



A Retrospective, Multicenter Analysis Within the National Network Genomic Medicine Lung Cancer in Germany to Detect RET Fusions as a Possible Mechanism of Resistance in Patients With EGFR Mutations

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Abbreviations: BRAF, B-Rapidly Accelerated Fibrosarcoma; CCDC6, Coiled-Coil Domain Containing 6; Cx, Chemotherapy; ddPCR, droplet digital Polymerase Chain Reaction; DEL19, Deletion in Exon 19 (EGFR mutation); EGFR, Epidermal Growth Factor Receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; FGFR3, Fibroblast Growth Factor Receptor 3; HER2, Human Epidermal Growth Factor Receptor 2; IASLC, International Association for the Study of Lung Cancer; ICI, Immune-Checkpoint Inhibition; KIF5B, Kinesin Family Member 5B; L858R, Leucine 858 Arginine (EGFR mutation); MET, Mesenchymal-Epithelial Transition factor; mPFS, median Progression-Free Survival; nNGM, National Network Genomic Medicine lung cancer (Germany); NCOA4, Nuclear Receptor Coactivator 4; NGS, Next Generation Sequencing; NTRK1, Neurotrophic Receptor Tyrosine Kinase 1; NSCLC, Non-Small Cell Lung Cancer; OS, Overall Survival; PFS, Progression-Free Survival; PI3K, Phosphoinositide 3-Kinase; RET, Rearranged during Transfection; SD, Standard Deviation; T790M, Threonine 790 Methionine (EGFR resistance mutation); yrs, Years.

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Abstract

Resistance to third-generation EGFR-TKIs in EGFR-mutated NSCLC may arise through oncogenic fusions, particularly RET. In this multicenter cohort from the German Network Genomic Medicine, clinical features, treatments, and outcomes of RET-positive EGFR-mutant patients were evaluated. Dual EGFR/RET inhibition demonstrated clinical activity, and personalized ddPCR enabled sensitive longitudinal plasma monitoring in selected cases.

Background: Resistance to third-generation EGFR tyrosine kinase inhibitors (TKIs) such as osimertinib remains a major challenge in the treatment of *EGFR*-mutated non-small cell lung cancer (NSCLC). While on-target and bypass mechanisms such as *MET* amplification are well-characterized, oncogenic fusions—particularly *RET* fusions—are emerging as relevant resistance mechanisms in a subset of patients. The feasibility of dual inhibition strategies and personalized monitoring through liquid biopsy remains underexplored in real-world clinical practice. **Materials and Methods:** This retrospective, multicenter study within the German national Network Genomic Medicine (nNGM) Lung Cancer identified patients with advanced *EGFR*-mutated NSCLC and co-occurring *RET* fusions between 2018 and 2024. Clinicopathological data, treatment history, progression-free survival (PFS), and overall survival (OS) were analyzed. *RET* alterations were either present at diagnosis or acquired under EGFR-TKI therapy. In 2 patients, a personalized droplet digital PCR (ddPCR) assay was developed to monitor *EGFR* and *RET* alterations longitudinally using liquid biopsy. **Results:** Nine patients met the inclusion criteria (median age: 54 years). *RET* fusions were detected at diagnosis in 2 patients and acquired in 7 patients during EGFR-TKI treatment, with a median time to detection of 11.4 months. *RET* fusion partners included *CCDC6* ($n = 4$), *KIF5B* ($n = 2$), *NCOA4* ($n = 2$), and one case with an unknown partner. The most common EGFR mutation was exon 19 deletion ($n = 6$), followed by L858R mutation ($n = 1$), with 2 patients harboring exon 20 insertions. First-line treatment consisted of *RET* inhibition with pralsetinib in the 2 patients with atypical EGFR mutations, third-generation EGFR-TKIs in 6 patients (median PFS: 13.5 ± 9.2 months), and a second-generation EGFR-TKI (afatinib) in one patient (PFS: 8.7 months). Following progression, all patients underwent re-biopsy, confirming persistence of the *EGFR* mutation and presence of *RET* fusions. Second-line therapies varied, including chemo-immunotherapy ($n = 4$, mPFS: 6.5 ± 2.5 months), chemotherapy ($n = 1$, PFS: 0.3 months), and best supportive care ($n = 1$). Three patients received the combined EGFR-TKI osimertinib and *RET*-TKI pralsetinib either in second- or third-line, with PFS ranging from 3.9 to 10.5 months. Median OS for the cohort was 27 months (range: 7 to >30 months), with 4 patients still alive at last follow-up. In 2 patients, a personalized ddPCR assay enabled non-invasive, longitudinal monitoring of *EGFR* and *RET* alterations, closely reflecting the clinical course of disease. In 1 patient, molecular recurrence in liquid biopsy preceded clinical and radiologic progression, underscoring the potential of this approach for early detection of therapeutic resistance. **Conclusion:** *RET* fusions represent a rare mechanism of acquired resistance in *EGFR*-mutant NSCLC. Combined *RET* and EGFR inhibition may offer clinical benefit, particularly in patients with co-occurring alterations. Personalized ddPCR-based liquid biopsy is a promising tool for real-time, non-invasive monitoring of treatment response and resistance evolution.

