Ultrasensitive gene fusion detection reveals fusion variant associated tumor heterogeneity

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Abstract (words 150)

Gene fusions are common drivers and therapeutic targets in cancers, but clinical-grade bioinformatics callers are lacking. Here we introduce a novel method SplitFusion, which is fast by leveraging BWA-MEM split alignments, can detect cryptic splice site fusions, and can infer frame-ness and exon-boundary alignments for functional prediction and minimizing false-positives. SplitFusion demonstrates superior sensitivity, specificity, accuracy and consumes minimal computing resources. In our study of 1,076 formalin-fixed paraffin-embedded lung cancer samples, SplitFusion detected not only common fusions (*ALK* 4.7%, *ROS1* 2.0% and *RET* 1.1%) with various partners, but also rare (*KLC1-ALK*, *CD74-NRG1*, and *TPR-NTRK1*) and novel (*FGFR3-JAKMP1*, *CLIP2-BRAF*, and *ITPR2-ETV6*) fusions. In 35 glioblastoma samples, SplitFusion-Target detected six (17%) *EGFR vIII* (exons 2-7 deletion) cases. Furthermore, we find that the *EML4-ALK* variant 3 is significantly associated with occurrence of multiple breakpoint-defined subclones, namely high intratumor heterogeneity. In conclusion, SplitFusion is well-suited for clinical use and for studying fusion-defined tumor heterogeneity.

Main

Chromosomal rearrangements or gene fusions that lead to novel or overexpressed oncoproteins have been identified across different human cancers¹. The cure for leukemia harboring *BCR-ABL*² and *PML-RARA*³ gene fusions represented the early success in the era of precision medicine. Beyond blood cancers, discoveries in gene fusions have continued to revolutionize the management of solid tumors, including *ALK*⁴ and *ROS1*⁵ in lung cancer, *TMPRSS2* in about 50% of prostate cancer⁶, *FGFRs* in diverse cancers⁷, and *NTRK* fusions with FDA approved drugs regardless of tissue type⁸.

Clinical diagnosis of gene fusions has been challenging in both wet lab and bioinformatics. The prevalence of gene fusions in The Cancer Genome Atlas study was estimated about 16% overall⁹ and 3% for those involving promising therapeutic targets (ranging from 0 to 12.9% across different tissue types)¹⁰. These RNA-seq analyses are powerful in gene fusion discoveries. However, the associated low specificities are problematic for clinical diagnosis because non-trivial efforts are needed to filter away clinically insignificant gene fusions (e.g. 37% of the identified 25,664 fusions could not be validated in genomic data⁹). Furthermore, alternative RNA splicing and exon deletion caused by alterations at the genomic level, which may be therapeutically important such as *EGFR vIII*¹¹, are not detectable by most fusion detection software. Numerous software have been developed to detect gene fusions for next-generation sequencing (NGS) data, such as *EricScript*¹², *Lumpy*¹³ and *STAR-Fusion*¹⁴, the latter being the best-performing one among 23 different software¹⁴. Most of these software are designed for analyzing high-quality RNA-seq data¹⁴, while none of them, to our knowledge, is able to infer fusion frame-ness and to judge exon-boundary alignment (for filtering functionally insignificant or clinically irrelevant fusions); to report targeted alternative splicing and exon deletion events; and also well-suited for clinical diagnosis where formalin-fixed paraffin-embedded (FFPE) samples are most accessible but often highly degraded.

We have previously designed a NGS target enrichment method, termed Anchored Multiplex PCR (AMP), for scalable and efficient detection of gene fusions, without prior knowledge of the fusion partners¹⁵. For broad clinical and research applications, a high-performing bioinformatics caller is still

required. Here, we introduce a novel algorithm named SplitFusion (<https://github.com/Zheng-NGS-Lab/SplitFusion>), a clinical-grade fusion detection algorithm with high research potentials. SplitFusion is fast by leveraging the chimeric alignment (split-read) of BWA-MEM¹⁷ for RNA sequencing data, and demonstrates higher sensitivity and specificity than current well-known software. Furthermore, SplitFusion features highly desirable abilities in clinical reporting, including the capabilities to infer fusion transcript frame-ness and exon-boundary alignments; to calculate number of unique DNA fragment ligation sites; and to customize target filtering and output. In 1,076 lung cancer and 35 glioblastoma FFPE samples, SplitFusion not only detected common, rare but also novel fusions, as well as intragenic exon deletions. Lastly, SplitFusion reveals that breakpoint hot-zone in *EML4-ALK* variant 3 (v3) is associated with a high intratumor heterogeneity, as defined by fusion breakpoint subclones. An intriguing question arises from recent clinical observations is that patients with lung tumors positive for the v3 of *EML4-ALK* fusion is associated with poorer clinical outcomes than lung cancer patients with other *EML4-ALK* variants^{18–20}. Using SplitFusion, we found that *EML4-ALK* v3 was significantly associated with a high tumor heterogeneity in terms of breakpoint-defined subclones – likely an underappreciated phenomenon that warrants further investigations.

Results

SplitFusion algorithm design

SplitFusion can use both regular RNA-seq and anchored multiplex targeted enrichment like the Anchored Multiplex PCR (**Fig. 1a**) NGS data. SplitFusion consists of the following major computation steps.

a. Anchored multiplex target enrichment

b. Reference alignment & de-duplication

c. CIGAR transformation & candidate breakpoint calling

Supplementary alignments						
QNAME	LEN	RNAME	START	END	STRAND	CIGAR
ReadID/1	117	chr2	42491846	42491871	+	24S26M67S
ReadID/1	117	chr2	42492057	42492091	+	48H35M34H
ReadID/1	117	chr2	29446361	29446396	-	36M81H
...						
ReadID/2	86	chr2	29446361	29446396	+	36M50S
ReadID/2	86	chr2	42492057	42492091	-	17H35M34H

