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Identification of *ALK*, *ROS1*, and *RET* Fusions by a Multiplexed mRNA-Based Assay in Formalin-Fixed, Paraffin-Embedded Samples from Advanced Non–Small-Cell Lung Cancer Patients

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BACKGROUND: Anaplastic lymphoma receptor tyrosine kinase (*ALK*), ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*), and ret proto-oncogene (*RET*) fusions are present in 5%–7% of patients with advanced non–small-cell lung cancer (NSCLC); their accurate identification is critical to guide targeted therapies. FISH and immunohistochemistry (IHC) are considered the gold standards to determine gene fusions, but they have limitations. The nCounter platform is a potentially useful genomic tool for multiplexed detection of gene fusions, but has not been validated in the clinical setting.

METHODS: Formalin-fixed, paraffin embedded (FFPE) samples from 108 patients with advanced NSCLC were analyzed with an nCounter-based assay and the results compared with FISH, IHC, and reverse transcription PCR (RT-PCR). Data on response to fusion kinase inhibitors was retrospectively collected in a subset of 29 patients.

RESULTS: Of 108 FFPE samples, 98 were successfully analyzed by nCounter (91%), which identified 55 fusion-positive cases (32 *ALK*, 21 *ROS1*, and 2 *RET*). nCounter results were highly concordant with IHC for ALK (98.5%, CI = 91.8–99.7), while 11 discrepancies were found compared with FISH (87.5% concordance, CI = 79.0–92.9). For *ROS1*, nCounter showed similar agreement with IHC and FISH (87.2% and 85.9%), but a substantial number of samples were positive only by 1 or 2 techniques. Of the 25 patients deriving clinical benefit from fusion kinase inhibitors, 24 were positive by nCounter and 22 by FISH.

CONCLUSIONS: nCounter compares favorably with IHC and FISH and can be used for identifying patients with advanced NSCLC positive for *ALK*/*ROS1*/*RET* fusion genes.

In the past decade, dramatic improvements in the outcomes of selected subgroups of patients have been achieved with targeted therapies for the management of non–small-cell lung cancer (NSCLC).[12] Comprehensive molecular profiling of lung adenocarcinoma *(1, 2)* has revealed a number of actionable driver alterations that are potential targets for inhibition in approximately 60% of this subtype of lung cancer *(3)*. Rearrangements in the anaplastic lymphoma receptor tyrosine kinase (*ALK*)[13] gene are the most common fusions identified in NSCLC (approximately 4%–6%) *(4)*. Their identification heightened in-

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[12] Nonstandard abbreviations: NSCLC, non-small cell lung cancer; FISH fluorescence in situ hybridization; IHC, immunohistochemistry; WT, wild-type; FFPE, formalin-fixed, paraffin embedded; RT-PCR, reverse transcription PCR; TKI, tyrosine kinase inhibitor; NGS, next generation sequencing.

[13] Human genes: *ALK,* anaplastic lymphoma receptor tyrosine kinase; *ROS1,* ROS proto-oncogene 1, receptor tyrosine kinase; *RET,* ret proto-oncogene; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *EML4,* echinoderm microtubule associated protein like 4; *EGFR,* epidermal growth factor receptor; *KRAS,* KRAS proto-oncogene, GTPase; *CD74* CD74 molecule; *EZR,* ezrin; *CCDC6,* coiled-coil domain containing 6; *KIF5B,* kinesin family member 5B; *SLC34A2,* solute carrier family 34 member 2 gene; *BRAF,* B-Raf proto-oncogene, serine/threonine kinase; *HER2,* also known as *ERBB2,* erb-b2 receptor tyrosine kinase 2.

terest in the field, leading to the discovery of other less common (up to 1%–2%) but also actionable fusion genes such as those involving ROS protooncogene 1, receptor tyrosine kinase (*ROS1*), and ret protooncogene (*RET*) *(5–8)*. All these oncogenic fusions have in common the expression of a constitutive active kinase protein with transforming potential suitable for targeted inhibition *(9–11)*. Soon after the identification of *ALK* fusions, crizotinib was licensed as the first kinase inhibitor for the treatment of advanced-*ALK*-positive NSCLC, overcoming the results derived from the standard chemotherapy *(12)*.

Actionable fusions are usually detected in clinical samples based on the results of single test techniques such as fluorescence in situ hybridization (FISH) or immunohistochemistry (IHC). Both the Vysis ALK Break Apart FISH Probe Kit and IHC with the VENTANA ALK (D5F3) CDx assay have been cleared by the US Food and Drug Administration as companion diagnostic tests to identify patients who are *ALK* positive and eligible for crizotinib treatment *(12–14)*, but there is still no recommendation regarding the optimal technique for identifying *ROS1* and *RET* fusions genes.

Alternative screening modalities are urgently needed to guide multiple biomarker-driven targeted therapies in patients with advanced NSCLC with rare molecular drivers. These modalities should be more practical, analytically sensitive, and cost-effective, and enable parallel gene fusion detection *(3, 15)*.

