**Peritoneum**	**2**	**0**	Urothelial sarcomatoid carcinoma	1	
Fallopian tube carcinosarcoma	1		Serous carcinoma of peritoneum	2		**Uterus**	**11**	**9**
Gall bladder	**1**	**0**	**Prostate**	**14**	**9**	Uterine Adenocarcinoma	1	
Gallbladder carcinoma	1		Prostate adenocarcinoma	11		Uterine Serous Carcinoma	1	
			Prostatic carcinoma	1		Endometrial Adenocarcinoma	4	
			Prostatic non-small cell carcinoma	1		Endometrial carcinoma	4	
			Prostatic small cell carcinoma	1		Uterine leiomyosarcoma	1	

Variants of uncertain significance (VUSs) are classified as such, primarily due to the absence of functional and biochemical information related to the variant, in terms of its impact on gene function. In an oncology setting, the absence of relevant therapeutic, prognostic, or diagnostic information in the context of the tumor type, additionally pushes the variants into the category of unknown significance. We identified 16 VUSs in our study, of which 10 were novel fusions (Table 1). Cancers of unknown primary had the highest number of fusion VUSs (4 cases), *EWSR1-PKM, GPR107-MUSK, PHIP-AKT3* and *TMPRESS2-ERG*, followed by fallopian tube cancers with 3 fusions, *DNASE2-MAST1, ESR1-MTHFD1L* and *TAF6L-AKT3* and ovarian cancers with 3 fusions, *ESR1-AKAP12, ESR1-MTHFD1L* and *EWSR1-WT1*. Two fusions, *ABCC4-ETV4* and *MSMB-NCOA4,* were identified in 1 case each of prostate adenocarcinomas, and two fusions, *FGFR3-SPDYE4 and TMEM66-NRG1,* were found in 1 case each of colorectal cancer. One fusion each was identified in adenosquamous carcinoma of lung (*EWSR1-GORASP2*) and glioblastoma multiforme (*ZNF84-BRAF*). The breakpoints (exons and chromosome coordinates) for known fusions *TMPRSS2-ERG* [14], *EIF3E-RSPO2, PTPRK-RSPO3* [15], *MSMB-NCOA4* [16], *EWSR1-WT1* [17], *ESR1-AKAP12,* and *ESR1-MTHFD1L* [18], as well as the novel fusions identified, are listed in Fig. 2 and Table 1 respectively. Based on literature and our evaluation of the fusions, 18/23 reported fusions were in-frame (Table 1).

Of the 30 fusions identified, two fusions (*DAZAP1-AKT3* and *FCGR3A-MAST2*) were classified as likely benign. The absence of functional information on these two fusions would have technically classified them as VUSs, but due to their detection in control samples, they were downgraded to likely benign and therefore were not reported. In 3 cases, we detected two fusions co-occurring, *TMPRSS2-ERG* and *MSMB-NCOA4* (prostate cancer), *PTPRK-RSPO3* and *FGFR3-SPDYE4* (colorectal cancer), and *QKI-BRAF* and *ETV1-ERG* (colorectal cancer). The *ETV1-ERG* fusion was ruled a false positive.

False positives called by the Archer software

Five fusions called by the Archer software (*ETV1-ERG, MYB-ESR1, DGKG-ETV5* and *ERG-EWSR1 (2 cases)*) were determined to be false positives by either RT-PCR or reprocessing the sample. The *ETV1-ERG, MYB-ESR1* and *ERG-EWSR1* fusions were determined to be due to mispriming, while the *DGKG-ETV5* fusion was due to wild-type read through. The *ERG-EWSR1* and *DGKG-ETV5* fusions were both detected in a single case of lung cancer.

Discussion

Fusions are thought to drive the onset and progression of cancer through a variety of mechanisms, resulting in a significant effort being invested in evaluating fusions in solid tumors for therapeutic, prognostic and diagnostic significance. Given the small number of gene targets (53 genes) being evaluated in our study, our observation of a 3.8% positive yield rate is therefore fairly significant. In addition to the identification of 3 well characterized fusions that are clinically actionable in solid tumors, we identified 11 novel fusions in our cohort, including one clinically actionable fusion, *QKI-BRAF* (Table 1 and Fig. 2).