S: soft clip; M: mapped; H: hard clip

d. Initial breakpoint filtering

e. Breakpoint gene annotation

f. In-frame calculation

g. Exon-boundary alignment calculation

h. Result reporting & visualization

Figure 1. The framework of SplitFusion algorithm design. (a) Next-generation sequencing (NGS) library construction using the anchored multiplex PCR. Double-stranded cDNA and gDNA were subjected to end-repair, dA-tailing, and ligation of a half-functional Y adaptor, which contains sample barcode and unique molecular identifier (UMI). Two rounds of hemi-nested multiplex PCR reactions were used to enrich any sequences (denoted in red question marks) downstream of the gene specific primers (GSPs), resulting in NGS library for sequencing. (b) NGS FASTQ data was de-multiplexed by sample barcodes, followed by adaptor trimming. Different colors represent different UMIs tagged to different molecules. The data was aligned to reference using BWA-MEM, and de-duplicated based on a combination of unique UMI and ligation site (UMI-LS). Supplementary alignments were assigned with the same UMI-LS as their Read 1 starting alignments. Reads with supplementary alignments (SA) were extracted. (c) The CIGARs of reads with supplementary alignments were transformed. For the same read ID, all alignments were sorted by their mapping positions (M) on the query read, and the candidate breakpoint(s) was inferred. (d) The initial candidate breakpoints were filtered by maximum overlapping length, maximum gap, minimum exclusive mapping lengths of partner alignments, and the minimum mapping lengths of ligation end and anchored end alignments; and further subjected to gene annotation (e), frame-ness calculation (f), exon-boundary alignment judgement (g), and reporting and visualization (h).

Reference alignment and deduplication.

SplitFusion leverages the fast and widely used genome mapping software BWA-MEM¹⁷, which generates supplementary alignments (the SA tag in SAM specification: other canonical alignments in a chimeric alignment) for a query sequencing read containing two or more parts mapped to different locations of the reference, i.e. multiple alignments (**Fig. 1b**). When genome (instead of transcriptome) is used as the reference for RNA-seq reads, these different parts can be different genes, neighboring exons or cryptic splice site containing sequences. SplitFusion recognizes FASTQ or BAM file in the specified directory as input. If the BAM file (BWA MEM aligned) exists, the Kickstart mode will be initiated, bypassing the FASTQ to BAM reference alignment step. Then, deduplication / consolidation of the BAM is performed based on a combination of a unique molecular identifier (UMI) and the aligned chromosomal position of the ligation site of a DNA fragment (LS). One unique UMI-LS read (with all its split alignments) will be kept among the duplicated UMI-LS reads, resulting in a consolidated BAM file (**Fig. 1b**).

CIGAR transformation

SplitFusion parses the CIGAR strings, orientates and sorts chimeric parts (split-read) by their segment order in the query read (**Fig. 1c**). To decipher the alignment construct of a query read, CIGAR is first parsed by soft clipped (S, unmapped but not trimmed), mapped (M), and hard clipped (H, unmapped and trimmed) segments, and their lengths. Short insertion and deletions (1D/1I) in CIGAR are removed and the mapped segment (M) length is adjusted accordingly. SplitFusion then uses these S/M/H lengths of all split alignments from the same read, taking their respective alignment orientations (plus or minus strand) into account, and query read lengths as available from BEDTools²¹ to calculate query read start and end positions of the different split alignments. SplitFusion uses the mapping chromosomal position of ligation starting site for deduplication (based on UMI-LS) and also for final reporting on the number of partner ends. However, for cDNA fragments, the heading sequences for adaptor ligation are often partial exonic sequences (followed by coding sequences of their upstream/downstream exons) and are too short to be mapped to the reference genome. SplitFusion uses the lengths of these unmapped sequences (soft-clipped length) to adjust for the

ligation site mapping position (encoded in read ID) for later accurate calculation of the number of partner ends (ligation sites). SplitFusion then sorts all the split alignments from the same query read by their original segment orders on the query read.

Candidate breakpoint calling.

From the transformed split-read alignments, SplitFusion infers breakpoint candidates. For any two neighboring alignments on the same query read, if they align to different chromosomes or the same chromosome but distant at >750,000 bases (the largest intron size used by BLAT²²), they are deemed candidate fusion partners and output to separate files (left and right). The partners are then merged by their read ID, and their junction-corresponding chromosomal alignment positions are deemed as candidate breakpoints (**Fig. 1c**). For a typical two-split alignment read, the merged new record has the construct “left query start – left query end [breakpoint 1] --- right query start [breakpoint 2] – right query end”. For a read consists of three or more alignments (as long as the length of sequencing read can accommodate), such as those involving intra-gene neighboring exons of fusion partner genes, the leftmost and rightmost alignments are merged and the new record has the construct “leftmost query start – nearest end to junction [breakpoint 1] --- nearest start to junction [breakpoint 2] – rightmost query end”. The middle alignments between the leftmost and rightmost are output to a separate file (mid) for downstream analyses.

Initial breakpoint filtering

As an initial step to minimize false positive results, SplitFusion filters the candidate breakpoints by minimum alignment length, minimum non-overlapping length of split parts, maximum gap length, and minimal number of ligation site (UMI-LS) (**Fig. 1d**). SplitFusion can apply different minimum mapping lengths on different partner alignments. Typically for anchored end NGS data, this feature allows for a high fusion calling specificity by specifying a high mapping length value (default 25; **Fig. 1d**) on the ligation end, and also for a high fusion calling sensitivity by specifying a short mapping length (default 18) on the anchored end (additional specificity on the anchored end was already

imposed in the wet lab protocol by using outer gene-specific primers [GSP1s; **Fig. 1a**] which were however not present in NGS data).

Breakpoint gene annotation, frame-ness, exon boundary, further filtering and target reporting

Next, SplitFusion annotates candidate breakpoints with gene names, exon numbers, and cDNA positions (**Fig. 1e**). It is common that split alignments of the same query read share a few identical ending DNA bases, which are “double counted” typically because they belong to Gene A exon and is also identical to partner Gene B intron (adjacent to the fusion exon of Gene B), largely due to the canonical splice junction sequences²³. These “double counted” overlapping alignment segments (as a result of BWA alignment seed-extension searching) need to be accounted for in order to accurately calculate fusion frame-ness. SplitFusion tackles this issue by temporally shifting the breakpoint positions by 6 bases (default) inwards to the center of the alignments, for both of the involved split alignments, in order to annotate the breakpoints with correct cDNA positions and exon number by annotation software such as ANNOVAR²⁴ or SnpEff²⁵. The middle alignments, if exist, are also annotated. If target gene panel BED file is specified in the initial configuration, at the beginning of this step, the ‘Target’ mode will be employed and only those alignments involving the targeted genes, together with any potential partners, will be annotated. Following annotation, SplitFusion infers the frame-ness of fusion transcript (in-frame/out-frame or not applicable; **Fig. 1f**) and judges whether the breakpoints are on known exon boundary (both/one/none) according to RefSeq²⁶ (**Fig. 1g**).

