Final amendment: A plausible explanation for *in silico* reporting of erroneous MET gene expression in tumor-educated platelets (TEP) intended for "liquid biopsy" of non-small cell lung carcinoma still refutes the TEP-study

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Abstract

Final amendment note: This paper had proposed a plausible way for detecting large quantities of MET, which the authors have clarified was not done :the possible explanation proposed for this erroneous MET gene expression does bypass the filtering step we perform in the data processing pipeline, i.e. selection of intron-spanning reads, as can be read in the main text" comments in <http://www.biorxiv.org/content/early/2017/07/02/146134>, where a continuing critique of the TEP study continues. Please consider this pre-print closed.

Original abstract:

The reported over-expression of MET genes in non-small cell lung carcinoma (NSCLC) from an analysis of the RNA-seq data from tumor-educated platelets (TEP), intended to supplement existing 'liquid biopsy' techniques [1], has been refuted recently (<http://biorxiv.org/content/early/2017/06/05/146134>, not peer-reviewed). The MET proto-oncogene (Accid:NG_008996.1, RefSeqGene LRG_662 on chromosome 7, METwithintrons) encodes 21 exons resulting in a 6710 bps MET gene (Accid: NM_001127500.2, METonlyexons). METwithintrons has multiple matches in the RNA-seq derived reads of lung cancer samples (for example: SRR1982756.11853382). Unfortunately, these are non-specific sequences in the intronic regions, matching to multiple genes on different chromosomes with 100% identity (KIF6 on chr6, COL6A6 on chr3, MYO16 on chr13, etc. for SRR1982756.11853382). In contrast, METonlyexons has few matches in the reads, if at all [2]. However, even RNA-seq from healthy donors have similar matches for METwithintrons - so the computation behind the over-expression statistic remains obscure, even if METwithintrons was used as the search gene. In summary, this work re-iterates the lack of reproducibility in the bioinformatic analysis that establishes TEP as a possible source for "liquid biopsy".

Introduction

Tumor tissue biopsy, the gold standard for cancer diagnostics, pose challenges that include access to the tumor, quantity and quality of tumoral material, lack of patient compliance, repeatability, and bias of sampling a specific area of a single tumor [3]. This has resulted in a new medical and scientific paradigm defined by minimal invasiveness, high-efficiency, low-cost diagnostics [4], and, whenever possible, personalized treatment based on genetic and epigenetic composition [5]. The presence of fragmented DNA in the cell-free component of whole blood (cfDNA) [6], first reported in 1948 by Mandel and Metais, has been extensively researched for decades, with extremely promising results in certain niches [7]. Additionally, cfDNA derived from tumors (ctDNA) [8] have tremendous significance as a cancer diagnostic tool [9], and for monitoring responses to treatment [10]. However, detection of ctDNA, and differentiation with cfDNA, remains a challenge due the low amounts of ctDNA compared to cfDNA [11].

Recently, tumor-educated blood platelets (TEP) were proposed as an alternative source of tumor-related biological information [1,12]. The hypothesis driving the potential diagnostic role of TEPs is based on the interaction between blood platelets and tumor cells, subsequently altering the RNA profile of platelets [13,14]. The study showed using RNA-seq data that tumor-educated platelets (TEP) can distinguish 228 patients with localized and metastasized tumors from 55 healthy individuals with 96% accuracy [1]. As validation, this study reported significant over-expression of MET genes in non-small cell lung carcinoma (NSCLC), and HER2/ERBB2 [15] genes in breast cancer, which are well-established biomarkers.

Previously, the TEP-study was refuted by an analysis of a subset of the samples (yet to be peer-reviewed) [2]. Here, an analysis based on the complete MET gene (both introns and exons, Accid:NG_008996.1) demonstrates that intronic non-specific sequences might mislead bioinformatic analysis. Moreover, considering that RNA-seq from healthy donors have similar matches with the complete MET gene, the computation behind the over-expression statistic remains obscure [1].

