# Non-Invasive Cancer Genotyping: A Single-Center Experience on the Challenges, Limitations, and Methods

## Noninvaziv Kanser Genotipleme: Zorluklar, Kısıtlamalar ve Yöntemler Üzerine Tek Merkezli Bir Deneyim

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**ABSTRACT**

**Background/aim**: A comprehensive liquid biopsy panel was performed on 242 patients to explain the challenges, limitations, and methods in liquid biopsy testing.
**Material and methods**: The majority of the patients who visited the clinic had advanced resistant cancer. There were four groups according to methods. The first group was Sophia (n = 100, 41.3%), the second was Qiagen (n = 100, 41.3%), the third was Archer-L (n = 30, 12.4%), and the fourth was Archer-T (n = 12, 5%). The patients underwent comprehensive liquid biopsy panels. Tier I-II-III variants have been discussed.
**Results**: The mean age was 61.4. T790M was detected in 6 patients (2.5%). *MET* amplification was detected in 5 patients (5%, only Qiagen group), and *NTRK* gene fusions were detected in 5 patients (5%, only Archer-T group). The most commonly mutated gene in patients was *TP53* (26%), and the most common mutations were *EGFR* exon 19 deletions and *EGFR* L858R.
**Conclusion**: Focusing on one gene and one mutation is not appropriate due to different resistance mechanisms. Discoveries regarding liquid biopsy applications will lead to more clinically meaningful therapeutic approaches for cancer patients and will play an essential role in improving individual risk prediction, therapy, and prognosis.

**Keywords**: Liquid biopsy, challenges, limitations, methods

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**ÖZET**

**Amaç**: Likit biyopsi testindeki zorlukları, sınırlamaları ve yöntemleri açıklamak için 242 hastada kapsamlı bir likit biyopsi paneli gerçekleştirildi.
**Yöntem**: Kliniği ziyaret eden hastaların çoğunda ileri evre ve dirençli kanser vardı. Yöntemlere göre hastalar dört gruba ayrildi: ilk grup Sophia (n = 100, % 41,3), ikincisi Qiagen (n = 100,% 41,3), üçüncü grup Archer-L (n = 30,% 12,4) ve dördüncü Archer-T ( n = 12,% 5). Hastalara kapsamlı likit biyopsi panelleri uygulandı. Tier I-II-III varyantları tartışıldi.
**Bulgular**: Ortalama yaş 61.4 idi. 6 hastada (% 2,5) T790M tespit edildi. 5 hastada (% 5, sadece Qiagen grubu) MET amplifikasyonu, 5 hastada (% 5, sadece Archer-T grubu) NTRK gen füzyonları tespit edildi. Hastalarda en yaygın mutasyona uğramış gen TP53 (% 26) ve en yaygın mutasyonlar EGFR ekson 19 delesyonları ve EGFR L858R idi.
**Sonuç**: Kanserde bir gen ve bir mutasyona odaklanmak, farklı direnç mekanizmaları nedeniyle uygun değildir. Likit biyopsi uygulamaları ile ilgili keşifler, kanser hastaları için klinik olarak daha anlamlı terapötik yaklaşımlara yol açacak ve bireysel risk tahmini, tedavisi ve prognozunun iyileştirilmesinde önemli bir rol oynayacaktır.

**Anahtar Sözcükler**: Likit biyopsi, zorluklar, sınırlamalar, yöntemler

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## INTRODUCTION

People are dying of cancer more than any other illness. Lung, breast, ovarian, colorectal, cervical, and gastric cancers are widely known. Physicians and scholars are dedicated to providing accurate control of illness, diagnosis, and prognosis and predicting resistance. The primary objective is to provide patients with appropriate care with the hope of restoring their former wellbeing. Lung cancer is the most common malignancy contributing to the largest number of cancer deaths (1).

Non-invasive cancer genotyping (Liquid biopsy, plasma genotyping) procedures have continued to be used in recent years to treat several various forms of cancer. Liquid biopsy is used in tumors to detect treatment, prognosis, and evaluation of the patient. Tumor cells shed biomarkers during apoptosis. Cellular components in these products may be screened for genetic aberration. This less invasive approach provides a more outstanding prospect of a favorable outcome and a better recovery rate (2,3).