Optionally, a backend “whitelist” database can be used for targeted output to report alternative splicing (e.g. for *MET* exon 14 skipping), exon deletion (e.g. *EGFR vIII* exons 2-7 deletion) and gene truncation (e.g. *FGFR1/2/3* exon 18 truncation), as well as a “blacklist” database to remove recurring false-positive calls.

Result reporting and visualization

SplitFusion outputs a final fusion summary, including exact breakpoints, number of unique supporting reads (according to UMI), number of partner ends (i.e. ligation starting site, LS), frame status, exon-boundary status, fusion gene names (with transcript ID) and exon numbers. Lastly, if a fusion is

detected, ten fusion-supporting split-reads (in FASTQ) will be randomly extracted from the consolidated BAM, saved as a text file that can be examined manually, and also converted into BAM file that can be visualized with a third party software or with an IGV API (**Fig. 1h**).

Benchmarking performance versus other software

To evaluate the performance of SplitFusion, we tested 46 cancer cases with known clinical diagnoses. FFPE tissue sections from 10 *ALK* positive lung cancers, 1 *EWSRI* fusion positive sarcoma, and 35 *ALK* fusion negative lung cancer cases (as confirmed by routine fluorescence *in situ* hybridization [FISH] assay. **Extended Data Fig. 1**) were subjected to a 19-gene lung panel (**Extended Data Tab. 1**) targeted RNA-seq assay using AMP¹⁵. We compared four fusion detection software, namely EricScript¹², Lumpy¹³, STAR-Fusion¹⁴ and the present SplitFusion. We down-sampled 11 fusion-positive sample datasets to different sizes of 3M, 1M, 500K, 100K, 50K, 10K, 7.5K, and 5K reads randomly, with 21 replicates of each size, generating a total of 1,848 datasets that were analyzed by the four software (**Fig. 2a**). With 500K or more reads, all software showed a sensitivity of 100% except EricScript (mean = 45%), but varied levels of unconfirmed or clinically insignificant fusion calls (EricScript and Lumpy: >100 per dataset as they do not include filtering steps by default; STAR-Fusion: 5 – 14; SplitFusion: 2 – 3) (**Fig. 2a**). By purposely reducing the size of datasets gradually, the sensitivities of the software started to differentiate (at 10K-read: EricScript 11%, Lumpy 57%, STAR-Fusion 74%, SplitFusion 85%; at 5K-read: EricScript 9%, Lumpy 37%, STAR-Fusion 61%, SplitFusion 78%); and unconfirmed or non-significant fusions were at the minimal level for STAR-Fusion and SplitFusion (**Fig. 2b**). For the overall accuracy based on the previous varying read-cutoff precision-recall area under curve (AUC)¹⁴, SplitFusion showed the highest AUCs among the software across all different dataset sizes (**Fig. 2c**). Indeed, in the smallest 5K-read datasets, mean AUCs for EricScript, Lumpy, STAR-Fusion and SplitFusion were 0.01, 0.18, 0.48, and 0.63, respectively. As for computing resources, STAR-Fusion requires ~30 GB RAM, which was much higher than those of Lumpy (~10 GB) and EricScript and SplitFusion (both at ~4 GB – a typical computation cluster configuration: 4 GB RAM per thread) (**Fig. 2d**). For the largest 3M-read datasets, the computation times were 0.65h, 0.51h, 0.46h and 0.42h for EricScript, Lumpy, STAR-Fusion and SplitFusion,

Figure 2. Benchmarking sensitivity, specificity, accuracy, computation memory use and speed among the four software – Ericscript, Lumpy, STAR-Fusion and SplitFusion. The datasets of the 11 positive samples were randomly down-sampled to different sizes (reads: 3M, 1M, 500K, 100K, 50K, 10K, 7.5K and 5K), each size with 21 replicates (random seeds), generating 1,848 datasets for analyses by the four software with their default parameters using one computation thread. (a) Sensitivity was calculated as the fraction of samples resulted in expected gene fusions (*ALK/EWSR1*) among the 11 samples of given size and given random seed. (b) Specificity was evaluated by the number of unconfirmed or non-significant fusions, i.e. other than *ALK* and *EWSR1*. (c) The overall accuracy was calculated by the area under the precision-recall curve (AUC) at varied supporting read thresholds (from minimum to maximum supporting reads) according to the previous method. The violin outlined the distribution (the solid circle was the mean and the lines denote median and upper and lower quartiles) of the statistics for each group (21 datasets). (d) Maximum RSS: the maximum resident set size (RSS: the portion of memory occupied by a process that is held in main memory) during the computation of the software. (e) User time: the amount of total CPU time for a given analysis.

respectively (**Fig. 2e**). It is worth noting that both STAR-Fusion and SplitFusion have Kickstart modes that can skip reference mapping and start from BAM, which reduced user time by >50% (**Fig. 2e**).

Among the 35 *ALK* fusion negative lung cancer samples, EricScript and Lumpy reported (by default without filtering) 39 and 46 unconfirmed or clinically insignificant fusions, respectively; whereas STAR-Fusion reported five such fusions involving tumor suppressor gene *CDKN2A*, and SplitFusion reported two *NCOA4-RET* cases with the *NCOA4* breakpoint aligned on exon boundary (**Fig. 3** and **Extended Data Fig. 2**). With the Target mode, SplitFusion can report targeted outputs, such as specific alternative splicing, exon-skipping and deletions. Moreover, SplitFusion can be tailored to filter recurrent false positives. To demonstrate this feature, we specified *EGFR vIII* (exons 2 – 7 deletion) – a known oncogenesis driver in glioblastoma – in the target output file when analyzing FFPE samples from 35 glioblastoma cases (mean age 54.8 y, male 65.7%). SplitFusion-Target reported six *EGFR vIII* cases (**Fig. 3 & Extended Data Fig. 3**).

