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Multicenter Evaluation of the Idylla GeneFusion in Non—Small-Cell Lung Cancer

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Targeted therapy in lung cancer requires the assessment of multiple oncogenic driver alterations, including fusion genes. This retrospective study evaluated the Idylla GeneFusion prototype, an automated and ease-of-use (<2 minutes) test, with a short turnaround time (3 hours) to detect fusions involving *ALK*, *ROS1*, *RET*, and *NTRK1/2/3* genes and *MET* exon 14 skipping. This multicenter study (18 centers) included 313 tissue samples from lung cancer patients with 97 *ALK*, 44 *ROS1*, 20 *RET*, and 5 *NTRKs* fusions, 32 *MET* exon 14 skipping, and 115 wild-type samples, previously identified with reference methods (RNA-based next-generation sequencing/fluorescence *in situ* hybridization/quantitative PCR). Valid results were obtained for 306 cases (98%), overall concordance between Idylla and the reference methods was 89% (273/306); overall sensitivity and specificity were 85% (165/193) and 96% (108/

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113), respectively. Discordances were observed in 28 samples, where Idylla did not detect the alteration identified by the reference methods; and 5 samples where Idylla identified an alteration not detected by the reference methods. All of the *ALK*-, *ROS1*-, and RET-specific fusions and *MET* exon 14 skipping identified by Idylla GeneFusion were confirmed by reference method. To conclude, Idylla GeneFusion is a clinically valuable test that does not require a specific infrastructure, allowing a rapid result. The absence of alteration or the detection of expression imbalance only requires additional testing by orthogonal methods. *(J Mol Diagn 2022, 24: 1021—1030; https://doi.org/10.1016/j.jmoldx.2022.05.004)*

The identification of targetable oncogene drivers has revolutionized the therapeutic landscape of non—small-cell lung cancer (NSCLC) with promising therapeutic targets. Recently, novel oncogenic drivers emerged as therapeutic targets besides the well-known *EGFR* mutations, and rearrangements in *ALK* and *ROS1* genes, considerably expanding the list of potential exploitable genetic aberrations. Those include fusions in *RET*, *NTRK1*, *NTRK2*, and *NTRK3* genes, and *MET* exon 14 skipping. According to updated US and European guidelines, *EGFR* mutation in exons 18 to 21, *BRAF* V600 mutation, and *ALK*, *ROS1*, *RET*, and *NTRK* rearrangement testing should be systematically analyzed in advanced NSCLC and should be available as early as first-line treatment.[1,2] Fusion genes involving *ALK*, *ROS1*, *RET*, and receptor family for NTRKs (*NTRK1*, *NTRK2*, *NTRK3*) are collectively rare and represent individually no more than 5% of NSCLC, and are usually found in adenocarcinoma.[3—13] *MET* exon 14 skipping mutations are as prevalent as *ALK* rearrangements, occurring in 3% to 4% of NSCLC.[14,15] Several techniques can be used to detect gene fusions, from single-test methods, including immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), to multiplex test methods, such as targeted DNA or RNA next-generation sequencing (NGS). Immunohistochemical detection of ALK, ROS1, RET, and TRK proteins with currently available antibodies is used to predict the presence of the respective fusion proteins, however with poor sensitivity and specificity in general except for ALK-fusion IHC, whereas sensitivity is not good enough for *RET* or *MET*.[16—21] To date, ALK IHC is the only test that has been recognized as an equivalent to FISH as a stand-alone test, meaning that no confirmation is required to initiate treatment except for cases with intermediate staining levels.[22] FISH detects gene rearrangements at the DNA level, primarily by using break-apart probes, and was considered as the gold standard, especially for *ALK* and *ROS1*.

Nevertheless, because most NSCLC patients are diagnosed at advanced stages, the limited material makes a multiplex test approach preferred over a single-test method. Among these, RNA-based NGS has been widely used for the simultaneous detection and typing of *ALK*, *ROS1*, *RET*, and *NTRK* fusions, as well as *MET* exon 14 skipping, because they can be better identified using RNA than DNA in small amplicon panels.[23] However, the long turnaround time of NGS may not be compatible with the therapeutic urgency of patients with NSCLC. To address this gap, Biocartis (Mechelen, Belgium) has developed a specific cartridge, the Idylla GeneFusion assay, to detect the presence of *ALK*, *ROS1 RET*, *NTRK1*, *NTRK2*, and *NTRK3* fusions and *MET* exon 14 skipping simultaneously, in only 3 hours. This assay has been optimized for use on formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections and automates the entire process from sample to result, including sample preparation, reverse transcription of mRNA, real-time PCR amplification, detection, and data analysis.

Our aim was to evaluate the technical performance of the Idylla GeneFusion prototype assay to detect *ALK*, *ROS1*, *RET*, *NTRK1*, *NTRK2*, and *NTRK3* fusions and *MET* exon 14 skipping previously defined by reference methods, including FISH, RNA-based NGS, and quantitative PCR (qPCR), on 313 FFPE tissue specimens.