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Keywords: NSCLC, *RET*-mediated EGFR resistance, Combined TKI-therapy, Personalized medicine, Digital droplet PCR

Background

NSCLC is the leading cause of cancer-related mortality worldwide with adenocarcinoma representing the largest part of the histologic subtypes¹ Molecular alterations occur most frequently in adenocarcinoma histology, with *EGFR* mutation occurring in approximately 12% to 15%^{2,3} of patients with metastatic disease in Europe and the United States. The most common EGFR mutations are exon 19 deletions and the exon 21 p.L858R substitution.^{1,3} These genetic alterations act as oncogenic drivers, leading to ligand-independent activation of EGFR downstream signaling, thereby promoting cell proliferation, survival, and migration.

Third-generation EGFR-TKIs, particularly osimertinib, are widely established as first-line therapy in *EGFR*-mutant NSCLC, demonstrating durable clinical benefit with an overall response rate (ORR) of approximately 80%, a median PFS of 18.9 months, and

a median OS of 38.6 months. Recent combination strategies—such as osimertinib plus chemotherapy or lazertinib with amivantamab—have shown further improvements in efficacy. In the FLAURA-2 trial, osimertinib plus chemotherapy achieved a median PFS of 25.5 months⁴ with a median OS of 47.5 months,⁵ while the MARIPOSA trial reported a PFS of 23.7 months and an OS of more than 42.97 months (HR 0.75) for amivantamab plus lazertinib.^{6,7}

However, despite the robust clinical activity of osimertinib or the newer combinations, resistance inevitably occurs—thus limiting a permanent clinical benefit achieved with this compound. On-target mechanisms involve *EGFR* tertiary mutations (such as p.C797S), while off-target alterations include *MET/HER2* amplifications, PI3K pathway activation, cell-cycle gene mutations, and histologic transformation.^{8,9} A less common but emerging resistance mechanism involves oncogenic gene fusions, identified in

3% to 10% of cases after second-line osimertinib.^{10–12} These include fusions involving *FGFR3*, *NTRK1*, *BRAF*, and notably *RET*, which is targetable and occurs in ~1% to 2% of NSCLC cases.^{13,14} Although infrequent, *RET* fusions have become a recognized mechanism of resistance following treatment with osimertinib. These fusion events result in constitutive activation of *RET* tyrosine kinase through fusion with upstream partner genes that typically harbor strong promoters and dimerization domains, thereby driving ligand-independent *RET* signaling. Among the various *RET* fusion partners identified in NSCLC, *KIF5B-RET* is the most common in newly diagnosed, treatment-naïve cases, representing around 70% to 80% of all *RET* fusion-positive tumors. In contrast, *CCDC6-RET* fusions occur less frequently—approximately 10% to 20%—and are more often detected in patients who develop resistance after targeted therapies. A smaller proportion of cases, roughly 5% to 10%, involve the *NCOA4-RET* fusion.^{15–17} Targeted therapies for patients with *RET* fusions have become available in recent years resulting in regulatory approval of *RET*-specific TKIs such as pralsetinib¹⁸ or selpercatinib.^{19,20} Recently, pralsetinib and selpercatinib were shown to be able to overcome *RET*-mediated resistance in *EGFR*-mutated patients. Smaller cohorts and case reports have demonstrated encouraging clinical responses and a manageable safety profile.^{10,21–25} The authors report a PFS ranging from 7.9 to 14 months, with some responses still ongoing, reinforcing the rationale for combined targeting of both pathways as a strategy to overcome resistance and improve treatment outcomes in this molecularly defined patient population.

Currently, the gold standard for managing disease progression in patients with *EGFR*-mutant NSCLC involves performing a repeat tissue biopsy followed by histopathological evaluation. However, this approach is often limited by the inaccessibility of tumor lesions or the patient's clinical status. In this context, liquid biopsy has emerged as a valuable noninvasive alternative, with recent advancements prompting organizations such as the International Association for the Study of Lung Cancer (IASLC) to advocate for its incorporation into routine clinical practice²⁶ For the detection of resistance mutations such as *EGFR* T790M, for example, liquid biopsy has been effectively employed using droplet digital PCR (ddPCR). DdPCR is a highly sensitive, quantitative PCR technology that enables absolute nucleic acid quantification and can detect rare variants with a variant allele frequency (VAF) as low as 0.01%, depending on the assay design²⁷ In this context, the development of personalized, tumor-informed assays targeting *EGFR* sequence variations and *RET* fusions in individual patients appears feasible. The application of liquid biopsy, as a less invasive and more practical approach, enables more accurate and timely monitoring of the emergence and potential clinical relevance of resistance mechanisms. The aim of this study is to retrospectively evaluate the occurrence of *RET* fusion as a possible resistance mechanism for patients with an *EGFR* alteration under TKI therapy within the nNGM and analyze the response to the selected therapies in a real-world population.

Moreover, this study sought to evaluate the feasibility of implementing a personalized molecular assay and to highlight its potential clinical utility for real-time therapeutic monitoring in individual patients.

Materials and Methods

Scope and Patients

We conducted a retrospective multicenter study within the nNGM lung cancer in Germany. The aim of the multisectoral nNGM is to improve diagnostics and treatment for lung cancer patients, conducting quality-assured Next-Generation Sequencing (NGS) panel diagnostics, therapy recommendations, and clinical trials in >20,000 newly diagnosed NSCLC patients with advanced disease per year.²⁸ Eight centers (Berlin, Dresden, Düsseldorf, Essen, Heidelberg, Loewenstein, Mannheim, Ulm) were able to contribute patients. Inclusion criteria included patients with locally advanced or metastatic NSCLC and present *EGFR* mutations under systemic therapy and concurrent *RET* alteration. This *RET* alteration could either be present at the time of initial diagnosis or emerge as an acquired resistance mechanism during therapy.