Results

The MET proto-oncogene (Accid:NG_008996.1, RefSeqGene LRG_662 on chromosome 7, METWITHINTRONS) encodes 21 exons leading to a 6710 bps MET gene (Accid: NM_001127500.2, METONLYEXONS). METWITHINTRONS has multiple matches in the RNA-seq derived reads (for example: SRR1982756.11853382) (Fig. 1). Unfortunately, these are non-specific sequences in the intronic regions: SRR1982756.M.11853382 (CTTCACGTAGTTCTCGAGCCTTGTTTTCAGCTCCATCAGCTCCTTTAAGCACTTCTCTGTA TTGGTTATTCTAGTTATACATTCTTCTAAATTTTTTCA) matches to multiple genes on different chromosomes (KIF6 on chr6, COL6A6 on chr3, MYO16 on chr13, etc). In contrast, METONLYEXONS has few matches in the reads, if at all [2]. Thus, it is erroneous to assign the intronic sequences being expressed to the MET gene. However, even RNA-seq from healthy donors have similar matches for METWITHINTRONS (Fig 3) - so the computation behind the over-expression statistic remains obscure, even if METwithintrons was used as the search gene.

Conclusion

Here, the absence of MET over-expression as reported in the TEP-study [1] is investigated in further detail by using the full MET gene including introns. It turns out that several intronic sequences have matches in the RNA-seq samples. However, these intronic sequences are non-specific (i.e. matching to several other genes with 100% identity). Further, there is to be large number of matches in healthy donor samples as well. This work re-iterates the lack of reproducibility in the bioinformatic analysis that establishes TEP as a possible source for "liquid biopsy".

Materials and methods

The BLAST interface suffices to demonstrate the presence of non-specific sequences from the intronic regions of the MET gene to the RNA-seq samples, and the non-specific nature of these sequences based on 100% identity to a plethora of genes in different chromosomes. These have been verified by a kmer-based version (KEATS [16]) of YeATS [17–21], as well.

Competing interests

No competing interests were disclosed.

References

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Table 1: **Raw counts of reads matching to the MET gene:** Although this is a subset (24 out of 60 NSCLC, and 20 out of 60 healthy), and the numbers are not normalized, its seems unlikely that any statistic will show MET over-expression in NSCLC. It does not even add up to one complete gene in most cases.

Healthy	SRR1982752	NM_001127500.2	ntranscripts=278+2
	SRR1982731	NM_001127500.2	ntranscripts=194+0
	SRR1982742	NM_001127500.2	ntranscripts=56+2
	SRR1982702	NM_001127500.2	ntranscripts=127+2
	SRR1982750	NM_001127500.2	ntranscripts=174+2
	SRR1982741	NM_001127500.2	ntranscripts=124+2
	SRR1982720	NM_001127500.2	ntranscripts=7+0
	SRR1982730	NM_001127500.2	ntranscripts=171+0
	SRR1982722	NM_001127500.2	ntranscripts=2+0
	SRR1982735	NM_001127500.2	ntranscripts=132+0
	SRR1982740	NM_001127500.2	ntranscripts=179+2
	SRR1982751	NM_001127500.2	ntranscripts=152+2
	SRR2095004	NM_001127500.2	ntranscripts=23+0
	SRR1982721	NM_001127500.2	ntranscripts=2+0
	SRR1982732	NM_001127500.2	ntranscripts=180+2
	SRR1982737	NM_001127500.2	ntranscripts=167+2
	SRR1982700	NM_001127500.2	ntranscripts=78+2
	SRR2095014	NM_001127500.2	ntranscripts=23+0
	SRR1982710	NM_001127500.2	ntranscripts=28+0
	SRR1982738	NM_001127500.2	ntranscripts=69+2
	SRR1982701	NM_001127500.2	ntranscripts=99+3
NSCLC	SRR1982781		
	SRR1982780	NM_001127500.2	ntranscripts=13+2
	SRR1982791	NM_001127500.2	ntranscripts=24+0
	SRR2096517	NM_001127500.2	ntranscripts=5+0
	SRR1982772	NM_001127500.2	ntranscripts=8+4
	SRR1982770	NM_001127500.2	ntranscripts=8+2
	SRR1982790	NM_001127500.2	ntranscripts=44+2
	SRR2096502	NM_001127500.2	ntranscripts=1+0
	SRR1982756	NM_001127500.2	ntranscripts=5+0
	SRR1982759	NM_001127500.2	ntranscripts=10+0
	SRR1982795	NM_001127500.2	ntranscripts=9+0
	SRR1982762	NM_001127500.2	ntranscripts=11+2
	SRR1982761	NM_001127500.2	ntranscripts=5+0
	SRR2096503	NM_001127500.2	ntranscripts=2+0
	SRR2096501	NM_001127500.2	ntranscripts=33+2
	SRR1982782	NM_001127500.2	ntranscripts=2+2
	SRR2096516	NM_001127500.2	ntranscripts=6+2
	SRR1982777	NM_001127500.2	ntranscripts=40+2
	SRR1982793	NM_001127500.2	ntranscripts=6+0
	SRR1982765	NM_001127500.2	ntranscripts=16+0
	SRR1982771	NM_001127500.2	ntranscripts=11+2
	SRR1982787	NM_001127500.2	ntranscripts=2+0
	SRR1982792	NM_001127500.2	ntranscripts=5+0
	SRR1982760	NM_001127500.2	ntranscripts=2+0