Physicians and care practitioners used tissue biopsy to provide optimal treatment for cancer patients. Another factor to note is that widely applied biopsy procedures are intrusive, and their success relies on the biopsy site (3). A biopsy is a standard procedure for somatic mutations but has high risks (4). Liquid biopsy is used as a minimally invasive procedure that can be administered repeatedly. Changes in circulating tumor DNA (ctDNA) may be used for cancer screening in asymptomatic patients, mutation identification for therapeutic purposes, tumor monitoring, and genetic evolution (2). Repeated examination and quantification of ctDNA may provide details on changes in clonal composition over time (5). Plasma-derived ctDNA is the most commonly used blood-based biomarker in clinics. The ctDNA is believed to be between 0.1-10% of the total cell-free DNA (cfDNA) present in human blood and could be linked to tumor burden, inflammation, and cancer cell accessibility to blood vessels (6). Global ctDNA analysis is a non-invasive, secure method of tracking tumors. A high correlation of biopsies and plasma samples suggests that the volume of ctDNA is related to the scale and function of the tumor lesions. Point mutations may occur weeks or months before disease progression (7,8).

In general, it is believed that gained resistance to selective therapy is linked to the accumulation of somatic mutation subclones. Resistance mechanisms include *MET/HER2* amplification, activation of the RAS–mitogen-activated protein kinase (MAPK) or RAS–phosphatidylinositol 3-kinase (PI3K) pathways, novel fusion events, and histological/phenotypic transition (9). Guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology published recommendations include *EGFR, ALK, ROS1* testing for all patients with adenocarcinoma; use of additional genes (*ERBB2*, *MET*, *BRAF*, *KRAS*, and *RET*) for laboratories performing next-generation sequencing panels (10). About half of patients with lung adenocarcinoma have at least one driver mutation, suggesting a reasonable target for therapeutic intervention (11). Historically, the approach to the advancement of selective therapies for oncogenic driver-positive cancers has been histology-specific. Examples include monoclonal antibodies or tyrosine kinase inhibitors (TKIs) for *ERBB2*, *EGFR*, and *ALK* in non-small-cell lung carcinoma (NSCLC) (12). In the vast majority of patients treated with an *EGFR*-inhibiting drug, resistance generally emerges after two years, and in 60% of cases, a secondary *EGFR* T790M mutation found in disorder hinders the access of the medication to the kinase. The *EGFR* T790M mutation predicts osimertinib sensitivity, and a positive mutation test is needed on prescription. T790M was more widespread in exon 19 deletion (10,13). C797S may occur in tumors that have progressed following osimertinib treatment with T790M disease. Most lung cancers will develop *EGFR-ALK-ROS* independent resistance after treatment (14). The most common cause of bypass pathway activation as an acquired resistance mechanism to *EGFR*-TKIs is *MET* gene amplification (15).

Increased activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway leads to many facets of cancer, including acquired growth signals, apoptosis inhibition, vessel development, and tolerance to anti-growth signals. In lung cancer, the PI3K/Akt/mTOR pathway is related to tumor growth and progression (16). The inhibition of the PI3K/Akt/mTOR oncogenic signaling mechanism is the most well-known anti-oncogenic effect of phosphatase and tensin homolog deleted in chromosome 10 (*PTEN*); other documented effects involve chromosomal integrity and DNA repair (17).

Oncogenic regulators of a number of adult and pediatric tumors are NTRK gene fusions involving NTRK1, NTRK2, or NTRK3 (encoding the neurotrophin receptors TRKA, TRKB, and TRKC, respectively). Patients with NTRK fusion-positive tumors that undergo a first-generation TRK inhibitor have a high response rate (>75%) regardless of tumor histology. TRK overexpression has been identified in the breast, cutaneous (such as basal cell carcinoma), lung cancers, neuroblastoma, cylindroma, and other cancers. Fusions involving NTRK1, NTRK2, or NTRK3 are the most typical pathways of oncogenic TRK activation (18). NTRK fusions are seen at far lower frequencies (5–25 percent or 5% ) in common tumors (such as breast, lung, colorectal cancers, and melanoma) (12).