Fusions in CUPs are observed at a frequency of ~4–20% [19,20], and our identification of 4 fusions across 46 samples of CUP does fall in the expected frequency at ~9%. Since CUP cases constituted the largest number of samples in our cohort [25%), this frequency was not significant when compared to the whole cohort ($p = 0.32$). Of the 4 fusions identified in the CUP cases, the most interesting was the presence of the *TMPRSS2-ERG* fusion, a well-established tumorigenic driver in prostate cancer, suggesting the prostate as a potential primary origin. The *TMPRSS2-ERG* fusion has been identified in a CUP cohort study by Ross et al. [20], which identified 19 fusions, including the *TMPRSS2-ERG* across 200 samples. However, based on the pathological information provided and the characterization of this fusion gene in epithelioid neoplasms being currently unknown, the fusion was classified as a VUS based on our clinical practice guidelines. The 3 other fusion genes (*GPR107-MUSK, EWSR1-PKM, EWSR1-GORASP2*) identified in CUP's were novel and also classified as VUSs (Table 2). At this time, we are not able to provide expansive information regarding the functionality of these fusions or to infer any possible mechanisms that could be involved with their role in CUP's and the associated pathological diagnosis. A small cohort study of CUPs identified 4 fusions across 17 samples, with *EWSR1* fusions comprising 3 of the 4 identified, similar to our observations [19], though the fusion partners were different (*ATF1, CREB1,* and *NR4A3)* [19]. Correlation of our observations with the published findings suggests that *EWSR1* fusions may play a significant role in CUP tumorigenesis.

Fusions are driver events in ~50% of prostate cancer, most frequently involving one of ETS family genes. Fusion partners of ETS family are often androgen regulated with few exceptions such as *C15ORF21, HNRPA2B1,* and *DDX5*, and can lead to the overexpression of these oncogenic transcription factors [21,22]. Of the 9 prostate cancer cases in our tested cohort, 3 fusions, *TMPRESS2-ERG* (actionable), *ABCC4-ETV4* (VUS), and *MSMB-NCOA4* (VUS) were identified in 6 cases (67%), reiterating the role of fusions as driver events in prostate cancer. *TMPRESS2-ERG* is well-established to be oncogenic while the functional significance of the other two fusions is yet unknown, although, *MSMB-NCOA4* has been reported in prostate cancer [16,21,23–25]. *MSMB-NCOA4* fusion is suggested to be a transcription induced chimera, with the transcriptional control of both *MSMB* and *NCOA4* being known to occur through androgen response elements, and the expression of the fusion product MSMB-NCOA4 being regulated by the *MSMB* promoter. Given the uncertainty of the significance of this fusion in prostate cancer, along with the fact that in our study the *MSMB-NCOA4* fusion was found in a patient, co-occurring with the *TMPRSS2-ERG* fusion, the fusion was classified as a VUS. The three 5 prime fusion partners of the fusions identified in the prostate cancer cases were all androgen dependent including the novel fusion, *ABCC4-*