Figure 3. Fusion calling results for the 46 lung cancer samples with clinical known diagnoses (11 positive and 35 *ALK*-negative) by the four software – Ericscript, Lumpy, STAR-Fusion and SplitFusion. Each column represented one sample. Different colors and symbols denoted different fusions. Total number of false-positive calls (other than *ALK/EWSR1*) for each software was plotted in the barplot. Out of 35 cases of glioblastoma (GBM), six were found with *EGFR vIII* using SplitFusion-Target. The GBM samples were not subjected for analysis by the other three software.

Expanded clinical analyses in 1,076 lung cancer FFPE samples

To evaluate the robustness of SplitFusion performance in clinical samples, we expanded SplitFusion analyses on targeted sequencing of 1,076 clinical lung cancer (mean age 59.6 y, male 57.8%) FFPE samples using a 62-gene panel (**Extended Data Tab. 2**). SplitFusion detected 86 (8.0%) fusion-positive cases in lung cancers (**Fig. 4a**). The prevalence of *ALK* (4.65%), diverse partner-*ROS1* (1.95%) and diverse partner-*RET* (1.12%) fusions were comparable to previously reported¹⁶. Rare oncogenic drivers and therapeutics relevant gene fusions *KLC1-ALK*, *CD74-NRG1* and *TPR-NTRK1* were also identified (**Fig. 4a & Extended Data Fig. 4**). Interestingly, SplitFusion detected new fusions that have not been reported in lung cancer previously, including one *FGFR3-JAKMP1* squamous cell carcinoma, one *CLIP2-BRAF* (two fusion isoforms) adenocarcinoma, and one *ITPR2-ETV6* adenocarcinoma (**Fig. 4a & Extended Data Fig. 5**). We did not attempt to call *MET* exon 14-skipping based on RNA-seq data alone, as the low-level RNA splicing donor and acceptor from non-consecutive exons that are spatially proximal could form exon-skipping mRNAs, which are however not substantial when the cells divide and should be distinguished from the true cancer drivers root at the DNA level. Combined RNA exon-skipping and DNA splicing site mutations analyses – simultaneous RNA and DNA target enrichment in wet lab and data analysis (e.g. using BWA for mapping both DNA and RNA reads) – await further developments.

Multiple gene fusion variants can co-occur in the same tumor¹⁵ and different variants may affect clinical outcomes^{18,27,28}. However, the frequencies of different variant co-existences are largely unknown. Using SplitFusion, we found that multiple gene fusion variants with in-framed exons of partner genes, with or without involving cryptic splice site sequences, co-occurred frequently in the same tumors (**Fig. 4b**). Among the kinase genes analyzed, *EML4-ALK* v3 and *CD74-ROS1* harboring lung tumors had the most frequent co-occurrences of multiple fusion variants.

Breakpoint-defined subclone

To dissect the intratumor clonal heterogeneity, fusion subclones were defined by the exact breakpoints inferred from genomic mapping of fusion RNA sequences (**Fig. 5a**). Our results showed

Figure 4. The analysis of 1,076 clinical lung cancer formalin-fixed paraffin-embedded samples using SplitFusion. **(a)** The prevalence of different target gene fusions were shown in the barplot. In total, 86 (8.0%) samples were fusion-positive. The numbers of target genes with partner genes (right column) were indicated in the cells. **(b)** The numbers of co-occurrence of fusions in the same samples were plotted in the matrix. The *EML4-ALK* fusion variants were denoted under each specific fusion.

Figure 5. The analysis of breakpoint-defined subclones in *EML4-ALK* positive lung tumors. **(a)** The schematic representations of different *EML4-ALK* fusion variants. Blue cells represent *EML4* exons and yellow cells represent *ALK* exons. Numbers inside the cells showed exon numbers. Cells with red strokes represents introns. **(b)** Each tile represents one patient. The tile color gradients denote the number of co-existing fusion subclones, defined by exact breakpoint, in the same tumor. **(c)** For one *EML4-ALK* variant 3 lung tumor sample, the Integrated Genome Viewer (IGV) showed 6 fusion breakpoints in *EML4* exon 6 and intron 6. Their gene partner *ALK* exon 20 was schematically shown on the right in red, and with nested gene specific primers 1 and 2.

that 55% (11 of 20) of *EML4-ALK* v3 tumors had co-existing heterogeneous fusion subclones in the same tumors (7 two-subclone, 3 three-subclone, and 1 six-subclone; **Fig. 5b & c**), which was significantly higher than 19% (6 of 31) in other variant tumors (4 of 19 v1, 2 of 8 v5 and none of 4 v2; *Fisher's* Exact Test two-sided *P* value = 0.0143).

Discussion

We demonstrate that SplitFusion is fast, highly sensitive, specific and computationally efficient in the detection of gene fusions. SplitFusion can infer fusion frame-ness and exon-boundary alignment, which are particularly desirable in the clinical setting so as to minimize non-trivial filtering of false-positive or functionally insignificant fusion candidates. SplitFusion-Target mode allows for continuous evidence-based improvement in clinical reporting. Furthermore, SplitFusion is agnostic to known coding transcripts and can profile multiple co-existing fusion subclones, providing a powerful tool for research studies.

In a previous matched tissue-blood analysis, 21% of ALK-positive patients showed different *EML4-ALK* variants between tissue and blood samples²⁰, indicating the existence of fusion-defined tumor heterogeneity evident at the genomic level. SplitFusion can comprehensively map cryptic splice site breakpoints and has identified up to six subclones co-existing in one *EML4-ALK* v3 tumor, with different breakpoints in *EML4* intron 6. Increasing evidences have suggested that lung cancer patients with *EML4-ALK* v3 tumors may have less favorable clinical outcomes compared to patients with other *EML4-ALK* variants^{18,19,29}. The most frequent resistance mutation *ALK* G1202R was shown to be present exclusively in the v3 of *EML4-ALK* tumors¹⁸. Heuckmann *et al.* showed that the v3 protein had a higher stability than other variant proteins³⁰. However, the expression of the v3 cDNA (homogenous) in Ba/F3 cells precluded the study of v3 subclones (heterogeneous) that may present in clinical tumors. When using clinical tumor derived cell lines, the study of ALK inhibitor on the v1-harboursing cell line H3122 showed the typical monotonic S-shape dose-response relationship (homogeneous)³¹. In contrast, inhibition of the v3b-harboursing cell line H2228 was observed across a wide range of ALK inhibitor concentration³¹, indicating heterogeneous responses among the H2228

cell population. Insertions (in-framed) could lead to more profound protein structure and property changes than do point mutations³². Therefore, v3a/b (c/d/e/f)-harboring tumors may contain structurally diverse ALK proteins and easier to develop resistances to a single targeting agent. Further studies are needed to investigate whether *EML4* intron 6 is a breakpoint ‘hot-zone’ that may provide a high genetic propensity for evolving resistance clones along the course of targeted therapy. Among *ROS1*-positive lung cancers, we also observed that, albeit a small number, *CD74-ROS1* tumors had more fusion subclones than *ROS1* tumors with other fusion partners. Interestingly, one recent study showed that lung cancer patients with *CD74-ROS1* had a lower survival rate compared to those with other *ROS1* partners³³. Our finding suggests that fusion-defined subclone analyses may be important in refining diagnosis and treatment for fusion-positive tumors in the future.