Somatic variants, which result in clonal hematopoiesis, may be present in seemingly healthy individuals (19). The detection of repeated somatic variants most commonly associated with peripheral blood hematological cancers distinguishes age-related clonal hematopoiesis, also known as clonal hematopoiesis of indeterminate potential (CHIP). DNMT3A, TET2, and ASXL1 are the most commonly mutated genes; however, TP53, JAK2, SF3B1, GNB1, PPM1D, GNAS, and BCORL1 mutations are also frequent. While most CHIP research has been performed on peripheral blood, these mutations often occur in plasma since hematopoietic cells are the source of most cell-free DNA in healthy people (19,20).

In this study, we performed a comprehensive panel on 242 patients to explain the challenges, limitations, and methods in liquid biopsy testing. Our data broadens the knowledge and provides insights for methods and interpretations used in non-invasive cancer genotyping.

## METHODS

### *Patients*

Consent for the publication of the study and any additional related information was taken from the patients or their parents involved in the study. Most of the patients visited the clinic with the diagnosis of advanced resistant cancers. Clinical histories and molecular results were reviewed for all unrelated patients examined at the Department of Medical Genetics, University of Health Sciences, Dışkapı Yıldırım Beyazıt Training and Research Hospital, and Department of Medical Genetics and University of Health Sciences, Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital, Ankara, Turkey. The patients underwent the comprehensive liquid biopsy panel between January 2018 and December 2020 at the Ankara Central Genetic Laboratory (Turkey). Moreover, patients with uncertain/missing data were filtered out.

In the study, a total of four groups were formed according to the methods. The first group was Sophia (n = 100, 41.3%), the second was Qiagen (n = 100, 41.3%), the third was Archer-L (n = 30, 12.4%), and the fourth was Archer-T (n = 12, 5%).

### *DNA Panels, NGS, and ddPCR*

From the blood samples collected in EDTA tubes, the patients' genomic DNA was extracted according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen Inc., Hilden, Germany) by QIAcube (Qiagen Inc., Mississauga, ON, Canada). The DNA samples were quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

Four different multigene panels have been used: ArcherDx Reveal ctDNA 28 Kit (Archer-L, *AKT1, CTNNB1, ESR1, IDH2, MAP2K2, NTRK1, RET, ALK, DDR2, FGFR1, KIT, MET, NTRK3, ROS1, AR, EGFR, HRAS, KRAS, MTOR, PDGFRA, SMAD4, BRAF, ERBB2, IDH1, MAP2K1, NRAS, PIK3CA, TP53),* ArcherDx FusionPlex CTL Kit (Archer-T, *AKT1, ALK, AXL, BRAF, CALCA, CCND1, CTNNB1, DDR2, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, GNAS, HRAS, IDH1, IDH2, KRAS, KRT20, KRT7, MAP2K1, MET, NRAS, NRG1, NTRK1, NTRK2, NTRK3, PIK3CA, PPARG, PTH, RAF1, RET, ROS1, SLC5A5, THADA, TTF1*), Qiagen GeneRead QIAact Lung DNA UMI Panel (*AKT1, ALK, BRAF, DDR2, EGFR, ERBB2/HER2, ESR1, FGFR1, KIT, KRAS, MAP2K1, MET, NRAS, NTRK1, PDGFRA, PIK3CA, PTEN, RICTOR, ROS1*), and Sophia Genetics 56G Oncology Solution *(ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF-1R, CTNNB1, DDR2, DNMT3A, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, STK11, SMAD4, SMARCB1, SMO, SRC, TP53, TSC1, VHL*). The Qiagen GeneRead QIAact Lung DNA UMI Panel and Sophia Genetics 56G Oncology Solution were used at the center from 2018 to 2020, and the ArcherDx Kits has been used since 2020.