The nCounter platform (Nanostring Technologies) is a novel technology that allows multiplexed identification of several aberrant transcripts using a dual approach based on the detection of both an imbalance in the 3′/5′ expression of the wild-type (WT) sequences and a fusion junction target. The assay has the potential to overcome the limitations of other massive screening techniques since it does not require any enzymatic reaction and identifies gene fusions by direct, digital transcript profiling using very small amounts of RNA. Despite its potential, nCounter has only been tested in series of surgically resected tumors, mostly in fresh-frozen samples *(16–20)*, and the results have not been compared with clinical outcomes.

In this study we have retrospectively validated a multiplexed nCounter assay for detection of *ALK, ROS1*, and *RET* fusion genes in a large set of formalin-fixed, paraffin embedded (FFPE) samples from patients with advanced NSCLC. The results obtained were compared with current standard FISH, IHC, and reverse transcription PCR (RT-PCR) techniques and correlated with clinical outcome data.

Material and Methods

PATIENT AND CELL LINE SAMPLES

Samples were obtained from the Quirón Dexeus University Hospital, Hospital Clínic, Barcelona, Spain; Hospital de Bellvitge, Hospitalet de Llobregat, Spain; Hospital Universitario Sanchinarro, Madrid, Spain; and the University Hospital Cologne, Germany, with prior full informed patient consent and approval from the ethical committees of each hospital. The study was conducted in accordance with the Declaration of Helsinki (Fig. 1A).

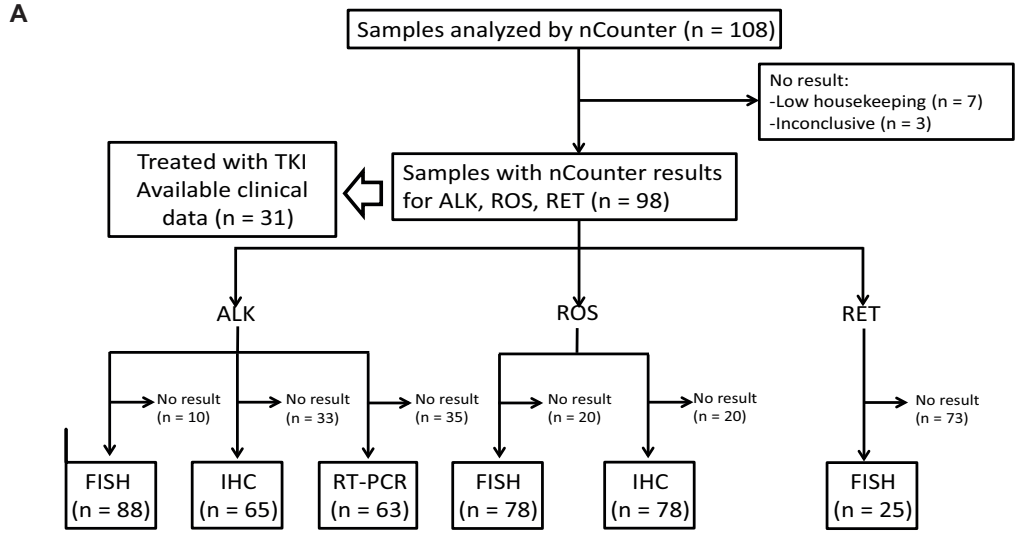
All cell lines (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue3) were purchased from the American Type Culture Collection and cultured in RPMI medium + 10% fetal bovine serum under standard conditions. Cell pellets from a minimum of 5 T-75 flasks were used to obtain FFPE blocks. Cells were counted using a Neubauer Chamber.

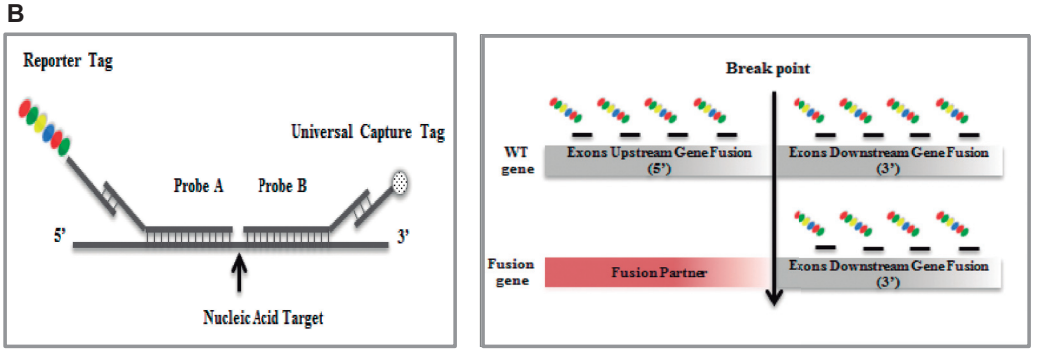
RNA PURIFICATION AND nCounter ELEMENTS ASSAY FOR *ALK*, *ROS1* AND *RET* GENE FUSIONS