Limitations of SplitFusion includes the inability to detect non-reference sequences as fusion partners, such as from viruses. In this case, one can combine human and suspected viral genome FASTA files, and use BWA (‘bwa index’) to build a combo reference genome. Our observation of the breakpoint ‘hot-zone’ in *EML4* intron 6 needs to be confirmed by larger studies. Further, whether this phenomenon could explain the resistances in the targeted treatment of the *EML4-ALK* v3 tumors requires further mechanism studies and prospective clinical investigations. In conclusion, SplitFusion is fast, sensitive, specific and well-suited for broad use in clinics for gene fusion detection, as well as in research for studying fusion-defined tumor heterogeneity.

Methods

Performance benchmarking with other software

We selected three other fusion detection software to benchmark the performance of SplitFusion for several reasons. In a recent comprehensive comparison of 23 different software, STAR-Fusion showed the highest accuracy (ranked #1) and was among the fastest software¹⁴. EricScript¹² was top-ranked among 12 well-known software in an earlier study³⁴. Lumpy was not among the software compared by the aforementioned two studies, but was able to integrate read-pair, split-read and read-depth jointly¹³, especially when structure variation (fusion) signal is reduced owing to either low

coverage data or low intra-sample variant (fusion) allele frequency, which are common in cancer samples.

Performance of the four software was compared using 46 RNA sequencing datasets generated from clinical FFPE samples (11 positive and 35 negative cancer cases; see *Clinical next-generation sequencing* for details). For the 11 positive sample datasets, each was randomly down-sampled using the seqtk tool (v1.0-r31; <https://github.com/lh3/seqtk>) to eight different sizes (3M, 1M, 500K, 100K, 50K, 10K, 7.5K, and 5K reads), each size had 21 replicates, generating a total of 1,848 datasets for benchmarking test. All of the software were run with one computing thread using human genome GRCh37-hg19 as the reference, and fusion analyses were run using default parameters (e.g. the default threshold for STAR-Fusion to call a fusion is one split-read [`'min_junction_reads'` default: 1]).

The performance among the four software was scored based on the following metrics: 1) Sensitivity: the fraction of samples with the correct fusion genes (*ALK* and *EWSRI*) detected among the 11 fusion-positive samples. 2) Unconfirmed or nonsignificant fusions: fusions involving genes other than the expected *ALK* and *EWSRI*. 3) AUC: the area under the precision-recall curve at varied supporting read thresholds (from minimum to maximum supporting reads) according to the previous method¹⁴. 4) Peak memory: the maximum resident set size (RSS: the portion of memory occupied by a process that is held in main memory) during the computation of the software. 5) User time: the amount of CPU time for a given analysis. Publically available database and tool versions used: human genome reference hg19, LUMPY v0.2.13, EricScript v2.1, STAR-Fusion v1.5, bwa v0.7.17, Samtools v1.10, BEDTools-v2.27.1, seqtk v1.0-r31, ANNOVAR (2019Oct24), PERL v5, R v3.6.3, Python v2.7/v3.

Clinical next-generation sequencing

In this study, 10 *ALK*-positive lung cancer and one *EWSRI*-positive sarcoma³⁵ samples, confirmed by clinical FISH assays, and 35 *ALK* FISH-negative lung cancer samples from Queen Elizabeth Hospital, Hong Kong, were included. The study was approved by the Research Ethics Committee, Hospital Authority, Hong Kong (KC/KE-16-0262/ER-1), institutional review boards at Zhejiang Cancer

Hospital (IRB-2018-24) and Shanghai Changzheng Hospital (2006LL022), China. Total nucleic acids (TNA) containing RNA and DNA were extracted from FFPE sections using the Agencourt FormaPure Kit for FFPE Tissue (Beckman Coulter, Indianapolis, IN). NGS libraries were constructed using the anchored multiplex PCR targeted RNA-seq method described previously¹⁵, with modifications for incorporating unique molecular identifier (UMI) in the adaptors and using an one-tube assay for simultaneous enrichment of RNA and DNA targets³⁶. Briefly, 100 ng of TNA was used for reverse transcription by SuperScript IV (ThermoFisher) using random hexamers, followed by second strand synthesis. The double strand cDNAs were purified using 1.8X SPRIselect (Beckman Coulter) and subjected to further library construction using the KAPA HyperPlus Kit (Roche). The purified ds-cDNAs were enzymatically fragmented at 37°C for 15 min, then end-repaired and A-tailed to produce 5'-phosphorylated and 3'-dA-tailed by incubating with the End-repair & A-tailing enzyme at 37°C for 15 min, followed by 65°C for 15 min. Adapters containing sample index and UMI³⁷ were then ligated to the End-repaired and A-tailed DNA fragments using DNA ligase with 25 pmol of annealed adapters at 16°C for 30 min, followed by 22°C for 30 min. Post-ligation cleanup was performed using 1.0X SPRIselect (Beckman Coulter). The size-selected ligation products were eluted in 20 µL nuclease-free water and amplified by two rounds of hemi-nested PCRs for target enrichment. In PCR1, the ligated DNA fragments were added to 3 µL of 10X PCR buffer, a final concentration of 2 mM of MgCl₂, 0.2 mM of dNTP (New England Biolabs), 25 pmol of panel-specific GSP1 enrichment primers, 3 Units of PlatinumTM Taq DNA polymerase (Invitrogen) and topped up to a final volume of 30 µL of nuclease-free water. The thermo cycling conditions were as follows: initial denaturation at 95°C for 5 min; followed by 20 cycles of 95°C for 30 sec and ramping down at -0.2°C/sec to 60°C for 1 min; hold at 4°C. The amplified PCR1 products were washed with 1.2 X SPRIselect and eluted in 20 µL nuclease-free water. In PCR2, 20 µL of the purified PCR1 products were mixed with 3 µL of 10X PCR buffer, a final concentration of 2 mM of MgCl₂, 0.2 mM of dNTP, 25 pmol of panel-specific GSP2 enrichment primers, 10 pmol of P5 primer, 10 pmol of P7 indexing primer³⁷, 3 Units of PlatinumTM Taq DNA Polymerase and topped up to 30 µL with nuclease free water. The thermo cycling parameters were as follows: initial denaturation at 95°C for 5 min; followed by 15 cycles of 95°C for 30 sec and 65°C for 5 min; hold at 4°C. The final library constructs were size selected by 0.7