Clinicopathological and outcome data were retrospectively collected, including patient characteristics, co-occurring gene mutations, type of systemic treatment (targeted therapy, chemo(immune)therapy, or a combination), PFS, and OS. Treatment response was assessed locally. If upon tumor progression a rebiopsy was performed, data was included as well as available data from liquid biopsy. Sample preparation and sequencing were carried out locally according to the guidelines within the nNGM Network (see Appendix).

The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). It received approval by the ethics committee II of the faculty of Mannheim (no. 2023-838). Informed consent was obtained when required by law.

Sample Collection and Processing

In addition to tumor tissue, serial peripheral blood samples were available from 2 patients for the detection of *EGFR* and *RET* alterations. Serum analyses beyond routine diagnostics included *EGFR* testing using a validated commercial assay and *RET* detection using patient-specific ddPCR assays that were specifically developed for the respective *CCDC6-RET* fusions (see details provided later in the text).

Cell-free DNA was extracted from plasma samples obtained from peripheral blood. The isolated DNA was then either used for analysis or stored for later use. Full protocol details are provided in the Appendix.

ddPCR Assays

For detection of *EGFR* exon 19 deletion, a ddPCR was performed using the ddPCR *EGFR* exon 19 Deletions Screening Kit (Bio-Rad) according to the manufacturer's instructions.²⁹ Tumor tissue and serial peripheral blood samples from 2 patients were analyzed. Oncogenic gene fusions were identified in tumor tissue at the RNA level and confirmed using the Integrative Genomics Viewer to define the fusion junctions. Analysis of the corresponding intronic regions allowed localization of the genomic breakpoints, consistent with balanced translocations. Based on the patient-specific fusion junctions, individualized PCR assays were developed and applied for sensitive detection of the fusions in peripheral blood.

A Retrospective, Multicenter Analysis

Table 1 Patients' Characteristics^a

	All Patients (n = 9) n [%]	
Median Age	[SD, y]	54 ± 14
Sex	Female	7 [77.8]
	Male	2 [22.2]
Smoking Status	Current smoker	1 [11.1]
	Ex-smoker	4 [44.4]
	Never-smoker	4 [44.4]
Clinical Stage	IVA	2 [22.2]
	IVB	7 [77.7]
Histology	Nonsquamous	9 [100]
Occurrence of RET fusion	At first diagnosis	2 [22.2]
	after TKI therapy	7 [77.8]

^a The participating centers reported a total of 23,304 molecular tests performed during the observation period. Based on the reported prevalence of EGFR alterations (15%) in Germany, this corresponds to an estimated 3496 patients with EGFR mutations. Among the 9 reported patients with combined alterations, 2 patients harbored a classical RET fusion with an exon 20 insertion at the time of initial diagnosis, whereas 7 patients developed RET alterations in the context of classical EGFR-mutated disease under targeted therapy. The estimated frequency of the occurrence of RET fusions as a mechanism of resistance is thus 0.2%.

The assay was validated for performance, and detailed validation results are summarized in Table 3 (Appendix).

Statistical Analysis

PFS was calculated from the day of initiation of the respective systemic therapy until disease progression or death. OS was calculated from the day of the first diagnosis to death. We analyzed PFS and OS as main outcome parameters because ORRs were only available for 2 patients. Statistical analyses were carried out using SPSS version 29.0.2.0(20) (IBM Corp., New York, NY).

Results

Between 2018 and 2024, 9 patients with *EGFR* and concurrent *RET* fusion could be identified with a median age of 54 ± 14 years. Seven (77.8%) were female, and 2 were male (22.2%). Four (44.4%) were never-smokers and ex-smokers, respectively, and 1 (11.1%) patient still smoked. Two patients (25%) had stage IVA disease, while 6 patients (75%) had stage IVB disease. The most frequent metastatic sites were bone (62.5%), brain (25%), liver (25%), and, less commonly, the adrenal glands and lung (12.5% each) (Table 1).

Histology: EGFR Mutation, RET Fusion, and Co-mutations

All patients were diagnosed with adenocarcinoma histology. Six patients (66.6%) harbored an *EGFR* exon 19 deletion (DEL19), and 1 patient (11.1%) had the p.L858R point mutation. Another 2 patients (22.2%) exhibited atypical *EGFR* mutations, namely exon 20 insertion (p.P772_H773dup and c.2367_2378dup, p.Leu792_Met793insIleThrGlnLeu). In the 2 patients with atypical *EGFR* mutations, the *RET* fusion was identified at the time of initial diagnosis, whereas in the remaining patients, the *RET* fusion emerged during EGFR-TKI therapy, with a median time to detection of 11.4 months.

RET fusion partners included *CCDC6* in 4 patients (44.4%), *KIF5B* and *NCOA4* in 2 patients each (22.2%), and in 1 patient

(12.5%) the fusion partner could not be determined (Figure 1). Co-occurring *TP53* mutations were detected in 5 patients (55.6%), and 1 patient (11.1%) harbored an additional *FGFR3* mutation.