FFPE slides (4 μm) were obtained by standard procedures and stained with hematoxylin and eosin. A pathologist determined the tumor areas and evaluated the percentage of tumor infiltration. For nCounter analysis, RNA was extracted with a high purity FFPET RNA isolation kit (Roche Diagnostics) according to the manufacturer's instructions. For RT-PCR, RNA was isolated using a proprietary procedure *(21)*. RNA concentration was estimated using the NanoDrop 2000 (Thermo Scientific). Total RNA was directly hybridized with a custom-designed multiplexed mixture of biotinylated capture tags and fluorescently labeled reporter probes located upstream (Elements Chemistry) complementary to *ALK*, *ROS1*, and *RET* target sequences. The mixture was designed and synthesized by NanoString Technologies Inc. The nCounter codeset used allowed for detection of gene fusions based on a dual strategy. The first strategy is based on 23 pairs of molecular-barcoding junction probes designed to bind to specific fusion transcripts, which enabled detection of a total of 27 rearrangements (8 *ALK*, 11 *ROS1*, and 8 *RET*). In the second strategy, 24 pairs of probes targeting WT *ALK*, *RET*, and *ROS1* (8 pairs each) allowed detection of imbalances between the 3′ and 5′ regions of mRNAs. This second set of probes allows recognition of any fusion, including those not identified with the first method (Fig. 1, B and C; also see online Supplemental Table 2). All processes of hybridization, capture, cleanup, and digital data acquisition were performed with nCounter Prep Station™ and Digital Analyzer™ (NanoString Technologies) according to the manufacturer's instructions. Reporter counts were collected with the nSolver analysis software version 2.6 and normalized as described later using R software version 3.2.2.

DATA ANALYSIS AND THRESHOLD DETERMINATION

Samples with glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) housekeeping gene counts lower than 600 were considered invalid. The geometric mean, arith-





C

	ALK (8 variants)	ROS (11 variants)	RET (8 variants)
Gene Fusion Probes (23 pairs)	EML4-ALK_E13:A20	CD74-ROS1_C6:R32	KIF5B-RET_K16:R12
	EML4-ALK_E20:A20	EZR-ROS1_E10:R34	KIF5B-RET_K22:R12
	EML4-ALK_E6:A20	TPM3-ROS1_T8:R35	KIF5B-RET_K23:R12
	EML4-ALK_E2:A20	LRIG3-ROS1 _L16:R35	KIF5B-RET_K24:R8*
	EML4-ALK_E18:A20	GOPC-ROS1_G4:R36	KIF5B-RET_K24:R11*
	TFG-ALK_T5:A20	GOPC-ROS1_G7:R35	KIF5B-RET_K15:R11*
	KIF5B-ALK_K17:A20	SLC34A2-ROS1_S13del2046:R32	KIF5B-RET_K15:R12*
	KIF5B-ALK_K24:A20*	SLC34A2-ROS_S4:R32*	CCDC6-RET_C1:R12
		SLC34A2-ROS_S4:R34*	
		SDC4-ROS1_S4:R34	
		SDC4-ROS1_S2:R32	
Imbalance Probes (24 pairs)	8 probes	8 probes	8 probes
Housekeeping genes	GAPDH , GUSB , OAZ1 , POLR2A		

Fig. 1. Selection of patients and nCounter assay.

(A), Flow chart of the patient cohort (n = 108). (B), nCounter assay strategies for fusion detection: (left) Junction Sequence detection (right) 3`/5` imbalance expression assay. (C), Target-specific oligonucleotide probes (n = 47) designed for simultaneous detection of aberrant transcripts and housekeeping genes. (*) Common probes for fusion variants [solute carrier family 34 member 2 gene (*SLC34A2*)-*ROS_S4*; *KIF5B-RET_K15*; *KIF5B-RET_K24*].

Table 1. Summary of results of gene fusion analyses in patients evaluable by nCounter (n = 98).

Technique	n = 98		*ALK*		*ROS1*		*RET*	Total positive
nCounter	Positive samples	32		21		2		55
	Only 3`/5`		6 (19%)		4 (19%)		0 (0%)	
	Only specific probes[a]		0 (0%)		4 (19%)		0 (0%)	
	3`/5` and specific probes		26 (81%)		13 (62%)		2 (100%)	
	Negative samples	66		77		96		
FISH	Positive samples	22		27		2		51
	Negative samples	66		51		23		
	No result	10		20		73		
IHC	Positive samples	29		18				47
	Negative samples	36		60				
	No result	33		20		98		

[a] Samples positive for *ALK* specific probes but negative for *ALK* 3`/5` imbalance were considered as not evaluable.

metic mean, SD and biological background thresholds for 3′/5′ imbalance were calculated as previously described *(17, 18)*. We subsequently developed an algorithm to define positivity based on 3′/5′ ratio and fusion-specific counts. We established the threshold value to determine the presence of a 3′/5′ imbalance as the mean plus SD of the normalized counts of the negative samples. For the junction probes, we considered a particular fusion to be present if the raw counts were higher than the mean + 3 SDs + 2 of the negative samples. We considered a sample positive for *ROS1* if the 3′/5′ ratio or 1 fusion probe count were above the preestablished thresholds. For *ALK* and *RET*, a sample was considered positive only if the 3′/5′ ratio was above the threshold.

FISH, IHC, AND RT-PCR

FISH and IHC for all ALK, ROS1, and RET and RT-PCR for *ALK* were performed by standard protocols (see online Supplemental Methods file).