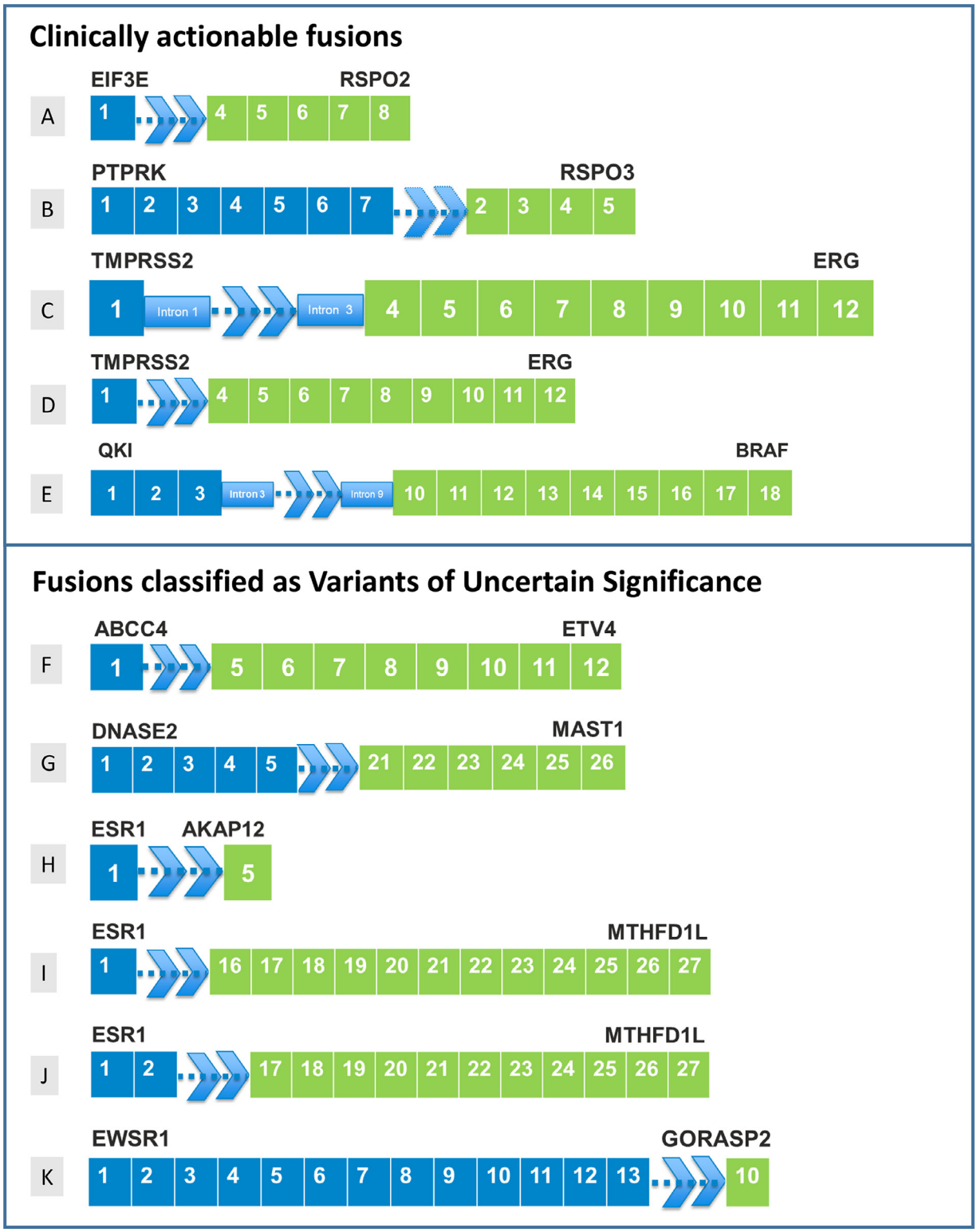

Fig. 2 Schematic of the breakpoints in the clinically actionable fusions (A–E) and the variants of uncertain significance (F–U). The details of the breakpoints and the transcript information are provided in Table 1.