Therapies and Clinical Outcome

For the 2 patients with atypical *EGFR* mutation and concomitant *RET* fusion (*KIF5B*) at initial diagnosis, pralsetinib was chosen as first-line treatment with a PFS of 2.76 and 14.95 months (ongoing), respectively. Among the remaining 7 patients harboring common *EGFR* mutations, 1 received the second-generation TKI afatinib and 6 received a third-generation EGFR-TKI as initial treatment—5 with osimertinib and 1 with either osimertinib or lazertinib within a clinical trial setting.

PFS in this subgroup ranged from 4.5 to 32.9 months, with a mPFS of 13.5 months (±9.8 months). The patient being treated with the second-generation TKI afatinib achieved a PFS of 8.71 months. Following disease progression, all patients underwent rebiopsy. In each case, an acquired *RET* fusion was detected, while the original *EGFR* mutation still remained detectable. Second-line treatments were heterogeneous and included chemotherapy (n = 4, mPFS 6.5 ± 2.5 months), chemotherapy alone (n = 1, PFS 0.3 months), and best supportive care (n = 1, PFS 3.0 months). One patient received osimertinib and pralsetinib with a PFS of 3.94 months, achieving a partial remission. In the further course of treatment, another 2 patients received the combination of dual-targeted therapy with osimertinib and pralsetinib as third-line therapy, leading to a PFS of 7.8 and 13.0 months (ongoing). Median overall survival in the nNGM cohort was 27 months, ranging from 7 to more than 30 months, with 4 patients being still alive, among them the 2 patients with *RET* fusion and atypical *EGFR* mutation (Table 2).

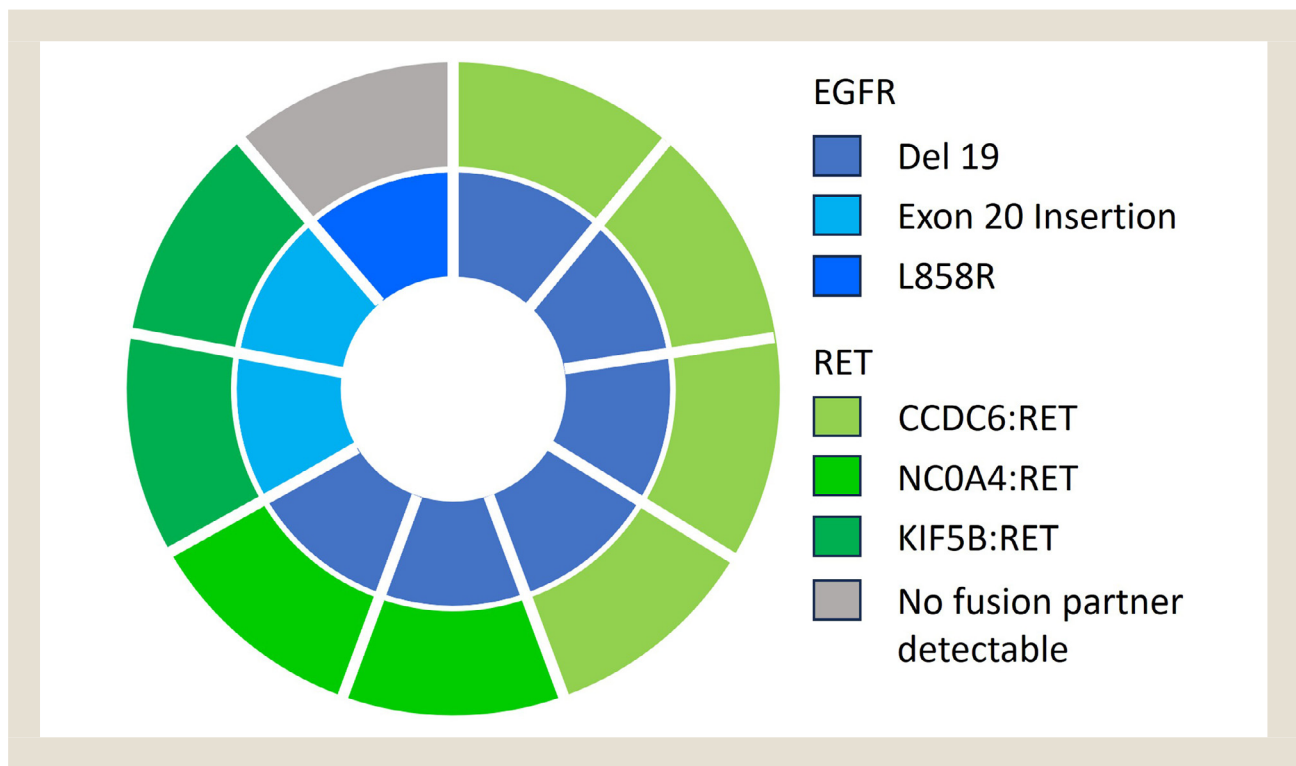
Personalized Liquid Biopsy Assay for Therapeutic Monitoring

For 2 patients, samples were available for the establishment of a personalized, tumor-informed assay. Figure 2 shows representative ddPCR results for an *EGFR* exon 19 deletion and a *RET-CCDC6* fusion, both detected via liquid biopsy.

In both patients for whom samples were available to develop an individualized droplet digital PCR (ddPCR) assay, the method enabled reliable detection of *EGFR* and *RET* alterations, demonstrating strong concordance with the clinical and radiological disease course.