Materials and Methods

Lung Cancer Tissue Sample Collection

This retrospective study was conducted to evaluate the analytical sensitivity and specificity of the Idylla GeneFusion prototype in archived FFPE tissue samples. A total of 313 NSCLC patient samples from primary tumor or metastatic sites were selected. All samples were previously tested using RNA-based NGS, FISH, and/or qPCR and included 198 positive and 115 negative fusion cases, in routine diagnostic laboratories of 18 clinical centers: Cochin Hospital (Paris, France), Institut Universitaire de Cardiologie et de Pneumologie de Québec (Québec, Canada), New Cross Hospital (Wolverhampton, United Kingdom), Queen Elizabeth Hospital (Birmingham, United Kingdom), Royal Hallamshire Hospital (Sheffield, United Kingdom), Hospital do Espírito Santo (Evora, Portugal), Hospital Del Mar (Barcelona, Spain), Hospital Universitario Virgen del Rocío (Seville, Spain), Marie Lannelongue Hospital and Gustave Roussy Cancer Campus (Le Plessis-Robinson and Villejuif, France), Center Hospitalier Régional de la Citadelle (Liege, Belgium), Universitätsklinikum Köln (Cologne, Germany), Universitätsklinikum Bonn (Bonn, Germany), Universitätsklinikum Frankfurt (Frankfurt, Germany), University Medical Center (Groningen, the Netherlands), European Institute of Oncology (Milan, Italy),

Regina Elena National Cancer Institute (Rome, Italy), Tan Tock Seng Hospital (Singapore), and Public Specialist Hospital of Lung Diseases (Zakopane, Poland). Each center tested its own samples with Idylla GeneFusion prototype cartridges supplied by Biocartis NV.

All FFPE tissue specimens came from surgical resection ($n = 82$) or biopsy ($n = 231$), excluding cytology specimens. Tumor cell content was determined on hematoxylin and eosin–stained slides by a pathologist, and macrodissection was performed on 87 of 313 cases (28%) to achieve a tumor cell content (TCC) of at least 10%. One to five FFPE tissue sections of 5- or 10-μm thickness were used, depending on tissue area and/or percentage of tumor cells. These FFPE tissue sections were placed directly into the Idylla GeneFusion prototype cartridge (Biocartis NV) following the assay instructions of the manufacturer.

Results Obtained by References Method

Idylla GeneFusion results were compared with those obtained earlier with routine diagnostic reference methods. Three techniques are considered as reference methods: FISH, qPCR, and RNA-based NGS. Some immunohistochemistry results (ALK or ROS1 IHC) were available, but IHC was not considered as a reference method. FISH analyses were performed ($n = 215$) on whole-tissue slides of FFPE samples with dual color break-apart probes, allowing the detection of *ALK* ($n = 75$), *ROS1* ($n = 34$), *RET* ($n = 10$), *NTRK1* ($n = 1$), and *NTRK3* ($n = 1$) rearrangements. qPCR ($n = 28$) was used to detect the *ALK* ($n = 7$) and *ROS1* ($n = 4$) fusions and *MET* exon 14 skipping ($n = 1$). For RNA-based NGS ($n = 194$), four different amplicon-based NGS panels were used: Oncomine Focus Assay ($n = 130$; Thermo Fisher Scientific, Waltham, MA), Archer FusionPlex ($n = 61$; ArcherDx, Boulder, CO), QIAseq RNA panel ($n = 1$; QIAGEN, Hilden, Germany), and customized ($n = 2$), allowing the detection of *ALK* ($n = 46$), *ROS1* ($n = 12$), *RET* ($n = 18$), *NTRK1* ($n = 3$), and *NTRK3* ($n = 2$) fusions and *MET* exon 14 skipping ($n = 32$). Only the RNA-based NGS method qualitatively detects fusion specific sequences.

Idylla GeneFusion Assay Description

In this study, an Idylla GeneFusion prototype cartridge was used, which is not a commercially available product. This prototype assay, performed on the Idylla System, is a fully integrated cartridge-based assay that provides automated sample processing including deparaffinization, tissue digestion, RNA extraction, reverse transcription of mRNA, real-time PCR amplification, and detection of the targeted sequences. Raw data generated by the Idylla GeneFusion prototype were sent to the manufacturer (Biocartis) for fusion-calling with the commercially available Idylla automated software version 1.0 (research use only). This assay qualitatively detects specific gene fusions for *ALK*, *ROS1*, and *RET*, as well as *MET* exon 14 skipping and $5'/3'$ imbalanced expression for *ALK*, *ROS1*, *RET*, and *NTRK1/2/3* transcripts (Table 1). More than 30 specific *ALK*, *RET*, and *ROS1* gene fusion transcripts can be detected (Table 1), but Idylla GeneFusion cannot discriminate between the different fusion gene partners. The $5'/3'$ imbalanced expression assays have the advantage that fusion transcripts with uncommon breakpoints or unknown fusions partners (other than those listed in Table 1) might still provide an indication of the presence of a fusion. To identify *MET* exon 14 skipping, the assay detects spliced isoforms with and without *MET* exon 14, corresponding to wild-type and mutant transcripts, respectively. RNA and DNA control genes are included in the cartridge. In this prototype version, the Ct values were not listed in the data reports and were not used in the data analysis.