Results

MINIMAL TUMOR AREA AND TUMOR CELL CONTENT

Frozen pellets from a panel of cell line cultures were used to determine the analytical sensitivity and specificity of the assay (see online Supplemental Table 1). As little as 5000 H2228, HCC78, and LC2ad fresh cells counted by Neubauer Chamber were found to be sufficient to detect an *ALK*, *ROS1*, and *RET* fusion transcript. In contrast, no signal was detected using 500 000 cells of nontranslocated cell lines. Next, FFPE blocks derived from cell lines were used to determine the minimal tumor area needed for fusion transcript detection. In the case of the echinoderm microtubule associated protein like 4 (*EML4*)-*ALK*–positive cell line H2228, 1.1 mm^2 were required, while 0.55 mm^2 was enough for detection in the *ROS1*- and *RET*-positive cell lines HCC78 and LC2ad. Finally, we prepared a series of tumor blocks by diluting *ALK*-, *ROS1*-, and *RET*-positive cells into WT cells at increasing percentages. We found that a tumor cell content of 10% was sufficient for successful detection of all fusion transcripts (see online Supplemental Fig. 2). Based on these findings, we selected a tumor area of ≥1.1 mm^2 with ≥10% tumor infiltration when testing clinical samples. Finally quantification of the purified RNAs revealed that 25 ng was sufficient to successfully determine fusion transcripts, although 200 ng was established as the optimum amount.

DETECTION OF FUSION TRANSCRIPTS BY nCounter IN CLINICAL SAMPLES

A total of 108 FFPE tumor samples from advanced NSCLC patients were profiled by nCounter (Fig. 1A). Most of the patients analyzed were epidermal growth factor receptor (*EGFR*) and KRAS proto-oncogene, GTPase (*KRAS*)-WT adenocarcinomas from patients who were never or former smokers presenting with stage IV disease (see online Supplemental Table 4).

According to the algorithm we developed, nCounter raw counts were transformed into a "positive (1)" or "negative (0)" result for each fusion gene. Only 7 samples had counts for the housekeeping genes below those considered acceptable and 3 samples positive for fusion-specific ALK probes but negative for the *ALK* 3′/5′ imbalance were considered "inconclusive" (Fig. 1A). Amongst the final set assessable by nCounter (n = 98), we identified a total of 55 fusion-positive samples: 32 for *ALK*, 21 for *ROS1*, and 2 for *RET* (Table 1). Positivity for *ALK*, *ROS1*, and *RET* was mutually exclusive in our patient population. The normalized counts for the *ALK*, *ROS1*, and *RET* 3′/5′ imbalances and results for fusion-specific probes in our final sample population are shown in Fig. 2 A–C. Six *ALK* and 4 *ROS1* patients were positive only for the 3′/5′ imbalance and consequently no variant