Clinical Course Patient #8

Osimertinib was initiated as first-line therapy in 2020. After 14 months, oligoprogression in the right lower lobe was detected. Rebiopsy revealed a *CCDC6* *RET* fusion, confirmed both histologically and by ddPCR. The patient underwent local radiotherapy, followed by continuation of osimertinib. Subsequently, increasing consolidation in the right lower lobe raised the differential diagnosis of tumor progression versus drug-induced pneumonitis. Based on the patient's clinical presentation and a negative liquid biopsy at that time, pneumonitis was favored, and corticosteroid therapy was initiated, resulting in rapid regression of the lesions and thereby confirming the diagnosis.

Figure 1 Distribution of EGFR mutations and RET fusions $n = 9$ patients.

After 24 months on osimertinib, the patient developed new brain metastases, which were treated with local radiotherapy. Shortly beforehand, a recurrent ddPCR signal had been detected, which subsequently reverted to negative. At 32.9 months, systemic progression was observed, again preceded by re-emergence of a positive ddPCR signal, and chemoimmunotherapy was initiated. After 6.6 months, further progression occurred, and combined treatment with osimertinib and seliprecatinib was commenced, which led to clearance of the previously positive ddPCR signal (Figure 3) and is still ongoing.

Clinical Course Patient #9

Osimertinib was initiated as first-line therapy in 2022. After 16 months, disease progression occurred, and rebiopsy revealed a CCDC6 RET fusion. Chemotherapy was administered as second-line treatment, but progression with new brain metastases was observed after 9 months. At that time, both EGFR and RET alterations were detected by ddPCR. Combined treatment with osimertinib and seliprecatinib was subsequently initiated, leading to ddPCR negativity within 14 days, which persisted at 3 months. After systemic progression at 7.8 months, fourth-line therapy with chemotherapy and amivantamab was commenced (Figure 4).

Discussion

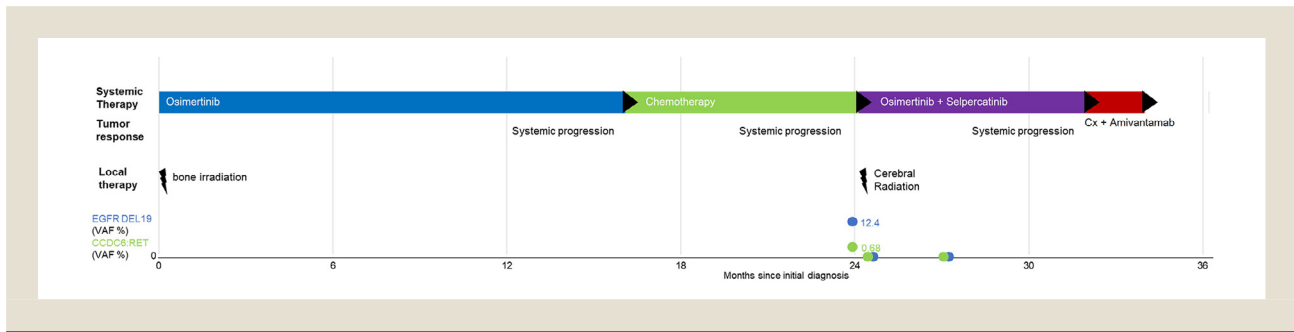
For patients with *EGFR*-mutant NSCLC, third-generation EGFR-TKIs and novel combination therapies, such as osimertinib/chemotherapy or amivantamab/lazertinib, have significantly

improved clinical outcomes, offering durable clinical benefit in many cases. Nonetheless, the development of drug resistance remains an inevitable challenge during the course of therapy. Both intrinsic and acquired resistance mechanisms have been described, with *RET* fusions emerging as a relatively recently identified bypass mechanism contributing to acquired resistance in a subset of patients.

In our cohort, 2 cases harbored combined EGFR and RET alterations at initial diagnosis, with the RET alteration considered clinically relevant, while 7 cases emerged during EGFR-TKI treatment. For 2 patients harboring classical RET fusions alongside atypical EGFR mutations, the RET fusion was considered the clinically dominant driver alteration. In these cases, both mutations were present at initial diagnosis, and first-line treatment with a selective RET-TKI was chosen.

The participating centers reported a total of 23,304 molecular tests performed during the observation period. Based on the reported prevalence of EGFR alterations (15%) in Germany, which represents the upper range reported in literature, this corresponds to an estimated 3496 patients with EGFR mutations. Among the 9 reported patients with combined alterations, 2 patients harbored a classical RET fusion with an exon 20 insertion at the time of initial diagnosis, whereas 7 patients developed RET alterations in the context of classical EGFR-mutated disease under targeted therapy. The estimated frequency of the occurrence of RET fusions as a mechanism of resistance is thus 0.2%. Given these figures, it can be concluded that the coexistence of EGFR and RET alterations represents a very rare molecular constellation. These data

Figure 4 Course of treatment and detection of EGFR and RET via ddPCR in patient #9. Abbreviations: Cx = chemotherapy; VAF = variant allele frequency.



EGFR inhibition and contributing to acquired therapeutic resistance.^{30,31}

This reinforces the importance of continuous molecular monitoring in patients with driver mutations, as tumor heterogeneity may lead to diverse resistance mechanisms. In this context, rebiopsy at the time of disease progression remains the gold standard to guide further treatment decisions.