Concordance Analysis

The Idylla GeneFusion prototype reports three types of results per gene for each of the two detection methods [specific fusion (spe) and expression imbalance (imb)]: i) detected, ii) not detected, and iii) invalid. A sample is classified as inconclusive with Idylla when more than one fusion transcript or *MET* exon 14 skipping transcript is detected (due to mutual exclusivity) or if the invalid gene is the one that has been detected as altered by the reference method. Results are considered discordant if one of the two detection methods (Idylla and reference method) found an alteration, which can be a specific fusion or expression imbalance with Idylla, and the other did not, or if it was an alteration in another gene. Discordant negative was used when Idylla did not detect the fusion or *MET* exon 14 skipping, neither via fusion-specific testing nor via expression imbalance, whereas the reference method did. Discordant positive was used when Idylla detected the fusion-specific or expression imbalance or *MET* exon 14 skipping, whereas the reference method did not. Sensitivity, specificity, positive percent agreement, negative percent agreement, and overall concordance were calculated using Prism software version 6 (GraphPad Software, San Diego, CA).

Ethics Approval and Consent to Participate

Human tissues were used according to "The Code for Proper Secondary Use of Human Tissue" and approved by the respective local ethics committee.

Results

Overall Performance of the Idylla GeneFusion Prototype

A total of 313 cases were tested in this multicenter evaluation study of the Idylla GeneFusion prototype. The

Table 1 Idylla GeneFusion Assay

Detection technologies							
Detection method	*ALK*	*ROS1*	*RET*	*METex14*	*NTRK1*	*NTRK2*	*NTRK3*
Specific fusion detection	X	X	X	X			
Expression imbalance	X	X	X		X	X	X

List of variants detected by specific fusions		
Gene	Partner gene	Variant
ALK	*EML4*	E2/6a/6b/13/15/17/18/20:A20
	KIF5B	K15/17/24:A20
	HIP1	H21/28/30:A20
	KLC1	K9:A20
	TFG	T4/6:A20
	TPR	T15:A20
ROS1	*CD74*	C6:R32
	SDC4	S2/4:R32
	SLC34A2	S4/13:R 32
	EZR	E10:R 34
	SDC4	S4:R34
	SLC34A2	S4:R34
	CD74	C6:R34
	TPM3	T8:R35
	GOPC	G8:R35
	LRIG3	L16:R35
	GOPC	G4:R36
RET	*KIF5B*	K15/24:R11
	KIF5B	K15/16/22/23:R12
	CCDC6	C1:R12

Combination of two detection technologies: specific fusion detection, allowing the detection of the most relevant gene fusions, directly from RNA transcripts by real-time PCR, and expression imbalance detecting gene fusions irrespective of fusion partner based on the 3′ kinase overexpression caused by the fused partner gene promoter.

average percentage of TCC was 50%. Among these cases, 198 were positive for a fusion in *ALK* ($n = 97$), *ROS1* ($n = 44$), *RET* ($n = 20$), *NTRK1* ($n = 3$), *NTRK3* ($n = 2$), or *MET* exon 14 skipping ($n = 32$), and 115 were negative for these alterations by routine clinical testing using either FISH, qPCR, or RNA-based NGS (Figure 1). Of the 313 samples, 306 valid results (98%) were generated using the Idylla GeneFusion prototype. Seven cases were classified as inconclusive, one in which Idylla GeneFusion detected an invalid ALK^{imb} in *ALK*-positive reference sample and six cases in which Idylla GeneFusion reported a double alteration, all with ALK^{imb}. One case with $ALK^{imb/spe}$ and *MET* exon 14 skipping, and one with $ALK^{imb/spe}$ and *NTRK3* in which *ALK* fusion was confirmed by FISH; two cases with ALK^{imb} and $RET^{imb/spe}$ in which *RET* fusion was confirmed by NGS; and one with ALK^{imb} and $ROS1^{imb}$ and another with ALK^{imb} and *MET* that were reported negative by reference method (qPCR).