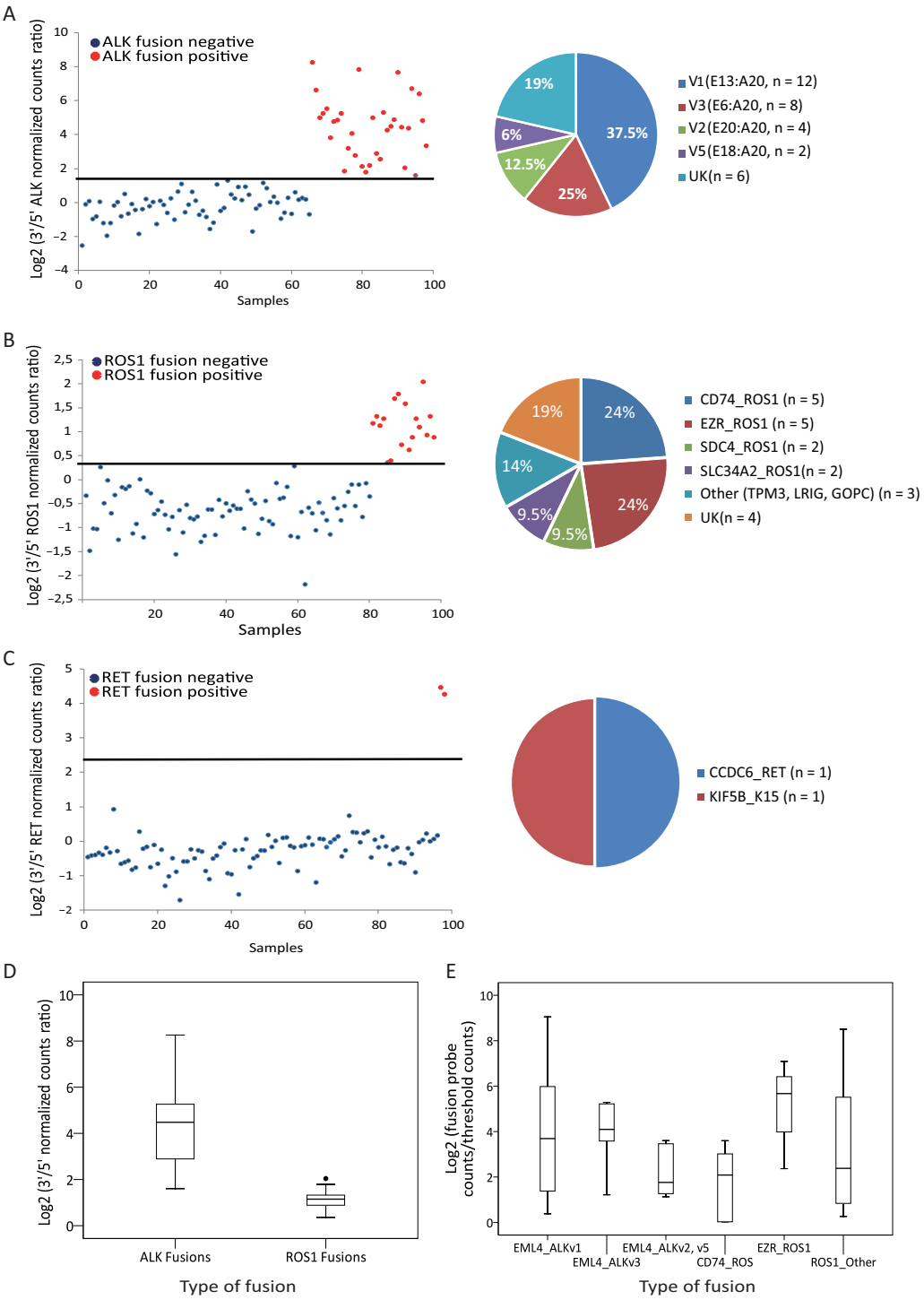


Fig. 2. Results of the 98 FFPE samples for *ALK* (A), *ROS1* (B), and *RET* (C).

Left, 3`/5` normalized counts, expressed as log2 imbalance ratios. The dotted lines indicate the threshold. Right, number of positive samples for each junction specific pair of probes. (D), Box plot of the medians of 3`/5` $\log_2$ ratios in *ALK*- and *ROS1*-positive patients (4.49 vs 1.14, $P < 0.005$). (E), Box plot of counts for the most common variants detected.

Table 2. Concordance of *ALK* IHC, FISH, and RT-PCR and *ROS1* IHC and FISH.

Gene fusion	*ALK*			*ROS1*
Variable	IHC vs FISH	RT-PCR vs FISH	RT-PCR vs IHC	IHC vs FISH
No. concordant samples	49	41	38	59
No discordant samples	8	17	7	15
Sensitivity	100% (CI = 83.9–100)	75.0% (CI = 53.1–88.8)	85.2% (CI = 67.5–94.0)	55.6% (CI = 37.3–72.4)
Specificity	78.4% (CI = 62.8–88.6)	68.4% (CI = 52.5–89.9)	83.3% (CI = 60.8–94.2)	96.1% (CI = 93.6–97.8)
Concordance	86.0% (CI = 74.7–92.7)	70.7% (CI = 58.0–80.8)	84.4% (CI = 71.2–92.3)	79.7% (CI = 69.2–87.3)
Cohen's κ	0.718 (CI = 0.47–0.97)	0.401 (CI = 0.15–0.65)	0.679 (CI = 0.39–0.97)	0.592 (CI = 0.31–0.75)

could be identified (Table 1). The most common *ALK* fusion variants were *EML4-ALK* v1 (37.5%), v3 (25%), and v2 (12.5%) whereas the most frequent partners of *ROS1* fusions were CD74 molecule (*CD74*) and ezrin (*EZR*; 24% each). For *RET*-positive patients, kinesin family member 5B (*KIF5B*) and coiled-coil domain containing 6 (*CCDC6*) were the partners identified. There was a statistically significant difference between the 3′/5′ imbalance medians in the *ALK*- vs *ROS1*-positive patients (22.3 vs 2.2-fold, $P < 0.001$) but not between the normalized counts of the junction probes (Fig. 2 D–E).

IHC, FISH, AND RT-PCR RESULTS FOR *ALK* AND *ROS1* IN CLINICAL SAMPLES

In the case of *ALK*, the concordance of IHC vs FISH was 86.0% (49/57), with 8 cases negative by *ALK*-FISH but positive by IHC. With RT-PCR, there was a good agreement with IHC (84.4% concordance) but only fair (70.7%) with FISH, with 12 of 27 RT-PCR–positive samples negative by FISH (Table 2 and online Supplemental Table 5).

Among *ROS1* FISH-positive samples, 18% showed moderate (2+) and 41% absent to weak/faint IHC staining (0–1+; also see online Supplemental Table 6). In *ROS1* FISH-negative samples, 26% had moderate and 6% intense membrane staining. Based on these results, we scored as ROS1 IHC-positive the 3+ samples and those with 2+ staining in ≥50% of tumor cells. This new cutoff improved agreement between the techniques, with an accuracy of 79.7% (CI= 69.2–87.3) and a Cohen's κ of 0,59 (CI = 0.31–0.75) *(22)* (Table 2 and online Supplemental Table 6).

COMPARISON OF *ALK* nCounter RESULTS WITH FISH, IHC, AND RT-PCR IN CLINICAL SAMPLES

The concordance of *ALK* and *ROS1* nCounter with FISH, IHC, and RT-PCR and the corresponding sensitivity, specificity, and Cohen κ values are shown in Table 3 and online Supplemental Table 7.