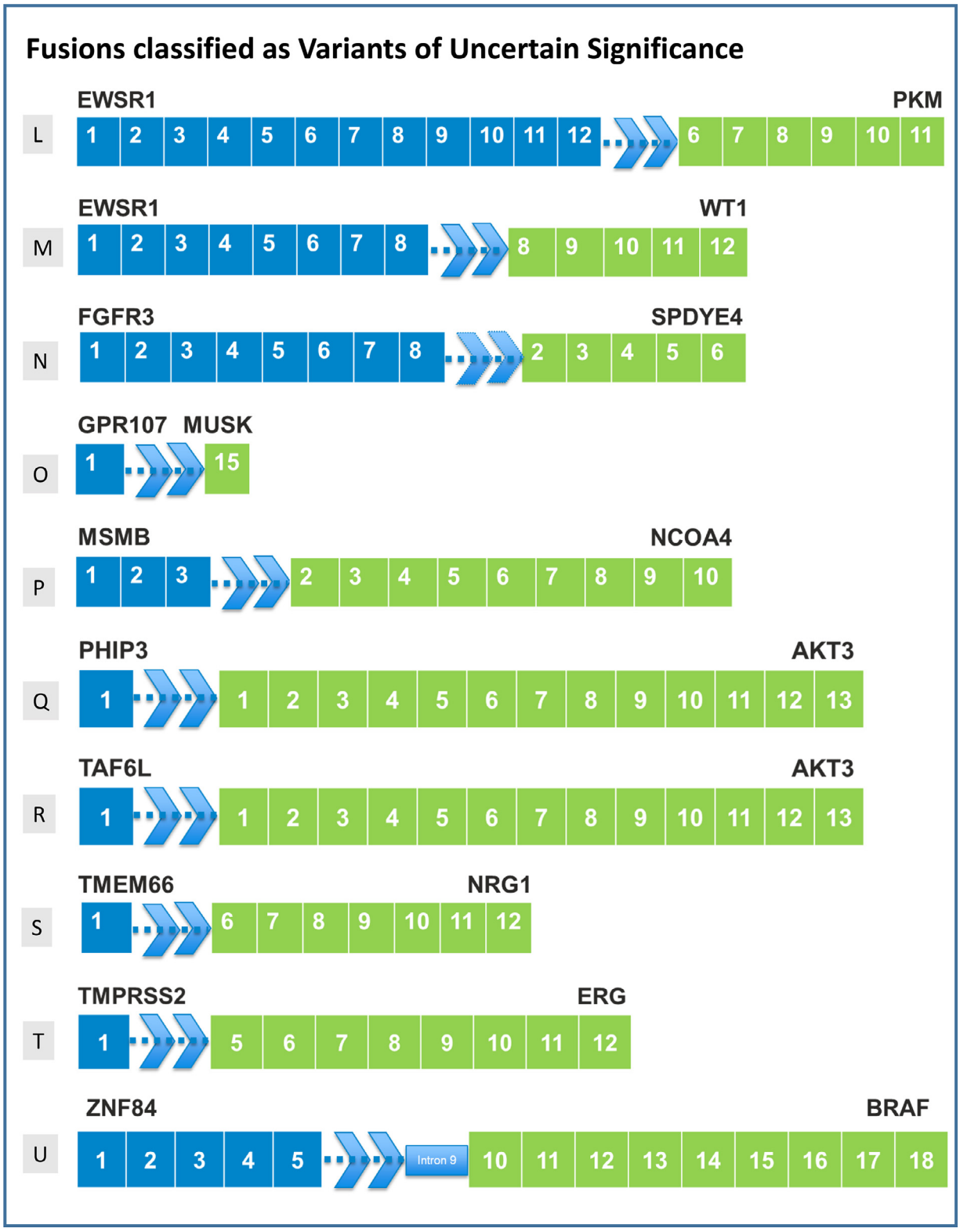

Fig. 2 Continued

ETV4 [26,27]. Although this suggests that the fusion of exon 1 of *ABCC4* gene to exon 5 of *ETV4* could be a driver event with a similar mechanism to TMPRESS2-ETS family gene fusions, the protein translation prediction in terms of being out of frame indicates otherwise, therefore it was reported as a VUS.

Fusions in colorectal cancer (CRC) on the other hand are not as well documented as prostate cancer. Five fu-

sions were identified in our CRC cohort of 18 cases (27%) and included fusions involving the R-spondin family members *RSPO2* and *RSPO3*, which are observed in about 10% of colon tumors. Two actionable R-spondin fusions, *EIF3E-RSPO2* and *PTPRK-RSPO3,* were identified in our study. These fusions were previously described to be tumorigenic through activation of WNT signaling [15]. Although at present there are no FDA approved drugs which target R-spondin fusions in colorectal cancers, an investigational drug LGK974 is being studied in a clinical trial (NCT01351103) for targeting upstream WNT signaling pathway alterations including RSPO fusions, resulting in this fusion being classified as actionable Tier II.

BRAF fusions on the other hand are relatively less frequent in colorectal cancers and are detected in only ~1% of cases [28]. The common oncogenic mechanism noted in *BRAF* fusions regardless of the 5′ fusion partner, is the loss of the auto-inhibitory region of *BRAF* and retention of kinase domain, leading to activation of downstream signaling [29]. This is similar to the *QKI-BRAF* fusion identified in our study, wherein the breakpoint occurs at intron 9 of *BRAF* resulting in loss of auto-inhibitory region and an in-frame fusion [29,30]. The 5′ fusion partner QKI, is an RNA binding protein belonging to the signaling transduction and activation of RNA protein family and is a known tumor suppressor. Similar to a known oncogenic fusion transcript *QKI-CRAF,* involving another RAF family member, *QKI-BRAF* could be predicted to function as a tumor driver and may be potentially targetable by MEK/RAF inhibitors [31–33]. Two novel fusion genes, *SARAF (TMEM66)-NRG1* and *FGFR3-SPDYE4,* were also seen in the colorectal cancer patients of our cohort, where *FGFR3-SPDYE4* co-occurred with *PTPRK-RSPO3* in the same case. Though *NRG1* and *FGFR3* are known genes to form fusion transcripts in cancers, the molecular pathogenicity of these novel fusions could not be inferred at the moment.