Of the 306 cases, 193 samples were fusion or *MET* exon 14 skipping positive as determined by reference method, and Idylla GeneFusion prototype confirmed the alteration in 165 cases [85.5% (165/193): 87% (82/94) *ALK*, 82% (36/44) *ROS1*, 94% (17/18) *RET*, 67% (2/3) *NTRK1*, 50% (1/2) *NTRK3*, and 84% (27/32) *MET* exon 14 skipping]. A total of 28 discordant negative cases were observed in which one *MET* exon 14 skipping–positive sample was detected as a *ROS1* fusion by expression imbalance on Idylla GeneFusion. Of the 28 discordant negative cases, Idylla GeneFusion failed to detect 23 fusions and 5 *MET* exon 14 skipping that were positive by FISH, qPCR, and/or RNA-based NGS (Tables 2 and 3). Of the 113 valid negative samples tested by reference method, no alteration was identified in 108 samples (96%, 108/113) using Idylla GeneFusion. A total of five discordant positive cases were observed, all with ALK^{imb} only, in which the reference method showed no *ALK* fusion [qPCR ($n = 2$), NGS ($n = 1$), and FISH ($n = 2$)] (Figure 1).

Overall, there was concordance between Idylla GeneFusion and reference methods for 273 of 306 samples (89.2%; 95% CI, 85.7% to 92.7%).

ALK Fusion Tumors

Of the 94 valid *ALK*-positive samples determined by reference methods, 82 cases were positive by Idylla GeneFusion. Among them, 58 were positive for both expression imbalance and specific fusion methods, 15 with ALK^{spe} only and 9 with ALK^{imb} only. There were 12 discordant samples,

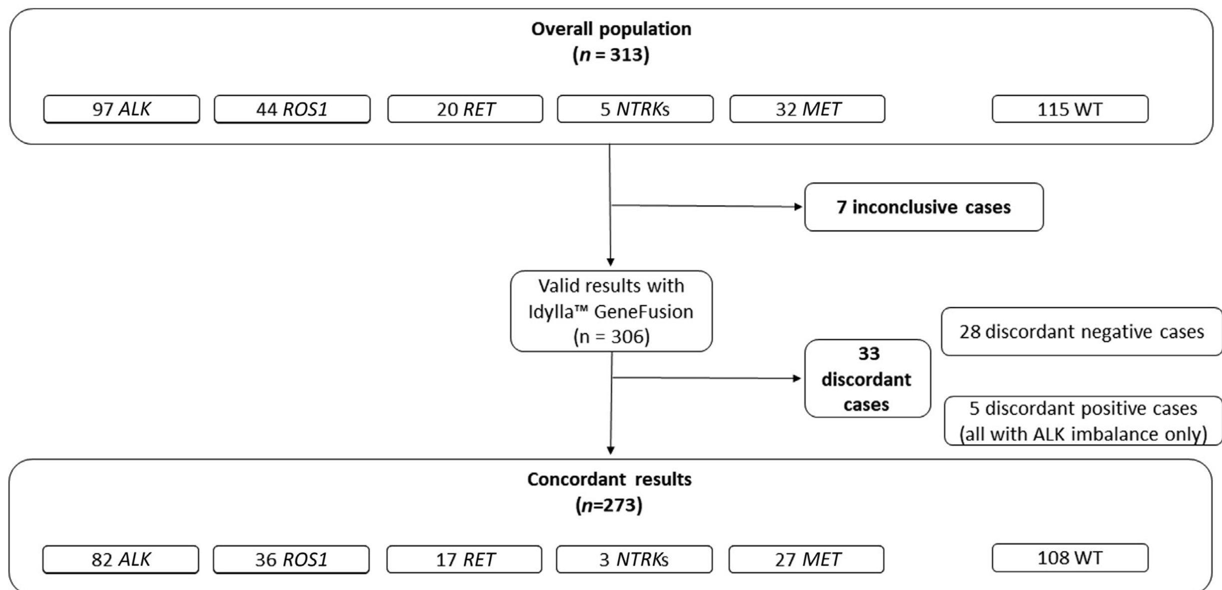

Figure 1 Flow chart of sample analysis. Results of the Idylla GeneFusion prototype were compared with the reference methods (RNA-based next-generation sequencing/fluorescence *in situ* hybridization/real-time quantitative PCR). WT, wild type.

for which one or more reference methods reported *ALK* alteration, whereas the Idylla GeneFusion did not (discordant negative cases). These 12 tissue samples include 11 biopsies and 1 resection, and the average percentage of TCC was 60%. Of these 12 samples, nine discordant negative cases were identified by only one reference method (five FISH, one NGS, and three qPCR), and it was impossible to confirm the true *ALK* fusion with a third method, even more if one sample was considered as an equivocal result on FISH (Table 4). Of these 12 discordant negative cases, the variant identification was only available for four samples and corresponded to the common *EML4::ALK (E6:A20)* fusion (Table 4).

All *ALK* fusions detected by the Idylla GeneFusion−specific fusion method were confirmed by reference methods. On the contrary, there were five samples identified by Idylla GeneFusion expression imbalance method as *ALK* rearrangement, but were *ALK*-fusion negative by reference methods, including two FISH, two qPCR, and one NGS. For these cases, only two ALK IHC were available and were negative.