X SPRIselect and eluted in 30 µL of EB buffer. After quantification by KAPA Library Quantification Kit for Illumina (Roche), the constructed libraries were subsequently pooled and sequenced by the Illumina NextSeq 500 System. Similarly, the lung cancer and glioblastoma cohort samples were analyzed as previously described³⁸.

Implementation and availability.

The code implementing all steps is included in the SplitFusion software package. SplitFusion is available on GitHub (<https://github.com/Zheng-NGS-Lab/SplitFusion>). User manual and example datasets are also included. The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

B.Z. and C.B. performed bioinformatics analyses and packaged the pipeline; Z.S., C.X., W.W., C.L. and Y.Z. designed and analyzed the large lung cancer cohort; A.H.Y.C performed bioinformatics analysis using benchmarking software; H.W., Z.G., and J.C. designed and analyzed the glioblastoma cohort; C.L., S.B., H.Y.K. and M.Z.C. performed NGS experiments; W.C., M.Y., and W.C.S.C designed and analyzed the initial clinical cohort study; M.Y., W.C.S.C., J.C. and Z.Z. supervised the research and obtained funding; Z.Z. designed SplitFusion; B.Z. and Z.Z. wrote the paper; all authors approved the paper.

Competing interests

Z.Z. holds equity in ArcherDX, a licensee of the AMP technology from Massachusetts General Hospital. Z.Z. and M.Y. are scientific advisors for and hold equity in Helitec, which interests are reviewed and regulated by institutional Outside Practice and Outside Work policies annually.

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Extended Data Figure 1. Fluorescence *in situ* hybridization (FISH) performed on a lung cancer formalin-fix and paraffin-embedded sample using Vysis ALK breakapart probes. The result indicated the presence of *ALK* gene translocation: separation of 3' (red) and 5' (green) signals were indicated by double arrows; narrow split of signals indicated by single arrows; isolated 3' signals indicated by short arrows.

P736-MIP5S36.NCOA4_exon4---RET_exon13

P716-MIP5S16.NCOA4_exon4---RET_exon13

Extended Data Figure 2. Visualization of *NCOA4-RET* gene fusions showed the alignment of detected partner gene *NCOA4* (colored in blue) on exon boundary.

EGFR exon1 --- EGFR exon8

Extended Data Figure 3. Visualization of EGFR *vIII* showed the transcript with exons 2 - 7 deletion in a glioblastoma sample (targeted exon 8 colored in red and detected parter exon 1 in blue).

Extended Data Figure 4. Visualization of rare fusions in lung cancer showed typical anchored multiplex PCR detected fusions involving targeted genes (colored in red; rectangle pileup) and detected fusion partners (colored in blue; open-end pileup) for *KLC1-ALK*, *CD74-NRG1*, and *TPR-NTRK1*. All of the fusion partners showed the alignments on exon boundary.

>**FGFR3 exon 17 --- JAKMIP1 exon 4**
GACCGTGTCTTACCGTGACGTCCACCGACATGGATGAGATCAAAGGGAAGACCGTGTGATTCTGGCCTTGGAGAAGGAACCTGGCGTGCAGGCTGGGCAGACC

>**CLIP2 exon 9 --- BRAF exon 9**
TTGAGACCTTGAGACCCAGACGCAGCTGGAGCACGCGCATTGGGGAGCTGGAACAGAGCCTGCTACTGGAGAAGGCCAGGCCGAGCGGCTGCTCCGAGAATTAGC
GGACAACAGGGACTTGATTAGAGACCAAGGATTCCTGGTGA

>**CLIP2 exon 8 --- BRAF exon 9**
CTGGAGGAGGAGAGGAGGAAGGTGGAGGATCTGCAGTTCGCGTGGAGGAGGAGTCCATCACCAGGGAGACCTGGAGGACTTGATTAGAGACCAAGGATTCCTGGTGA

>**ETV6 exon 2 --- ITPR2 exon 37**
ACTGGGCTCTCTGGAGGTGTATATGAAATTCGTTCCCTGCTCTCCACAAAGCTGTCTTTCTCTCTAACATTCTCGTAATATCTGAAGAATTTAATGCACAGTTTTCTTC
TTTCTCCATTAGTTTCTTTGTATGATTAATCAACTTC

Extended Data Figure 5. Visualization of new fusions in lung cancer showed typical anchored multiplex PCR detected fusions involving targeted genes (colored in red; rectangle pileup) and detected fusion partners (colored in blue; open-end pileup) for *FGFR3-JAKMIP1*, *CLIP2-BRAF*, and *ETV6-ITPR2*. All fusion partners showed the alignments on exon boundary.

Table 1. The list of RNA and DNA targets in a 19-gene lung cancer panel.