In the case of *ALK*, we observed excellent agreement when comparing nCounter vs IHC (98.5%, CI = 91.8–99.7). The only discordant case was positive for nCounter and negative for IHC as well as FISH and RT-PCR. The concordance of *ALK* nCounter with FISH was substantial (87%, CI = 79.0–92.9), but there were 11 discordant cases. Ten *ALK* FISH-negative samples were positive by nCounter. Of those, 9 were also positive by IHC and 1 could not be immunostained due

Table 3. Concordance of nCounter (nC) with IHC, FISH for *ALK* and *ROS1*.

Gene	ALK			ROS1	
Variable	nC vs FISH	nC vs IHC	nC vs RT-PCR	nC vs FISH	nC vs IHC
No. concordant samples	77	64	54	68	67
No. discordant samples	11	1	9	10	11
Diagnostic sensitivity	95.5% (CI = 78.2–99.2)	100% (CI = 88.3–100)	85.7% (CI = 68.5–94.3)	70.4% (CI = 51.5–84.2)	77.8% (CI = 54.8–91.0)
Diagnostic specificity	84.9% (CI = 74.3–91.6)	97.2% (CI = 85.8–99.5)	85.7% (CI = 70.6–93.7)	96.1% (CI = 86.8–98.9)	88.3% (CI = 77.8–94.2)
Concordance	87.5% (CI = 79.0–92.9)	98.5% (CI = 91.8–99.7)	85.7% (CI = 75.0–92.3)	87.2% (CI = 78.0–92.9)	85.9% (CI = 76.5–91.9)
Cohen's κ	0.707 (CI = 0.5–0.91)	0.969 (CI = 0.73–1.21)	0.712 (CI = 0.46–0.96)	0.701 (CI = 0.48–0.92)	0.625 (CI = 0.40–0.85)

to insufficient material remaining. The only sample positive for FISH and negative by nCounter was also negative by RT-PCR and again could not be evaluated by IHC due to lack of material (Table 3 and online Supplemental Table 7).

Finally, we also found a substantial agreement (85.7%, CI = 75.0–92.3) between *ALK* nCounter and RT-PCR, with 9 discordant cases. The 4 patients positive for RT-PCR but negative for nCounter were also negative for IHC and FISH. Finally, 4 of the 5 tumor samples negative for RT-PCR and positive by nCounter were also positive for IHC and FISH. Three of them showed exclusively a 3′/5′ imbalance by nCounter, suggesting that they harbored *ALK* fusion transcripts not detectable either by our nCounter specific set or our RT-PCR test (v1-v3).

COMPARISON OF *ROS1* nCounter RESULTS WITH FISH AND IHC IN CLINICAL SAMPLES

In line with the results observed when comparing *ROS1* IHC and FISH, a high percentage of nCounter-positive samples showed moderate (24%) or absent-weak/faint (28%) ROS IHC staining and 21% of nCounter-negative samples had moderate staining (see online Supplemental Table 6). By comparing nCounter and IHC based on the cutoff for *ROS1* positivity mentioned above (3+ or 2+ in at least 50% of tumor cells), we obtained an accuracy of 85.9% (CI= 76.5–91.9) with 11 discordant cases (Table 3). Two of the 4 cases negative by nCounter and positive by IHC were confirmed as negative by FISH. Likewise, 6 of the 7 nCounter-positive samples scored as negative by IHC were also positive by FISH.

The comparison of *ROS1* nCounter vs FISH revealed an agreement of 87.2% (CI 78.0–92.9; Table 3 and online Supplemental Table 7). Among the 10 discrepant cases, 2 were nCounter positive and FISH negative; 1 was also negative (1+) by IHC. Six out of 8 samples scored negative by nCounter and positive by FISH were also negative by IHC, with 5 failing to show any staining.

FISH, IHC, AND nCounter RESULTS AND CLINICAL OUTCOME TO TYROSINE KINASE INHIBITORS

We retrospectively collected clinical information on 29 patients included in our cohort who were treated with tyrosine kinase inhibitors (TKIs) targeting fusions, based on the results of standard techniques (FISH, IHC). Response data was collected and clinical benefit was defined as partial response or stable disease for at least 6 months.

Twenty patients identified as *ALK* positive were treated with TKIs and 18 derived clinical benefit. All of them were nCounter positive while 3 were negative or not evaluable by FISH (see online Supplemental Fig. 3). One of the patients identified as nCounter positive and FISH negative showed a remarkable response to *ALK* inhibition that lasted more than 3 years (Fig. 3). Two patients identified as *ALK* positive did not respond to therapy; 1 of them was negative by FISH but positive by nCounter. This patient also showed ALK staining by IHC.

Nine patients identified as *ROS1* positive based on FISH or IHC and treated with crizotinib were also evaluated. The 7 patients who attained clinical benefit were FISH positive and 6 were also positive by nCounter. Reexamination of the remaining sample revealed a very low (5%) tumor infiltration. The 2 patients who did not derive clinical benefit from crizotinib were positive by FISH, while 1 of them was negative by nCounter (see online Supplemental Fig. 3).