Finally, the sensitivity for Idylla GeneFusion *ALK* fusion was 87% (82/94; 95% CI, 83.4% to 90.9%), specificity 98% (193/198; 95% CI, 95.8% to 99.2%), positive percentage agreement 94% (82/87; 95% CI, 91.7% to 96.8%), and negative percentage agreement 94% (193/205; 95% CI, 91.3% to 96.6%). The overall concordance between Idylla GeneFusion and reference method for *ALK* was 94% (275/292; κ = 0.86; 95% CI, 91.3% to 96.6%) (Table 3).

ROS1 Fusion Tumors

Of the 44 *ROS1* valid positive samples determined by reference method, 35 cases were positive by Idylla GeneFusion. Among them, 16 were positive with both specific fusion and expression imbalance methods, 19 with $ROS1^{spe}$ and none with $ROS1^{imb}$. There were eight discordant samples (six biopsies and two resected samples), for which one reference method reported *ROS1* alteration, whereas the Idylla GeneFusion did not (discordant negatives cases). All of these eight discordant negative cases could not be tested with a third method to confirm the true *ROS1* fusion,

Table 2 Overall Concordance and Kappa Correlation Coefficient of Idylla GeneFusion Prototype Compared with Reference Methods (RNA-Based NGS, FISH, and/or qPCR)

Idylla versus ref. method	*ALK*	*ROS1*	*RET*	*METex14*	*NTRKs*
Sn	87% (82/94)	82% (36/44)	94% (17/18)	84% (27/32)	60% (3/5)
Sp	98% (193/198)	99% (256/257)	100% (289/289)	100% (275/275)	100% (288/288)
PPA	94% (82/87)	97% (36/37)	100% (17/17)	100% (27/27)	100% (3/3)
NPA	94% (193/205)	97% (256/264)	99% (289/290)	98% (275/280)	99% (288/290)
Overall concordance	94%	97%	99%	98%	99%
Kappa correlation coefficient	0.86	0.86	0.97	0.91	0.75

FISH, fluorescence *in situ* hybridization; NGS, next-generation sequencing; NPA, negative percentage agreement; PPA, positive percentage agreement; qPCR, quantitative PCR; ref., reference; Sn, sensitivity; Sp, specificity.

Table 3 Performance of the Idylla GeneFusion Assay for Each Gene

Gene	Idylla result	Reference methods		Total
		Alt	No alt	
ALK	Alt	82	5	87
	No alt	12	193	205
	Invalid	3	18	21
	Total	97	216	313
RET	Alt	17	0	17
	No alt	1	289	290
	Invalid	2	4	6
	Total	20	293	313
NTRKs	Alt	36	1	37
	No alt	8	256	264
	Invalid	0	12	12
	Total	44	269	313
ROS1	Alt	36	1	37
	No alt	8	256	264
	Invalid	0	12	12
	Total	44	269	313
METex14	Alt	27	0	27
	No alt	5	275	280
	Invalid	0	6	6
	Total	32	281	313

Alt, alteration; no alt, no alteration.

especially because one sample was considered as an equivocal result on FISH, and another one was tested negative by IHC (Table 4). These samples were detected positive by five FISH, one RNA-based NGS (*SLC34A2::ROS1*, S4:R32), and two qPCR, with an overall average TCC of 51% (95% CI, 47% to 55%). There was 1 sample identified as *ROS1* rearranged by Idylla GeneFusion expression imbalance method only (biopsy with 100% of TCC), in which a *MET* exon 14 skipping mutation was detected with the reference method (classified as discordant negative) (Table 4).

Finally, the sensitivity for Idylla GeneFusion *ROS1* fusion detection was 82% (36/44; 95% CI, 77.4% to 86.1%), specificity 99.6% (256/257; 95% CI, 98.2% to 100%), positive percentage agreement 97% (36/37; 95% CI, 95.4% to 99.1%), and negative percentage agreement 97% (256/264; 95% CI, 95% to 98.9%). Idylla GeneFusion and reference methods showed an overall concordance of 97% for *ROS1* fusion detection (292/301; κ = 0.86; 95% CI, 95% to 98.9%) (Table 3).

RET Fusion Tumors

Of the 18 valid *RET*-positive samples determined by reference method, 17 cases were positive by Idylla GeneFusion. Among them, 12 were $RET^{spe/imb}$, 1 was RET^{imb} only, and 4 were RET^{spe} only. One discordant negative sample was found for which *RET* fusion was detected using FISH, but not by Idylla GeneFusion. This sample was old (from 2001), and the second technique (NGS) used to confirm failed (Table 4).

There were no discordant positives observed because all *RET*-specific fusion identified by Idylla GeneFusion was confirmed by reference method.