The sequencing was performed on GeneReader (Qiagen Inc., Hilden, Germany) system for the Qiagen GeneRead QIAact Lung DNA UMI Panel (Qiagen Inc., Hilden, Germany) and for the others the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA). The data were analyzed on the Archer Analysis Platform (ArcherDX, Inc., CO, USA) for the ArcherDx panels, Qiagen Clinical Insight (QCI) for Qiagen GeneRead QIAact Lung DNA UMI Panel, and Sophia DDM software (Sophia Genetics, Saint-Sulp) for the Sophia Genetics 56G Oncology Solution (Table 1). Visualization of the data was performed with IGV 2.7.2 (Broad Institute) software. Table 1 provides relevant information used and observed in this study.Droplet Digital PCR (ddPCR) (Bio-Rad Laboratories, Inc., Ca, USA) was used for detecting EGFR T790M mutation in 7 patients with acquired EGFR-TKI resistance

**Table 1**. The NGS methods and platforms used in this study.

| Features | ArcherDx Reveal ctDNA 28 | Sophia 56G oncology solution | Qiagen GeneRead QIAact Lung DNA UMI Panel | ArcherDx Fusionplex CTL |
|---|---|---|---|---|
| Sample count | 30 | 100 | 100 | 12 |
| Platform | Illumina MiSeq/NextSeq | Illumina MiSeq/NextSeq | GeneReader | Illumina MiSeq/NextSeq |
| Read depth | ~1000 | ~30000 | ~200 | ~100-500 |
| UMI | + | - | + | + |
| Hotspot/whole gene | hotspot | hotspot | hotspot | hotspot |
| CNV | + | - | - | + |
| Fusion | - | - | - | + |
| Amplification / Expression | + | - | + | + |
| Platform for analyzing | Archer Analysis Platform (Online) | Sophia DDM v.4 | QCI | Archer Analysis Platform (Online) |
| FFPE | - | + | - | + |
| Plasma | + | + | + | - |

'+': yes, '-': no, UMI: Unique molecular identifier, CNV: copy number variation, FFPE: formalin-fixed paraffin-embedded.

***Interpretations, Descriptive Statistics and Graphics***

In compliance with the recommendations issued by the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists, variants were categorized as tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants with unknown clinical significance; and tier IV, variants that are benign or likely benign(21). Tier I-II-III variations have been included in the study. Further, descriptive statistical calculations have been done, and the figure has been prepared with Python 3.9.2 (IPython 7.19.0).

**Ethical Publication Statement**

We confirm that we have read the journal's position on issues concerning ethical publication, and we affirm that this report is consistent with the guidelines. The Ethics Committee approved (2021-03/1072) the study at the University of Health Sciences, Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital, and informed consent was obtained from the patients or their parents (mentioned within the subsection "Patients")

## RESULTS

A large percentage of our patients were suffering from advanced lung cancer (80%). The mean and the median ages were 61.4 and 62, respectively. There were more males (145, 59.9%) than females (97, 40.1%).

The majority of the variant allele fractions (VAFs) were between 1-10%. The most commonly mutated genes were *TP53, EGFR, PIK3CA, RET, PTEN, MET, ATM,* and *KRAS.* When combined, different *EGFR* exon 19 deletions exceeded other mutations (n=9). *EGFR* L858R and T790M mutations followed exon 19 deletions. T790M was detected in 6 patients (1.75%). *NTRK* fusions in 5 (5/12, only Archer-T group) and *MET* amplifications were detected in 5 (5/100, only Qiagen group) patients. In 61 (25.2%) of the patients, we could not find any responsible mutation.

No mutation was detected with Droplet Digital PCR (ddPCR) (Bio-Rad Laboratories, Inc., Ca, USA), and it did not increase the sensitivity in this study.

## DISCUSSION

Clinical research has recently embraced circulating cell-free DNA (cfDNA), which has resulted in the discovery of druggable *EGFR* mutations and treatment control of responses to targeted therapy.