Discussion

Gene fusions involving *ALK* protein kinase and *EGFR* mutations are the more accurately validated predictive biomarkers of response to first-line targeted treatment in advanced nonsquamous NSCLC *(13, 23)*. Fusion genes involving *ROS1* and *RET* have shown great promise for targeted inhibition but are not widely tested for since clinical trials are still in progress and the available drugs are not licensed by all relevant agencies *(24–26)*. As patients with these actionable alterations have a number of clinical characteristics (never smokers, younger age) and pathological patterns in common *(27)*, there are no specific features that can be relied upon for preferential testing of particular genes. In consequence, samples are usually screened for the most common alterations (*EGFR, KRAS, ALK*), whereas *ROS1* and *RET* are only analyzed in triple-negative cases. However, this sequential strategy has many drawbacks in the management of advanced NSCLC patients as samples are frequently obtained by nonsurgical, minimally invasive procedures providing insufficient material to complete this sequential approach.

In this study, we have developed an nCounter assay that allows simultaneous evaluation of *ALK, ROS1*, and *RET* fusion genes and it has been validated in a large cohort of 108 archival FFPE tumor specimens from advanced NSCLC patients.

We found that areas of $\geq$1.1 mm^2 with $\geq$10% tumor content obtained from 4 μm FFPE slides were sufficient for successful detection of fusion transcripts, results in line with previously reported with snap-frozen and FFPE samples from cell lines and surgically resected tumors *(18, 20)*. There was only 6% screening failure due to low housekeeping counts (n = 7), although the amount of RNA analyzed was in some cases as low as 25 ng. We did not verify the quality of the RNA extracted, but the poorer quality of RNA extracted from archival FFPE tumor blocks as compared to fresh samples is well known *(28)*. The nCounter technology uses 50-mer oligonucleotide probes and, in contrast with other next gen-

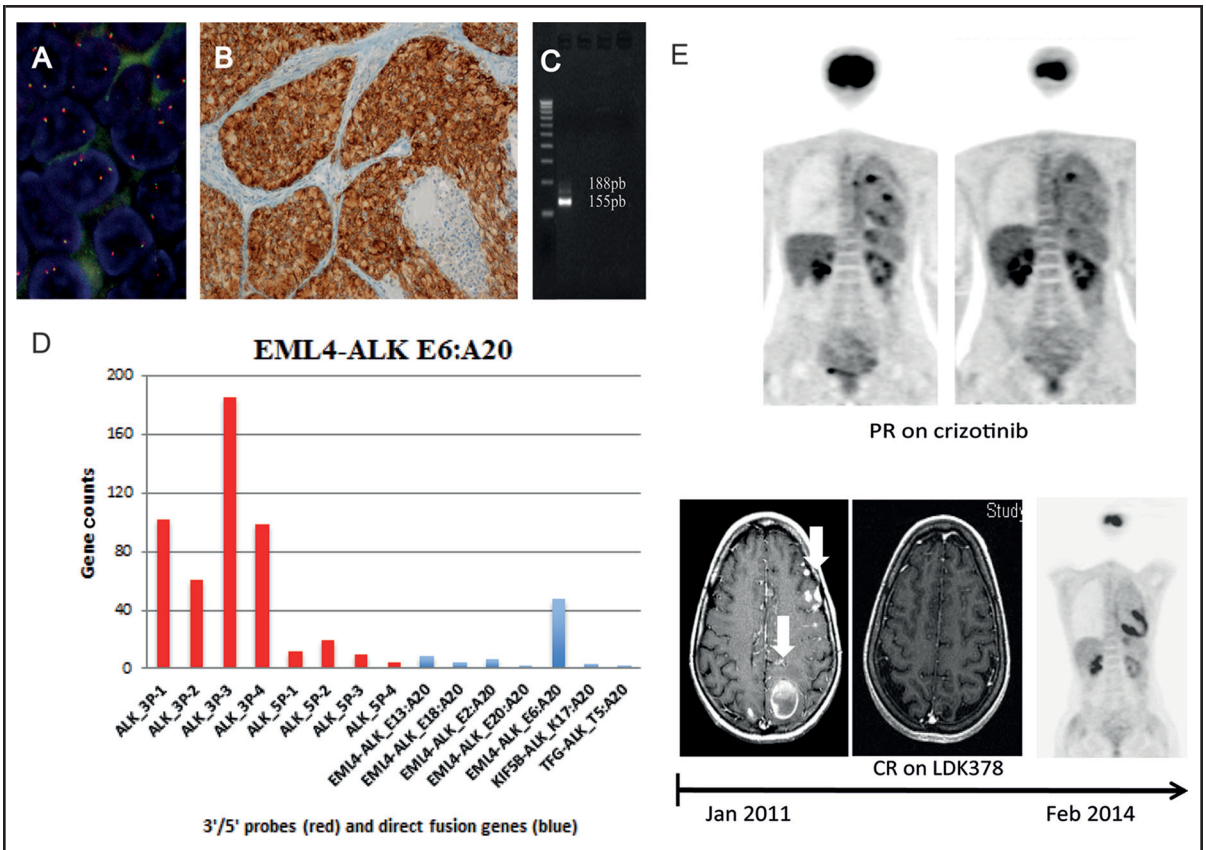


Fig. 3. Case report of a 29-year-old woman, never smoker, diagnosed with stage IV lung adenocarcinoma (T3 N2 M1a pleural effusion), *EGFR*, B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), *HER2* (also known as *ERBB2*, erb-b2 receptor tyrosine kinase 2) and *KRAS* WT.