Finally, the sensitivity for Idylla GeneFusion *RET* fusion detection was 94% (17/18; 95% CI, 91.3% to 96.6%), specificity 100% (289/289) and positive percentage agreement 100% (17/17), and negative percentage agreement 99% (289/290; 95% CI, 97.9% to 100%). Idylla and reference method overall concordance for *RET* fusion detection was 99% (306/307; κ = 0.97; 95% CI, 97.9% to 100%) (Table 3).

NTRKs Fusion Tumors

Of the five *NTRK*1/2/3-positive samples determined by reference method, Idylla GeneFusion detected three cases by expression imbalance method. There were two discordant negative samples, for which one or more reference methods reported *NTRK1* (NGS) or *NTRK3* (NGS and FISH) fusion, whereas the Idylla GeneFusion did not (Table 4). These samples were two tissue biopsies with an average TCC of 50%. *NTRK1* fusion was detected by NGS only. No discordant positive results were observed.

Finally, the sensitivity for Idylla GeneFusion *NTRK1/2/3* fusion detection was found to be 60% (3/5; 95% CI, 54.5% to 65.5%), specificity 100% (288/288) and positive percentage agreement 100% (3/3), and negative percentage agreement 99% (288/290; 95% CI, 98.5% to 100%). The Idylla GeneFusion and reference method overall concordance was 99% (291/293; κ = 0.75; 95% CI, 97.9%-100%) (Table 3).

MET Exon 14 Skipping Tumors

Of the 32 valid *MET* exon 14 skipping−positive samples determined by reference methods, 27 cases were positive by Idylla GeneFusion. The five discordant negative samples were tissue biopsies, with an average TCC of 60%, and were identified using only one reference method (RNA-based NGS). Of these five discordant negative samples, the target reads of three cases were very low (<800) and could correspond to false-positive cases of the reference method (Table 4). Moreover, one of the five discordant negative cases that reported no *MET* exon 14 skipping showed *ROS1*-positive expression imbalance using Idylla GeneFusion. No sample was identified falsely as *MET* exon 14 skipping positive with the Idylla GeneFusion assay.

Finally, the sensitivity for Idylla GeneFusion *MET* exon 14 skipping detection was found to be 84.4% (27/32; 95% CI, 80.3% to 88.5%), specificity 100% (275/275) and positive percentage agreement 100% (27/27), and negative percentage agreement 98% (275/280; 95% CI, 96.9% to 99.7%). Idylla GeneFusion and reference method overall

Table 4 Characteristics of Discordant Negative Cases (N = 28)

Case	Alteration	Variant	Ref methods	IHC	Idylla	Number of slides used for Idylla	Sample type	Tumor cell content, %
1	*ALK*	*EML4(E6)::ALK(A20)*	FISH and NGS-RNA	ALK pos	Neg	3 (5 μm)	Tissue resection	40
2	*ALK*	*EML4(E6)::ALK(A19)*	FISH and NGS-RNA	ALK pos	Neg	1 (5 μm)	Tissue biopsy	40
3	*ALK*		qPCR	Not available	Neg	1 (10 μm)	Tissue biopsy	50
4	*ALK*		qPCR	Not available	Neg	1 (10 μm)	Tissue biopsy	50
5	*ALK*		qPCR	Not available	Neg	5 (10 μm)	Tissue biopsy	30
6	*ALK*		FISH	ALK pos	Neg	4 (5 μm)	Tissue biopsy	60
7	*ALK*		FISH	ALK pos	Neg	1 (5 μm)	Tissue biopsy	70
8	*ALK*	*EML4(E6)::ALK(A20)*	FISH and NGS-RNA	Not available	Neg	3 (5 μm)	Tissue biopsy	70
9	*ALK*		FISH (equivocal, 20%)	Not available	Neg	3 (5 μm)	Tissue biopsy	60
10	*ALK*		FISH	ALK pos	Neg	3 (5 μm)	Tissue biopsy	70
11	*ALK*		FISH	Not available	Neg	1 (10 μm)	Tissue biopsy	20
12	*ALK*	*EML4(E6)::ALK(A19)*	NGS-RNA	Not available	Neg	3 (10 μm)	Tissue biopsy	50
13	*ROS1*		qPCR	Not available	Neg	5 (10 μm)	Tissue biopsy	30
14	*ROS1*		qPCR	Not available	Neg	5 (10 μm)	Tissue biopsy	20
15	*ROS1*	*SLC34A2(S4)::ROS1(R32)*	NGS-RNA	Not available	Neg	5 (10 μm)	Tissue resection	50
16	*ROS1*		FISH	ROS1 neg	Neg	1 (5 μm)	Tissue biopsy	70
17	*ROS1*		FISH (equivocal, 20%)	Not available	Neg	1 (5 μm)	Tissue biopsy	50
18	*ROS1*		FISH	Not available	Neg	3 (5 μm)	Tissue biopsy	80
19	*ROS1*		FISH	Not available	Neg	3 (5 μm)	Tissue biopsy	20
20	*ROS1*		FISH	Not available	Neg	1 (5 μm)	Tissue resection	30
21	*MET*ex14		NGS-RNA		ROS^{imb}	4 (5 μm)	Tissue biopsy	90
22	*MET*ex14		NGS-RNA		Neg	5 (10 μm)	Tissue biopsy	30
23	*MET*ex14		NGS-RNA (<800 reads)		Neg	3 (5 μm)	Tissue biopsy	70
24	*MET*ex14		NGS-RNA (<800 reads)		Neg	3 (5 μm)	Tissue biopsy	50
25	*MET*ex14		NGS-RNA (<800 reads)		Neg	1 (5 μm)	Tissue biopsy	70
26	*NTRK1*	*TPM3(T7)::NTRK1(N12)*	NGS-RNA		Neg	5 (5 μm)	Tissue biopsy	40
27	*NTRK3*	*NTRK3::ETV6*	FISH and NGS-RNA		Neg	3 (5 μm)	Tissue biopsy	50
28	*RET*		FISH (NGS failed)		Neg	3 (5 μm)	Tissue resection	40