Clinical Outcome and Potential Benefit

In our case series, patients with common *EGFR* mutations, 6 patients received a third generation TKI, leading to a median PFS of 13.5 (± 9.8 ; 4.5-32.9) months, and one second generation TKI (PFS 8.0 Mon). This is significantly less than what is predicted in the literature and can be attributed to the real-world nature of our cohort.^{32,33} Among more recent combination strategies, a median PFS of 25.5 months was observed for osimertinib plus chemotherapy, and 23.7 months for the combination of amivantamab and lazertinib. Notably, the respective control arms with osimertinib monotherapy also yielded better outcomes, with a median PFS of 16.7 and 16.6 months, respectively.^{4,34} These findings suggest that the emergence of a *RET* fusion constitutes a clinically relevant resistance mechanism, ultimately compromising the efficacy of third-generation TKI monotherapy.

Second-line treatment strategies for *EGFR*-mutant NSCLC include various chemotherapy-based regimens as well as the combination of amivantamab and chemotherapy. These approaches, however, are typically associated with intravenous administration schedules and increased toxicity profiles. In this context, continuation of a third-generation *EGFR*-TKI in combination with a *RET* inhibitor emerges as a more patient-friendly, well-tolerated, and fully oral therapeutic alternative.

Recent case reports and small cohort studies have highlighted this combination as a promising strategy, demonstrating rapid and durable responses alongside a favorable safety profile.^{10,22,25} In a multicenter cohort study by Rotow et al., 13 patients with acquired *RET* fusions following progression on osimertinib were treated with the combination of osimertinib and selpercatinib. The reported median duration of response was 7.9 months (range, 0.8-25 months), with the median duration of response among confirmed responders reaching 11 months.²¹ In our cohort, patients treated with osimertinib and pralsetinib exhibited a PFS of 3.9,

7.8, and 13.0 months (ongoing), further underscoring the clinical relevance of the combined treatment strategy—particularly given its concurrently well-characterized and manageable safety profile.

While encouraging clinical activity was observed in selected patients receiving combined *EGFR* and *RET* inhibition, these observations must be interpreted with caution. Owing to the small cohort size, heterogeneous prior treatments, and variable treatment sequences, the present data do not allow definitive conclusions regarding clinical efficacy or superiority compared to established therapeutic strategies. Rather, the observed outcomes support the biological plausibility and clinical feasibility of this approach in carefully selected cases.

Fusion Partners

While the most common *RET* fusion partners in NSCLC patients are *KIF5B* and *CCDC6*, other reported partners include *NCOA4*, *TRIM33*, etc.^{10,14} In our study, *CCDC6* was the most common, followed by *NCOA4*. Of note, in patients with an atypical *EGFR* mutation and *RET* fusion already present at initial diagnosis only *KIF5B* was found. Due to the small number of cases, it is not possible to draw any conclusions about the possibility of a better response to different types of treatment.

Monitoring Using Droplet Digital PCR

In 2 patients, the identification of the specific *RET* fusion partner (*CCDC6*) enabled the development of a personalized ddPCR assay.

In the context of gene fusions, ddPCR assays are inherently limited to patient-specific designs, as fusion events are defined by unique breakpoint combinations and fusion partners. Unlike point mutations, for which standardized, mutation-agnostic assays can be applied across patients, fusion detection by ddPCR requires prior identification of the exact fusion partner and breakpoint, typically through tissue-based or comprehensive NGS analysis. Consequently, ddPCR is not suitable as a patient-agnostic screening tool for fusion detection. Customized assays are covered by a flexible accreditation and are routinely implemented in clinical diagnostics. With correct variant annotation, assay design and validation are well feasible, requiring up to 0.5 working days for design and approximately 2 weeks for validation when tumor DNA is available as a positive control, with limited personnel effort. Its clinical utility lies

Table 2 Patients' Characteristics, Treatment Lines, and Duration of Response

Pat no	Age	Sex	EGFR-mutation	RET-fusion at time of progression	Time to occurrence of RET-fusion [Mon]	1st line therapy	PFS 1st line [mon]	2nd line therapy	PFS 2nd line [mon]	3rd line therapy	PFS 3rd line [mon]	4th line therapy	PFS 4th line [mon]	OS [mon]
1	64	male	atypical	KIF5B:RET	at initial diagnosis	Prasertinib	14.9*							13.9*
2	42	female	atypical	KIF5B:RET	at initial diagnosis	Prasertinib	2.8	Cx + ICI	20.5*					26.5*
3	88	female	L858R	no fusion partner detectable	6.3	Osimeritinib	4.5	BSC						7.4
4	46	female	DEL19	CCDC6:RET	11.4	Afatinib	8.7	Cx + ICI	6.4	Osimeritinib	10.2			33.8
5	56	female	DEL19	CCDC6:RET	19.7	Osimeritinib	17.0	Cx	0.3	BSC				21.6
6	54	female	DEL19	NC0A4:RET	11.1	Osimeritinib or Lazeritinib**	9.3	Cx + ICI	1.7					13.2
7	58	male	DEL19	NC0A4:RET	11.0	Osimeritinib	11.0	Osimeritinib + Pralsetinib	3.9	Cx	3.2	Osimeritinib+ Bevacizumab	3.9	27.0
8	42	female	DEL19	CCDC6:RET	13.2	Osimeritinib+ local Tx	32.9	Cx + ICI	6.6	Osimeritinib+ Selpercatinib	13.0*			28.0*
9	52	female	DEL19	CCDC6:RET	12.5	Osimeritinib	16.3	Cx	7.6	Osimeritinib+ Selpercatinib	7.8	Cx+ Amivantamab	NR	

Blue indicates activating EGFR mutations, green indicates oncogenic RET gene fusions, and purple indicates combined treatment with EGFR and RET tyrosine kinase inhibitors. Abbreviations: Cx = chemotherapy; ICI = immunotherapy; BSC = best supportive care; * ongoing response, ** in a clinical trial setting.

in focused longitudinal monitoring once a defined fusion has been identified.