(A), FISH showing no split fluorescence patterns. (B), ALK IHC positive (C), RT-PCR positive for *ALK* variant 3 (E6:A20). (D), nCounter results. (E), Response to ALK inhibitors.

eration sequencing (NGS) platforms, is based on direct hybridization without cDNA synthesis or PCR amplification. Our results demonstrate that nCounter does not require the high quality samples necessary for other NGS techniques and can be successfully used in FFPE-extracted RNA at low concentrations. At this respect, as cytological samples are the only source of material in a significant number of patients, we are planning to validate nCounter in this setting.

Our cohort was enriched with patients positive for ALK and ROS by FISH or IHC as well as with *EGFR-KRAS*–negative samples. By using the 2 nCounter strategies, we successfully identified 55 fusion-positive samples: 32, 17, and 2 for *ALK, ROS1*, and *RET* respectively. In agreement with previous reports *(29, 30)*, the most common *ALK* variants were *EML4-ALK* v1 (37.5%), v3 (25%), and v2 (12.5%). Regarding *ROS1*, among 11 positive patients the most frequent fusion partners were *CD74* and *EZR* (n = 5 each). These results are in good agreement with those obtained in early stage NSCLC samples *(18, 20)*. Regarding *RET*, both *KIF5B* and *CCDC6* have been cited as the most frequent partners in surgically resected and advanced NSCLC *(18, 20, 31)*. In our enriched cohort, we were able to identify 2 RET-positive cases, 1 of each. The identification of specific fusion partners and variants is a clear advantage of nCounter over FISH and IHC and there is increasing evidence that fusion partners can have an influence on outcome to targeted therapy *(29, 31)*.

In our cohort of FFPE samples from advanced NSCLC, nCounter showed an excellent agreement with ALK IHC (Cohen's κ 0.97) and substantial concordance with *ALK* FISH (Cohen's κ 0.70). These results differ from the absolute agreement and complete absence of discordant cases of nCounter vs *ALK* FISH-IHC previously described in surgical samples *(18)*. However, they are in line with the widely reported discrepancies described between ALK IHC and *ALK* FISH *(32, 33)*,

with several studies finding a higher number of patients positive by IHC *(34)*. This observation can be explained by several factors. For instance, FISH positivity criteria are evaluated by pathologist and can have some degree of subjectivity. In contrast, nCounter offers a quantitative results and positivity is determined by a mathematical algorithm. Regarding IHC, there is no expression of ALK protein in fusion-negative tumors and evaluation of the staining is usually straightforward. It is noteworthy that in our study, nCounter allowed for the identification of 10 positive cases that were scored as negative by FISH. Previous reports using new molecular platforms with NGS techniques have also reported higher sensitivity rates than FISH in detecting clinically relevant ALK rearrangements *(35)*. This observation, along with the significant number of responses observed in patients with IHC-positive/FISH-negative results *(32, 34, 36)*, illustrate the clinical relevance of identifying *ALK* gene expression instead of the chromosomic alteration, which is one of the advantages of the transcript-based nCounter technology.

A complex picture emerged in the case of *ROS1*, with 86%–87% concordance between nCounter vs IHC and FISH and a significant number of samples positive for only 1 or 2 techniques. In the only study published so far comparing ROS1 FISH, IHC, and mRNA-based techniques *(37)*, of the 20 samples positive for *ROS1* RT-PCR, 7 (35%) were FISH-negative and 4 (25%) IHC-negative. In our study, of 21 patients *ROS* positive by nCounter, 2 (10%) were negative by FISH and 7 (33%) by IHC. Taken together, these data do not support the use of IHC as the standard technique to determine *ROS1* fusions. In concordance with the IHC results, we observed frequent expression of endogenous WT *ROS1* mRNA in negative tumors, with 808 (177) raw counts [mean (SE)] of the 3′ and 5′ *ROS1* probes. In contrast WT *ALK* and *RET* mRNA levels were very low or undetectable in negative tumors [13 (2) and 52 (12) counts, respectively]. The absence of WT ALK expression in negative samples can explain the high concordance of nCounter and IHC in *ALK* fusions testing.

Of the 25 patients deriving clinical benefit to targeted therapies (partial response or stable disease for more than 6 months), 24 were positive by nCounter and 22 by FISH. In the group of patients benefitting from ALK fusion inhibitors (n = 18), all were nCounter positive while 3 were negative or not evaluable by FISH. Complete clinical follow-up was available for 1 FISH-negative and nCounter-positive patient, who had a remarkable response to crizotinib lasting more than 3 years. Again, these results are in line with several reports showing responses to TKIs in ALK FISH-negative/IHC-positive patients *(32, 34, 36)* questioning FISH as the optimal technique for identifying ALK-positive lung cancer patients. One limitation of our work is that clinical data was retrospectively collected from a minor subset of patients (n = 29) and tumor assessment was not centrally analyzed. Therefore, we cannot comment on the techniques' performance to predict response to fusion gene inhibition in the remaining group.

In summary, we have validated a multiplexed, nCounter assay to detect *ALK*, *ROS1*, and *RET* fusion transcripts. The assay requires minimal amounts of FFPE-derived RNA, has high sensitivity, is time-efficient and has proved to be more practical than current standard diagnostic assays to determine gene fusions.

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