Highly sensitive methods (i.e., digital droplet PCR, Next Generation Sequencing) were used to test *EGFR*-dependent (T790M and C797S mutations) and independent (MET gene amplification, *KRAS*, *PI3KCA*, and *BRAF* gene mutations) pathways of resistance to *EGFR* tyrosine kinase inhibitors (TKIs) in plasma samples from NSCLC patients, allowing for the move to other therapies. Liquid biopsy is a non-invasive way to observe improvements related to therapy and cancer heterogeneity (22). Another critical obstacle in the use of ctDNA in clinical choices is the common occurrence of co-mutations or copy number alterations. Many options exist to classify drug resistance pathways to targeted therapy via plasma ctDNA research, including the presence of conflicting mutations that can influence treatment decisions for multiple cancers (23,24).

*EGFR* T790M mutation is responsible for more than half of the EGFR-TKI resistance mechanisms, but mutation frequency was low according to expected results in this study. However, when fastq files have been scanned for the specific mutation patterns with MutScan, *EGFR* T790M mutation was detected in nearly half of the patients (25). Due to very low reads (<0.1%), it is not easy to distinguish false mutations and report them; but liquid biopsy testing could be repeated in 3-6 months periods if subclonal driver mutations are suspected, or tissue biopsy could be performed. It was reported that patients with *EGFR* exon 19 deletions who receive long-term EGFR-TKI therapy show a high prevalence of T790M mutation (26). In this study, the T790M mutation was detected in addition to the exon 19 deletion.

The tumor suppressor gene with the most mutations was *TP53*. *TP53* controls the cell cycle, apoptosis, senescence, and metabolism. According to our findings, the most frequently mutated genes were *TP53, EGFR, PIK3CA, PTEN, RET, MET, ATM, KRAS*, and *DNMT3A*. The most widespread mutations observed were *EGFR* exon 19 deletions. *PTEN* is a tumor suppressor gene that is often deactivated in a number of cancers. In patients with non-small cell lung cancer, *PTEN* deficiency and PI3K/AKT/mTOR signaling activation have been related to *EGFR*-TKI resistance (NSCLC). The overwhelming majority of *APC* mutations were nonsense or frameshift. Although the bulk of *APC* mutations in the germline are missense, somatic *APC* mutations are not. It may help distinguish between germline and somatic mutations.

Limitations could be described in six points. First, coexisting mutations are confusing; however, they are essential, particularly when searching for new resistance mechanisms. Second, the reported mutations varied according to a VAF. Most of the variants with <1% VAF were not reported due to false-positive risk. Third, not all somatic variants identified in circulating cell-free DNA originate from cancer. Somatic variants may be found in apparently healthy people, arising in part from clonal hematopoiesis, and the most commonly involved genes include *DNMT3A*, *TET2*, *ASXL1*, *TP53*, *JAK2*, *SF3B1*, *GNB1*, *PPM1D*, *GNAS*, and *BCORL1*. *DNMT3A* was one of the commonly mutated genes in this study, and these mutations may be due to clonal hematopoiesis (Figure 1).

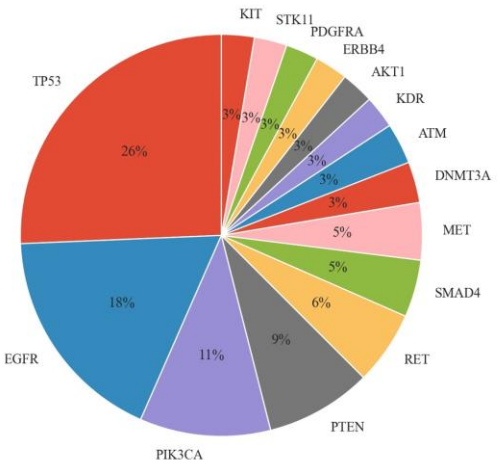


**Figure 1.** Pie chart showing the most common mutated genes in the study.

Due to the insufficient evidence, interpretations are needed to figure out how to view and explain ctDNA variants in these genes (19,20). Fourth, each platform provides thousands of mutations. Actionable or potentially actionable mutations were chosen, but many of them currently unknown. It should be kept in mind that there could be novel resistance mechanisms. Therefore, each patient's data were collected for further analyses. Fifth, *RET* translocations, other rearrangements, copy number variations, and *MET* amplification could not be checked for all patients depending on the methods. There are many different methods to choose from, and all of them try to create more robust, complex multigene panels to answer the questions regarding treatment options. More complex tests may combine tumor mutational burden (TMB) and microsatellite instability (MSI). Even though the number of patients was low, ddPCR did not help increase the sensitivity in this study. Sixth, little data are available on the effects of patient-related factors such as pregnancy, smoking, exercise, and various non-malignant conditions that might affect cfDNA levels in blood (27). Tumor status also affects the success, such as treatment, size, apoptosis/necrosis, and shedding DNA into the vessels (28).