DdPCR offers highly sensitive, allele-specific quantification, allowing for precise detection and monitoring of emerging resistance mutations across a heterogeneous tumor landscape, especially in EGFR-mutant NSCLC³⁵. The technique is rapid and cost-effective, providing results within days while substantially reducing the need for invasive procedures and thereby minimizing patient discomfort and biopsy-related risks. In our case, retrospective analysis demonstrated a clear correlation between clinical tumor dynamics—response and progression—and the allele frequencies of EGFR and RET mutations as measured by ddPCR. This suggests that, in future clinical practice, ddPCR could serve as a valuable real-time tool for treatment monitoring when a defined resistance mechanism is known.

Currently, therapeutic decisions are typically based on radiographic progression observed on CT imaging or clinical deterioration—markers that usually indicate a substantial increase in tumor burden. DdPCR, by contrast, offers the ability to detect molecular changes earlier, potentially preceding radiological or symptomatic progression. This opens the door to more proactive treatment adaptations, such as extended imaging beyond standard thoracic CT if tumor progression at another site is suspected, escalation of local therapy in cases of oligoprogression, or timely modification of the systemic therapy. In our case, the differential diagnosis of pneumonitis was considered more likely based on the patient's good clinical condition and the negative ddPCR result, already underscoring the relevance of ddPCR. Our findings highlight the growing clinical relevance of RET fusions as an emerging resistance mechanism in EGFR-mutant NSCLC. While rare, the coexistence of EGFR mutations and RET rearrangements—particularly in the setting of acquired resistance - poses a significant therapeutic challenge, compromising the efficacy of third-generation EGFR-TKIs. In this context, combining EGFR and RET inhibition represents a rational, biologically guided treatment approach that may offer meaningful clinical benefit, particularly given its favorable safety profile and oral administration route.

Although data are still limited, recent case series and early cohort studies—including our own—demonstrate that this dual-targeted strategy can induce durable responses, even in heavily pretreated patients. The use of droplet digital PCR for molecular monitoring further adds value, allowing for sensitive, real-time tracking of resistance dynamics. As demonstrated in our cases, fluctuations in EGFR and RET allele frequencies closely mirrored clinical tumor behavior, underscoring the potential of ddPCR to detect resistance earlier than conventional imaging or clinical deterioration would allow.

Taken together, these insights underscore the importance of continuous molecular surveillance and personalized therapeutic adaptation in EGFR-mutant NSCLC. Integrating tools such as ddPCR into routine clinical workflows could facilitate earlier intervention, guide treatment intensification or modification, and ultimately improve outcomes in this evolving landscape of precision oncology.

Study Limitations and Interpretative Framework

The present analysis is narrative and descriptive in nature and based on a small number of cases derived from a real-world, retrospective cohort. Given the limited sample size, the heterogeneity of applied treatment strategies, and the nonrandomized design, no definitive conclusions regarding the superiority or comparative efficacy of specific therapeutic approaches can be drawn. The findings should therefore be interpreted as hypothesis-generating and illustrative of clinical feasibility rather than as confirmatory evidence for a particular treatment strategy.

Conclusion

In summary, this multicenter real-world analysis demonstrates the feasibility of detecting RET fusions as a rare resistance mechanism in EGFR-mutant NSCLC using routine molecular diagnostics, including tissue- and plasma-based approaches, in a real-world setting. While dual targeting of EGFR and RET represents a biologically rational strategy and was applied in selected patients with encouraging individual clinical responses, the present study does not provide evidence for a proven therapeutic benefit or superiority of this approach. Rather, our findings highlight the potential of combined targeted strategies and emphasize the clinical feasibility of their implementation, underscoring the importance of systematic molecular reassessment at disease progression. Prospective studies are required to further define the clinical value, optimal timing, and appropriate patient selection for dual EGFR and RET inhibition.

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CRediT authorship contribution statement

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Appendix

Sample Collection and Processing

For each liquid biopsy (LB), 10 to 20 mL of peripheral blood was collected in either Streck Cell-Free BCT CE tubes or PAXgene ccfDNA tubes, transported to the Institute for Clinical Chemistry, Medical Faculty Mannheim of the University of Heidelberg, and processed within 72 hours of collection. Upon receipt, plasma was separated using a two-step centrifugation protocol. Initially, blood samples were centrifuged at 1600 x g for 10 minutes at room temperature without brake. The resulting supernatant was transferred to a fresh tube and centrifuged at 6000 x g for 10 minutes at room temperature without brake. Plasma was either processed immediately for cfDNA isolation or stored at -80°C until use.