RNA targets

Gene	NM	Exon	Sense /antisense
ALK	NM_004304	19,20,21,22	antisense
EGFR	NM_005228	1,8,9	antisense
FGR	NM_005248	1,2,3	antisense
MET	NM_000245	13,14,15	antisense
NRG1	NM_004495	1,2,3,4,6	antisense
NTRK1	NM_002529	8,10,11,12,13, 14,15	antisense
NTRK2	NM_006180	11,12,13,14,1 5,16,17	antisense
NTRK3	NM_002530	13,14,15,16	antisense
ROS1	NM_002944	31,32,33,34,3 5,36,37	antisense
RET	NM_020630	8,9,10,11,12,1	antisense

DNA targets (Reference: hg19)

Gene	NM	start	end	chr	Gene	NM	start	end	chr
ALK	NM_004304	29432648	29432747	2	FGFR1	NM_015850	38285866	38285956	8
ALK	NM_004304	29443568	29443704	2	CDKN2A	NM_000077	21968225	21968244	9
ALK	NM_004304	29445206	29445277	2	CDKN2A	NM_000077	21970897	21971210	9
MSH2	NM_000251	47630328	47630544	2	CDKN2A	NM_000077	21974673	21974828	9
MSH2	NM_000251	47635536	47635697	2	KRAS	NM_004985	25362726	25362848	12
MSH2	NM_000251	47637229	47637514	2	KRAS	NM_004985	25378544	25378710	12
MSH2	NM_000251	47639549	47639702	2	KRAS	NM_004985	25380164	25380349	12
MSH2	NM_000251	47641404	47641560	2	KRAS	NM_004985	25398204	25398320	12
MSH2	NM_000251	47643431	47643571	2	POLE	NM_006231	133250157	133250296	12
MSH2	NM_000251	47656877	47657083	2	POLE	NM_006231	133253128	133253242	12
MSH2	NM_000251	47672683	47672799	2	TP53	NM_000546	7572924	7573011	17
MSH2	NM_000251	47690166	47690296	2	TP53	NM_000546	7573923	7574036	17
MSH2	NM_000251	47693793	47693950	2	TP53	NM_000546	7576849	7576929	17
MSH2	NM_000251	47698100	47698204	2	TP53	NM_000546	7577015	7577158	17
MSH2	NM_000251	47702160	47702412	2	TP53	NM_000546	7577495	7577611	17
MSH2	NM_000251	47703502	47703713	2	TP53	NM_000546	7578173	7578292	17
MSH2	NM_000251	47705407	47705661	2	TP53	NM_000546	7578367	7578557	17
MSH2	NM_000251	47707831	47708013	2	TP53	NM_000546	7579308	7579593	17
MSH2	NM_000251	47709914	47710090	2	TP53	NM_000546	7579696	7579724	17
ROS1	NM_002944	117638302	117638438	6	TP53	NM_000546	7579835	7579914	17
ROS1	NM_002944	117639347	117639418	6	ERBB2	NM_004448	37871535	37871615	17
ROS1	NM_002944	117641027	117641196	6	ERBB2	NM_004448	37880975	37881167	17
EGFR	NM_005228	55241610	55241739	7	ERBB2	NM_004448	37881298	37881460	17
EGFR	NM_005228	55242411	55242516	7	ERBB2	NM_004448	37882811	37882915	17
EGFR	NM_005228	55248982	55249174	7	SMAD4	NM_005359	48573414	48573668	18
EGFR	NM_005228	55259408	55259570	7	SMAD4	NM_005359	48575052	48575233	18
MET	NM_000245	116411899	116412046	7	SMAD4	NM_005359	48575661	48575697	18
MET	NM_000245	116417439	116417526	7	SMAD4	NM_005359	48581147	48581366	18
MET	NM_000245	116423354	116423526	7	SMAD4	NM_005359	48584491	48584617	18
MET	NM_000245	116435937	116436180	7	SMAD4	NM_005359	48584706	48584829	18
BRAF	NM_004333	140453071	140453196	7	SMAD4	NM_005359	48586232	48586289	18
BRAF	NM_004333	140481372	140481496	7	SMAD4	NM_005359	48591789	48591979	18
FGFR1	NM_015850	38271432	38271544	8	SMAD4	NM_005359	48593385	48593560	18
FGFR1	NM_015850	38272073	38272150	8	SMAD4	NM_005359	48603004	48603149	18
FGFR1	NM_015850	38279311	38279462	8	SMAD4	NM_005359	48604622	48604839	18

Table 2. The list of RNA and DNA targets in a 62-gene lung cancer panel.

RNA targets						DNA targets		
Gene	Exon	Sense /antisense	Gene	Exon	Sense /antisense	Selected hotspot exons		
ALK	18	antisense	FGFR4	2	antisense	ABL1	FLT3	MPL
ALK	19	antisense	FGFR4	8	antisense	AKT1	FOXJ2	NRAS
ALK	20	antisense	FGFR4	9	antisense	APC	GNA11	PIK3CA
ALK	21	antisense	FGFR4	10	antisense	ATM	GNAQ	POLE
B2M	2	antisense	FGFR4	17	sense	AURKA	GNAS	PTEN
B2M	2	sense	FGR	1	antisense	CDK4	H3F3A	PTPN11
BRAF	8	antisense	FGR	2	antisense	CDKN2A	HRAS	RHOA
BRAF	9	antisense	FGR	3	antisense	CTNNB1	IDH1	SMAD4
BRAF	10	antisense	GZMA	2	antisense	ERBB2	IDH2	TERT
BRAF	11	antisense	GZMA	3	antisense	ERBB3	JAK2	TP53
BRAF	12	antisense	GZMA	4	antisense	EZH2	KIT	
CD274	2	antisense	GZMA	5	antisense	FBXW7	KRAS	
CD274	3	antisense	MET	13	antisense			
CD274	4	antisense	MET	14	antisense			
CD274	4	sense	MET	15	antisense			
CD274	5	sense	NRG1	1	antisense			
CD274	6	antisense	NRG1	2	antisense			
CHMP2A	3	antisense	NRG1	3	antisense			
CHMP2A	3	sense	NRG1	4	antisense			
CSF1R	9	antisense	NRG1	6	antisense			
CSF1R	10	antisense	NTRK1	8	antisense			
CSF1R	11	antisense	NTRK1	10	antisense			
CSF1R	12	antisense	NTRK1	11	antisense			
CSF1R	13	antisense	NTRK1	12	antisense			
CSF1R	14	antisense	NTRK1	13	antisense			
CTLA4	3	antisense	NTRK1	14	antisense			
EGFR	1	antisense	NTRK1	15	antisense			
EGFR	8	antisense	NTRK2	11	antisense			
EGFR	9	antisense	NTRK2	13	antisense			
EGFR	23	sense	NTRK2	14	antisense			
EGFR	24	sense	NTRK2	15	antisense			
EGFR	25	sense	NTRK2	16	antisense			
ESR1	3	sense	NTRK2	17	antisense			
ESR1	4	sense	NTRK3	13	antisense			
ESR1	5	sense	NTRK3	13	sense			
ESR1	6	sense	NTRK3	14	antisense			
ETV6	1	sense	NTRK3	14	sense			
ETV6	2	antisense	NTRK3	15	antisense			
ETV6	2	sense	NTRK3	15	sense			
ETV6	3	antisense	NTRK3	16	antisense			
ETV6	3	sense	PDCD1	2	antisense			
ETV6	4	sense	PDCD1	3	antisense			
ETV6	5	antisense	PDGFRA	9	antisense			
ETV6	5	sense	PDGFRA	10	antisense			
ETV6	6	antisense	PDGFRA	11	antisense			
ETV6	6	sense	PDGFRA	12	antisense			
ETV6	7	antisense	PDGFRA	13	antisense			
FGFR1	2	antisense	PDGFRA	14	antisense			
FGFR1	3	antisense	PRF1	2	antisense			
FGFR1	4	antisense	PRF1	3	antisense			
FGFR1	5	antisense	RET	8	antisense			
FGFR1	6	antisense	RET	9	antisense			
FGFR1	7	antisense	RET	10	antisense			
FGFR1	8	antisense	RET	11	antisense			
FGFR1	9	antisense	RET	12	antisense			
FGFR1	10	antisense	RET	13	antisense			
FGFR1	11	antisense	ROS1	31	antisense			
FGFR1	17	sense	ROS1	32	antisense			
FGFR2	2	antisense	ROS1	33	antisense			
FGFR2	8	antisense	ROS1	34	antisense			
FGFR2	9	antisense	ROS1	35	antisense			
FGFR2	10	antisense	ROS1	36	antisense			
FGFR2	17	sense	ROS1	37	antisense			
FGFR3	8	antisense	TRAC	1	antisense			
FGFR3	9	antisense	TRBC	1	antisense			
FGFR3	10	antisense						
FGFR3	17	sense						