Inherited (familial) cancers are responsible for 5–10% of all cancers; they are significantly associated with tumor growth and appear early in life (29). In this study, particularly patients with high variant fraction mutations in *APC, ATM, MLH1, MSH6, PTEN, PTPN11, RB1, RET, STK11, TP53, TSC1, VHL* underwent testing with familial cancer panel. There is a probability of genetic abnormalities passing on to the next generation (germline inheritance), even if the VAFs were between 1-10%. Genetic counseling and family screening are required for possible germline mutations.

There is no agreed classification methodology. As Joint Consensus of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists (AMP-ASCO-CAP) recommends: variants were categorized as tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants with unknown clinical significance; and tier IV, variants that are benign or likely benign(21). European Society for Medical Oncology (ESMO) recommends the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT) classification. ESCAT defines six levels of clinical evidence for molecular targets according to the implications for patient management: tier I, targets ready for implementation in routine clinical decisions; tier II, investigational targets that likely define a patient population that benefits from a targeted drug but additional data are needed; tier III, a clinical benefit previously demonstrated in other tumor types or for similar molecular targets; tier IV, preclinical evidence of actionability; tier V, evidence supporting co-targeting approaches; and tier X, lack of evidence for actionability(30). National Center for Tumor Diseases (NCT) in Heidelberg recommends level M1-2-3-4 classification, similar to AMP-ASCO-CAP classification. AMP-ASCO-CAP classification is more commonly used.

It is difficult to identify actionable variants within hundreds of mutations. Many bioinformatics techniques have been developed to characterize and annotate gene mutations; however, none offers target drug details for gene variants. CKB (https://ckb.jax.org/), OncoKB (https://www.oncokb.org/), cBioPortal (https://www.cbioportal.org/), CIVIC (https://civicdb.org/home), MyCancerGenome (https://www.mycancergenome.org/), Precision Medicine Knowledgebase (PMKB, https://pmkb.weill.cornell.edu/), and Cosmic (https://cancer.sanger.ac.uk/cosmic) are several databases/tools that might be useful. The variants and descriptions in the databases can vary. OncoKB is a freely accessible information base and tool that curates and annotates the biological, prognostic, and predictive significance of somatic molecular alterations linked to cancer. Clinically insignificant variants need not be published.

Although a tiny proportion of false-positive test findings distributed through a national population will significantly boost demand for confirmatory imaging and biopsy sampling of imaging-detected benign anomalies; as a result, false positives have clear consequences on both healthcare services and patient wellbeing. False-negative findings, on the other side, would have severe effects related to diagnostic delays.

## CONCLUSIONS

To the best of our knowledge, this is the first research to concentrate on the challenges, limitations, and methods of liquid biopsy in the context of Turkish population and evaluate both the genetic variability and treatment. These findings open up the possibility of using liquid biopsy for different types of cancers. *MET* amplification, one of the most common *EGFR*-independent mechanisms of resistance, was frequently observed in patients with advanced resistant cancers. NTRK fusions were also common in the selected group. Detecting these changes will lead to more clinically meaningful therapeutic approaches for cancer patients. The discovery of effective therapeutic targets across cancer forms is a top priority. The findings of the present study show that patients with solid tumors should also undergo testing for *MET* amplification, *NTRK* fusions to assess clinical characteristics and prognosis. Moreover, continued developments in assessing and researching new variants of known cancer genes will play an essential role in improving individual risk prediction, therapy, and prognosis. The emergence of different cancer targets provides hope that all patients will profit from precision medicine in the future.

**Conflict of interest**

No conflict of interest was declared by the authors.

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