Cell-free DNA (cfDNA) was isolated from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions, with the exception of an extended proteinase K incubation period of 60 minutes instead of 30 minutes. For ddPCR, cfDNA was eluted in 70 µL of AVE buffer (proprietary elution buffer supplied with the kit) and was either used immediately for LB assays or stored at -20°C until analysis.

ddPCR Assays

In brief, 18 to 30 µL of isolated cfDNA was used in 3 to 5 multiples of 20 µL reactions for emulsion PCR. The optimal annealing temperature of emulsion PCR was determined to be 56.0°C. Droplets were generated by mixing 20 µL of ddPCR master mix with 70 µL of generation oil in a cartridge of an Automated Droplet Generator (QX200TM, Bio-Rad) and analyzed using the QX200TM Droplet Reader (Bio-Rad). Results were evaluated using QuantaSoft analysis software version 1.7.4 (Bio-Rad). Within each run, 1 nontemplate control, 1 negative control (wild-type), and 1 positive control (mutant) were included. Validation studies revealed a limit of blank (LOB) of <1copy/µL, a VAF of 0.05% as limit of detection (LOD), and a VAF of 0.2% as limit of quantification (LOQ) based on a coefficient of variation <25% for quantitative results, as recommended by the Guidelines for Validation of qPCR-based methods.³⁶

For the detection of *RET-CCDC6* fusions, a personalized tissue-informed ddPCR assay was developed for each eligible patient and performed using 2xddPCR Supermix for Probes (no UTP) (Bio-Rad) according to the manufacturer's instructions. Except for probe design, the assay was performed as specified above. Probe design was based on available patient-specific tissue NGS datasets and followed probe design guidelines from Bio-Rad (Bulletin 6407 Rev A, 13-05790214Sig 1213, Bio-Rad). For assay validation, optimal annealing temperature, LOB, LOD, and LOQ were determined. A summary of the specific validation details is provided in Table 3.

Table 3 Probes Used for and Assay Details of Individualized ddPCRs

Diagnostic Panels used in the nNGM network

Version 1: ALK (exon 22 - 25), BRAF (exon 11, 15), CTNNB1 (exon 3), EGFR (exon 18 - 21), FGFR1 (exon 4, 5, 6, 7, 10, 12, 13, 14, 15), FGFR2 (Tr-A*: 6, 7, 8, 10, 11, 13, 14, 15; Tr-B*: 8, 9, 12, 18), FGFR3 (exon 2, 5, 6, 8, 9, 11, 13, 15, 17), FGFR4 (exon 3, 6, 9, 12, 13, 15, 16), HER2 (exon 2, 19, 20), IDH1 (exon 4 (R132X)), IDH2 (exon 4 (codon 140, 172)), KRAS (exon 2 - 4), MAP2K1 (exon 2, 3), MET (exon 16 - 19), NRAS (exon 2 - 4), PIK3CA (exon 10, 21), PTEN (exon 1-8), ROS1 (exon 34 - 41), TP53 (exon 4 - 8).

Version 2: ALK (exon 22 - 25), BRAF (exon 11, 15), CTNNB1 (exon 3), EGFR (exon 18 - 21), FGFR1 (exon 4, 5, 6, 7, 10, 12, 13, 14, 15), FGFR2 (Tr-A*: 6, 7, 8, 10, 11, 13, 14, 15; Tr-B*: 8, 9, 12, 18), FGFR3 (exon 3, 6, 7, 9, 10, 12, 14, 16, 18), FGFR4 (exon 3, 6, 9, 12, 13, 15, 16), HER2 (exon 8, 19, 20), HRAS (exon 2 - 4), IDH1 (exon 4 (R132X)), IDH2 (exon 4 (codon 140, 172)), KEAP1 (exon 2 - 6), KRAS (exon 2 - 4), MAP2K1 (exon 2, 3), MET (exon 14, 16 - 19 / Intron 13, first 100 bp of intron 14), NRAS (exon 2 - 4), NTRK1 (exon 13 - 17), NTRK2 (exon 14 - 19), NTRK3 (exon 15 - 20), PIK3CA (exon 8, 10, 21), PTEN (exon 1-8), RET (exon 10 - 18), ROS1 (exon 34 - 41), STK11 (exon 1 - 9), TP53 (exon 4 - 8).

Version 3.2: ALK (exon 20, 21, 22 - 28), BRAF (exon 11, 12, 14, 15), CTNNB1 (exon 3), CUL3, EGFR (exon 18 - 21), ERBB2 (exon 8, 19 - 21), FGFR1 (exon 4 - 7, 10, 12 - 16), FGFR2 (Tr-A*: 6, 7, 8, 10, 11, 13, 14, 15; Tr-B*: 8, 9, 12, 18), FGFR3 (exon 3, 6, 7, 9, 10, 12 - 16, 18), FGFR4 (exon 3, 6, 9, 12, 13, 15, 16), HRAS (exon 2 - 4), IDH1 (exon 4), IDH2 (exon 4), KEAP1 (exon 2 - 6), KRAS (exon 2 - 4), MAP2K1 (exon 2, 3), MET (exon 14, 16 - 19 / intron 13, first 100 bp of intron 14), NFE2L2 (exon 1 - 5), NRAS (exon 2 - 4), NTRK1 (exon 13 - 17), NTRK2 (exon 14 - 19), NTRK3 (exon 15 - 20), PIK3CA (exon 8, 10, 21), PTEN (exon 1-9), RB1 (all exons), RET (exon 10 - 18), ROS1 (exon 34 - 41), SMARCA4 (all exons), STK11 (exon 1 - 9), TP53 (exon 2 - 11).