Fusion genes with *ESR1* are frequently identified in metastatic estrogen receptor (ER) positive breast cancers even-though they are rare events in solid tumors. Commonly, ESR1 fusion genes reported in literature lose their ligand binding domain and have breakpoints clustered between exon 6 and 7 of *ESR1* gene, resulting in the ESR1 fusion protein acquiring ligand independent activation with slight involvement of the 3′ fusion partners [18]. In our cohort, we identified two *ESR1* fusion genes, *ESR1-AKAP12* and *ESR1-MTHFD1L*, in three cases (1 fallopian tube cancer and 2 ovarian cancers). Both fusions have been previously reported in metastatic breast cancers [18,34]. Since the breakpoints of these fusions were at the transactivation (AF1) domain which results in loss of both DNA binding and ligand binding transactivation (AF2) domains, the function significance of these fusions is not yet known and needs further studies, resulting in a VUS classification.

In addition to the *ESR1-MTHFD1L* fusion, the fallopian tube cancer cases had two other novel VUS fusions, *DNASE2-MAST1* and *TAF6L-AKT3*, of which the functional significance in cancer is currently unknown. Interestingly, we also found a well-known oncogenic fusion, *EWSR1-WT1*, commonly seen in desmoplastic small round cell tumors (DSRCT), in a patient with poorly differentiated ovarian carcinoma. While the pathological diagnoses favored ovarian cancer, a differential diagnosis of DSCRT should not be ruled out given the clinical presentation as a pelvic mass, the difficulty in further subtyping the pathological diagnosis, and the higher frequency of the *EWSR1-WT1* fusion in DSCRT tumors.

Gliomas are known to have fusion genes in ~30–50% of cases, of which only a certain number of fusions are well characterized and are targetable [35]. *BRAF* associated fusions are predominant events in pilocytic astrocytoma, where the *KIAA1549-BRAF* fusion is the most common [29]. In our cohort, we found a novel *ZNF84-BRAF* fusion in a case of glioblastoma multiforme. The breakpoint on the 3′ partner of this fusion lies at intron 9 of BRAF gene similar to the other *QK1-BRAF* fusion identified in the colorectal case, indicating the fusion to be in-frame (Fig. 2). Based on previous studies with *BRAF* fusions in gliomas, it could be hypothesized that the *ZNF84-BRAF* fusion could be activating mutation due to the loss of its upstream auto-inhibitory region. Since the fusion was in-frame, we could have reported it at a Tier II fusion, however functional studies are not currently available, therefore we classified this fusion as a VUS.

Fusions have been demonstrated to be important driver mutations in soft tissue tumors (STT) and about 142 fusions have been described in STTs, with 78 of these being reported in at least two cases of the same morphological STT subtype and are defined as recurrent [36]. The absence of any fusions in the 2 STT samples in our cohort could be a limitation of the panel, in that genes known to be involved in forming fusions in STT were absent in our 53 gene panel, possibly underestimating the positive yield rate of our cohort.

In summary, we note that our study had a 3.8% diagnostic yield. Overall, 68% (17 of 25) of both known and novel fusions in our cohort were identified in 3 main tumor types; prostate (6 fusions), colorectal (5 fusions) and gynecological cancers (6 fusions), which suggests that the frequency of fusions is dependent on specific tumor type, similar to previous observations [37]. These clinical laboratory observations are consistent with established findings in the literature and underscore the need to establish indication-specific testing algorithms in order to optimize precision medicine costs, manage patient expectations, and mitigate extraneous results reporting, as is currently done for *RET* or *ROS* fusions in lung cancer [38]. The small cohort size and the number of fusion partners evaluated are indeed limitations of this study as discussed for the STT cases in our cohort. Fusion variants, like variants of other types commonly reported by testing laboratories, should be deliberated with the same risk/benefit considerations to determine appropriate gene panel content and test ordering criteria. Though not entirely sufficient to address the clinical utility of evaluating fusions for precision medicine, the high number of novel fusions identified in our study does suggest that evaluation of fusions in solid tumors is translationally relevant.

Conflict of Interest

The authors declare no relevant conflict of interest.

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Supplementary materials

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