Figures

Figure 1

The framework of SplitFusion algorithm design. (a) Next-generation sequencing (NGS) library construction using the anchored multiplex PCR. Double-stranded cDNA and gDNA were subjected to end-repair, dA-tailing, and ligation of a half-functional Y adaptor, which contains sample barcode and unique molecular identifier (UMI). Two rounds of hemi-nested multiplex PCR reactions were used to enrich any

sequences (denoted in red question marks) downstream of the gene specific primers (GSPs), resulting in NGS library for sequencing. (b) NGS FASTQ data was de-multiplexed by sample barcodes, followed by adaptor trimming. Different colors represent different UMIs tagged to different molecules. The data was aligned to reference using BWA-MEM, and de-duplicated based on a combination of unique UMI and ligation site (UMI-LS). Supplementary alignments were assigned with the same UMI-LS as their Read 1 starting alignments. Reads with supplementary alignments (SA) were extracted. (c) The CIGARs of reads with supplementary alignments were transformed. For the same read ID, all alignments were sorted by their mapping positions (M) on the query read, and the candidate breakpoint(s) was inferred. (d) The initial candidate breakpoints were filtered by maximum overlapping length, maximum gap, minimum exclusive mapping lengths of partner alignments, and the minimum mapping lengths of ligation end and anchored end alignments; and further subjected to gene annotation (e), frame-ness calculation (f), exon-boundary alignment judgement (g), and reporting and visualization (h).

Figure 2

Benchmarking sensitivity, specificity, accuracy, computation memory use and speed among the four software – Ericscript, Lumpy, STAR-Fusion and SplitFusion. The datasets of the 11 positive samples were

randomly down-sampled to different sizes (reads: 3M, 1M, 500K, 100K, 50K, 10K, 7.5K and 5K), each size with 21 replicates (random seeds), generating 1,848 datasets for analyses by the four software with their default parameters using one computation thread. (a) Sensitivity was calculated as the fraction of samples resulted in expected gene fusions (ALK/EWSR1) among the 11 samples of given size and given random seed. (b) Specificity was evaluated by the number of unconfirmed or non-significant fusions, i.e. other than ALK and EWSR1. (c) The overall accuracy was calculated by the area under the precision-recall curve (AUC) at varied supporting read thresholds (from minimum to maximum supporting reads) according to the previous method. The violin outlined the distribution (the solid circle was the mean) of the statistics for each group (21 datasets). (d) Maximum RSS: the maximum resident set size (RSS: the portion of memory occupied by a process that is held in main memory) during the computation of the software. (e) User time: the amount of total CPU time for a given analysis.

Figure 3

Fusion calling results for the 46 lung cancer samples with clinical known diagnoses (11 positive and 35 ALKnegative) by the four software – Ericscript, Lumpy, STAR-Fusion and SplitFusion. Each column represented one sample. Different colors and symbols denoted different fusions. Total number of false-positive calls (other than ALK/EWSR1) for each software was plotted in the barplot. Out of 35 cases of glioblastoma (GBM), six were found with EGFR vIII using SplitFusion-Target. The GBM samples were not subjected for analysis by the other three software.

Figure 4

The analysis of 1,076 clinical lung cancer formalin-fixed paraffin-embedded samples using SplitFusion. (a) The prevalence of different target gene fusions were shown in the barplot. In total, 86 (8.0%) samples were fusion-positive. The numbers of target genes with partner genes (right column) were indicated in the cells. (b) The numbers of co-occurrence of fusions in the same samples were plotted in the matrix. The EML4-ALK fusion variants were denoted under each specific fusion.

Figure 5

The analysis of breakpoint-defined subclones in EML4-ALK positive lung tumors. (a) The schematic representations of different EML4-ALK fusion variants. Blue cells represent EML4 exons and yellow cells represent ALK exons. Numbers inside the cells showed exon numbers. Cells with red strokes represents introns. (b) Each tile represents one patient. The tile color gradients denote the number of co-existing fusion subclones, defined by exact breakpoint, in the same tumor. (c) For one EML4-ALK variant 3 lung tumor sample, the Integrated Genome Viewer (IGV) showed 6 fusion breakpoints in EML4 exon 6 and intron 6. Their gene partner ALK exon 20 was schematically shown on the right in red, and with nested gene specific primers 1 and 2.

Supplementary Files

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