Figure 1: Graphical representation of matches to the complete MET gene (including introns) to a lung cancer sample (SRR1982756): This shows the first 100 matches, out of thousands of significant matches using the online BLAST interface. However, as demonstrated previously [2], very few of these matches are in the exonic region. Also, these sequences (an example is SRR1982756.11853382) are very non-specific and match to many genes in different chromosomes (Fig 2).

	Description	Max score	Total score	Query cover	E value	Ident	Accession
▫	Homo sapiens kinesin family member 6 (KIF6), RefSeqGene on chromosome 6	187	358	100%	3e-44	100%	NG_054928.1
▫	Homo sapiens collagen type VI alpha 6 chain (COL6A6), RefSeqGene on chromosome 3	187	187	100%	3e-44	100%	NG_054914.1
▫	Homo sapiens uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA), RefSeqGene on chromosome 1	187	187	100%	3e-44	100%	NG_054898.1
▫	Homo sapiens NLR family pyrin domain containing 11 (NLRP11), RefSeqGene on chromosome 1	187	187	100%	3e-44	100%	NG_054722.1
▫	Homo sapiens 12p13 proximal LINE-mediated recombination region (LOC108178987) on chromosome 12	187	187	100%	3e-44	100%	NG_050935.1
▫	Homo sapiens 11p14.2 proximal LINE-mediated recombination region (LOC108178984) on chromosome 11	187	187	100%	3e-44	100%	NG_050933.1
▫	Homo sapiens 9q21.12 distal LINE-mediated recombination region (LOC108175350) on chromosome 9	187	187	100%	3e-44	100%	NG_050923.1
▫	Homo sapiens 9q21.12 proximal LINE-mediated recombination region (LOC108175349) on chromosome 9	187	187	100%	3e-44	100%	NG_050922.1
▫	Homo sapiens diacylglycerol kinase beta (DGKB), RefSeqGene on chromosome 7	187	730	100%	3e-44	100%	NG_029494.2
▫	Homo sapiens myosin XVI (MYO16), RefSeqGene on chromosome 13	187	187	100%	3e-44	100%	NG_053147.1
▫	Homo sapiens collagen type XXIV alpha 1 chain (COL24A1), RefSeqGene on chromosome 1	187	557	100%	3e-44	100%	NG_053093.1
▫	Homo sapiens regulating synaptic membrane exocytosis 2 (RIMS2), RefSeqGene on chromosome 1	187	369	100%	3e-44	100%	NG_053027.1
▫	Homo sapiens neurexin 3 (NRXN3), RefSeqGene on chromosome 14	187	369	100%	3e-44	100%	NG_052991.1
▫	Homo sapiens POU class 6 homeobox 2 (POU6F2), RefSeqGene on chromosome 7	187	187	100%	3e-44	100%	NG_016022.2
▫	Homo sapiens potassium voltage-gated channel interacting protein 4 (KCINP4), RefSeqGene on chromosome 1	187	187	100%	3e-44	100%	NG_052969.1
▫	Pongo abelii genomic sequence	187	722	100%	3e-44	100%	KX224531.1

Figure 2: **Non-specific nature of the intronic sequences of the MET gene that match to the RNA-seq reads from the platelets:** The sequence SRR1982756.M.11853382 matches to several genes across different chromosomes - KIF6 on chr6, COL6A6 on chr3, MYO16 on chr13, etc..

Figure 3: Graphical representation of matches to the complete MET gene (including introns) to a healthy donor sample (SRR1982730): This shows the first 100 matches, out of thousands of significant matches, as found in lung cancer samples as well (Fig. 1). Thus, any statistic showing over-expression must be validated against these raw numbers. These sequences are very non-specific and match to many genes in different chromosomes (Fig 2).