FISH, fluorescence *in situ* hybridization; NGS, next-generation sequencing; IHC, immunohistochemistry; METex14, *MET* exon 14 skipping; neg, negative; pos, positive; qPCR, quantitative PCR.

concordance for *MET* exon 14 skipping detection was 98% (302/307; κ = 0.91; 95% CI, 97.9% to 100%) (Table 3).

Discussion

Gene fusion analysis is crucial for the clinical management of lung cancer patients. Patients with tumors harboring *ALK*, *ROS1*, *RET*, or *NTRK1/2/3* gene rearrangements or *MET* exon 14 skipping can benefit from treatment with tyrosine kinase inhibitors.[1] Due to the diversity in these alterations, multiplex assays, such as NGS, are preferred over single tests, such as FISH or qPCR. From test request to result, the turnaround time of NGS is often too long for patients with NSCLC who require rapid treatment. Compared with NGS, the Idylla GeneFusion assay employs a faster technology, which multiplexes the targets and provides results in only 3 hours. Idylla technology has been already validated in NSCLC with a CE-IVD certification for the detection of clinically relevant *EGFR* mutations.[24–28]

In this study, the performance of the Idylla GeneFusion prototype assay was tested in real-world conditions, using 313 FFPE lung cancer tissue samples, including 198 cases with fusion or *MET* exon 14 skipping. Idylla GeneFusion allowed the detection of 87% *ALK* (82/94), 82% *ROS1* (36/44), 94% *RET* (17/18), and 60% *NTRK1/2/3* (3/5) fusions and 84% *MET* exon 14 skipping (27/32) of previously tested positive cases. A good overall concordance was observed, but with a difference between genes of 94% (275/292) for *ALK*, 97% (292/301) for *ROS1*, 98% (302/307) for *MET* exon 14 skipping, 99% (306/307) for *RET*, and 99% (291/293) for *NTRKs*. Seven cases were excluded, including one *ALK*-positive sample detected as *ALK* invalid and six cases with double fusion detected, all involving *ALK*^{imb}. Among the six double fusion−positive cases, Idylla GeneFusion detected the known fusion in four (two *ALK*$^{spe/imb}$ and two *RET*$^{spe/imb}$) of them. The driver alterations in NSCLC are conventionally considered mutually exclusive, especially for *ALK* fusion,[29] thus the detection of two rare alterations in the same tumor should be confirmed by another testing method. Likewise, the presence of at least one invalid gene, without alteration reported by Idylla GeneFusion, should be completed by another test.

Regarding the discordant cases, Idylla GeneFusion failed to detect the alteration in 28 cases (12 *ALK*, 8 *ROS1*, 1 *RET*, 1 *NTRK1*, 1 *NTRK3*, and 5 *MET* exon 14 skipping), which were tested positive using FISH (8 *ALK*, 5 *ROS1*, 1 *RET*, and 1 *NTRK3*), NGS ($n = 12$), or qPCR ($n = 5$) methods (Table 4). The majority of the discordant results have been tested by only one valid reference method [$n = 24/28$, including five FISH *ALK*, five FISH *ROS1*, one FISH *RET*, five qPCR (three *ALK*, two *ROS1*), and eight RNA-based NGS (five *MET* exon 14 skipping, one *NTRK1*, one *ALK*, and one *ROS1*)]. It is not excluded that these discrepancies corresponded to false-positive cases from the reference method, as already described for *RET* FISH,[18,30,31] qPCR,[32] and *MET* exon 14 skipping RNA-based NGS[33,34] (Table 4). Notably, three *MET*-positive cases had <800 reads of *MET* exon 14 skipping, and two FISH (for *ALK* and *ROS1* genes) had a low percentage of rearranged tumor cells (20%) (Table 4). However, discordant negative cases can also be explained by the presence of atypical or rare variants or by the low RNA quality and/or quantity, related to the age of the sample or the insufficient number of sections loaded. Unfortunately, in this multicenter study, it was not possible to confirm these discrepancies, either by a third assay or by the clinical response data, and future comparative studies should confirm the discrepant cases by another reference method.

If the specific detection method does not allow the identification of fusion, the assessment of expression imbalance method should have identified them. Nevertheless, cases in which a specific fusion was detected did not always reveal a 5′/3′ expression imbalance: 38 specific fusion detections (15 *ALK*, 19 *ROS1*, and 4 *RET*) were not detected by expression imbalance. Expression imbalance might be more sensitive to RNA quality and/or quantity than specific detection. Thus, these discordant negative cases highlight the need to confirm negative results with Idylla GeneFusion by another multiplex assay, such as RNA-based NGS.

Five false-positive cases (five ALK^{imb}) were detected with the Idylla GeneFusion assay reporting expression imbalance only, but not by reference methods. An expression imbalance−only call occurs when a specific fusion event cannot be detected with the primers and probes in the current version of the assay. Alternatively, the incorrect assessment of imbalance may result from the potentially higher level of physiological RNA degradation at the 5′-RNA terminus compared with degradation of the 3′ terminus.[35] As a result, the amount of template for 5′ and 3′ primers is skewed due to the higher level of 5′ degradation rather than overexpression of the kinase domain-containing 3′ end. As mentioned above, six cases were found in which Idylla GeneFusion reported a double alteration, all with ALK^{imb}, including only two *ALK*-positive reference samples. It is unclear why the 5′/3′ ratio for *ALK*, but not *ROS1*, *RET*, and *NTRK1/2/3*, can result in a false-positive result. Setting the cutoff higher decreases the false-positive rate, but also decreases the sensitivity. As such, calling an *ALK* fusion based on an ALK^{imb}-only result, should be interpreted with caution and preferably confronted with ALK IHC. Overall, an expression imbalance without a call for a specific fusion should always be confirmed with an orthogonal method.

Currently, Idylla GeneFusion is commercialized as a reference use only version (updated from a prototype version). Additional comparative studies are required to confirm its performance as reported for the prototype version tested in the current study, as well as its clinical validity prior to implementation. The place of Idylla GeneFusion in routine practice is not yet well-defined, and it may replace conventional methods, once evidence has been provided that Idylla performs at least as well as FISH/RNAseq/qPCR. In a less comprehensive rapid-type test such as Idylla, reduced sensitivity is acceptable, but false positives are not; this is consistent with this study's results in which no false positive−specific fusion was found in contrast to false-negative cases. Because this is a rapid test, it could be integrated into the diagnostic workflow in case of urgent molecular analysis for patients with metastatic NSCLC who require a rapid therapeutic decision, or when a platform for multiplex DNA and RNA analysis is not available. The proposal would be to first screen for frequent hotspot *KRAS* and *EGFR* mutations with a rapid single-gene test and then perform Idylla GeneFusion for negative cases. In the event that Idylla GeneFusion detects specific fusion or *MET* exon 14 skipping, targeted therapy could be prescribed, but DNA/RNA NGS would remain necessary to either confirm expression imbalance alone or to complete a negative result with Idylla GeneFusion. This strategy could provide fast and accurate results (<24 hours' turnaround time) covering main targets with available first-line therapy. The repetition of techniques is not cost effective and needs sufficient material, which is why this strategy should be limited to emergency cases or when NGS analysis is not possible within a reasonable time (<2 weeks) or fails.

In conclusion, this current multicenter comparative study demonstrated that Idylla GeneFusion assay is a rapid method to detect *ALK*, *ROS1*, *RET*, and *NTRK1/2/3* fusions and *MET* exon 14 skipping. As with other Idylla assays, it can be easily implemented in pathology laboratories, because it is a fully automated test, not requiring RNA extraction, and easy to interpret. The absence of alteration or positive result with expression imbalance only on the Idylla GeneFusion test requires additional testing by orthogonal methods.

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Author Contributions

T.D., S.G., and A.M.-L. analyzed the data and performed statistical analysis; L.C.V.K., E.S., S.C., B.B., C.E., S.B., J.S., S.M.-B., V.T., M.-C.D., H.P., C.S., V.T.d.M., E.R., A.B., M.P.-L., R.Y.W.T., K.L.C., M.Ba., C.Q., M.Bi., M.D.-G., D.V., A.R., M.C., M.S., P.J., A.M., and P.D. provided technical and material support and analyzed their samples; T.D., L.C.V.K., E.S., B.T., and A.M.-L. wrote and revised the manuscript; and all authors read, reviewed, and approved the final paper. A.M.-L. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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