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REVIEW

Application of the conventional and novel methods in testing *EGFR* variants for NSCLC patients in the last 10 years through different regions: a systematic review

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Abstract

Variants in the epidermal growth factor receptor (EGFR) gene are recognized as predictors of therapy response and are correlated with progression-free and overall survival in non-small cell lung cancer (NSCLC) patients. Molecularly guided therapy needs precise and cost-effective molecular tests. This review focused primarily on screening or target methods for the *EGFR* variants detection with diagnostic and prognostic potential in the clinical research published papers. Concerning the inclusion and exclusion criteria, the search interval comprised available articles published from 2010 until 2020 in three electronic databases, ISI Web of Science, Pub Med, and Scopus. The analysis of eligible studies started with 5647 and obtained the final 987 full-text articles analyzed as clinical research. The regions comprised were Africa, America, Australia, Asia, Euro-Asia, Europe, or a consortium of different countries. All of the tested methods were applied prevalently in Asia. In clinical research, the polymerase chain reaction (PCR), followed by sequencing methods have been involved mostly over the years. The identified high-through output approaches evolved to improve the survival and quality of the NSCLC patient's life becoming more sensitive, specific, and cost-effective.

Keywords EGFR · Methods · Variants · Polymorphism · Mutation · NSCLC

Abbreviations

'Immuno'- Immunohisto/cytochemistry

TMA	Tissue microarray
FP	Fluorescence polarization
AQUA	Automated quantitative analysis system

Hybridization techniques

FISH	Fluorescence in situ hybridization
CISH	Chromogenic in situ hybridization
SISH	Silver-enhanced in situ hybridization
ISH	In situ hybridization

Proteomics

MALDI TOF–MS	Mass spectrometry, Matrix assisted laser desorption/Ionization time of flight mass spectrometry, Nucleotide mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
DHPLC	Denaturing high performance liquid chromatography
HPLC	High performance liquid chromatography

'Seq': Sanger sequencing

NGS	Next generation sequencing (NGS) (with the advances), Deep sequencing (CAPP-Seq), SEQUENOM MassARRAYiPLEX assay
PCR	Polymerase chain reaction (End point, classical PCR)

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RD	Recently developed: Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), Gold nanoparticle-based microarray, Liquid-chip array, Fluorescence resonance energy transfer-based preferential homoduplex formation assay (F-PHFA), Mach–Zehnder Interferometer (MZI) sensor and isothermal solid-phase DNA amplification (IDA) technique (MZI-IDA sensor system), Microfluidic paper-based electrochemical DNA biosensor (-PEDB)
PLA	Proximity ligation assay
PNA microarray-based fluorometric assay	
MaMTH	Mammalian-membrane two-hybrid assay
HRMA	High-resolution melting analysis
RT-PCR	Reverse transcription PCR
qRT PCR	Real-time quantitative reverse transcription PCR
qPCR	Quantitative PCR or real time PCR: 'BEAMing', 'Idylla', 'Cobas' real time PCR, Peptide nucleic acid (PNA)-mediated PCR clamping
ARMS	Amplification refractory mutation system, or allele specific PCR
dPCR	Digital PCR or digital droplet PCR
SPM	Specific PCR methods: Restriction fragment length polymorphism PCR (RFLP-PCR), PCR Invader method, Single-strand conformation polymorphism (PCR-SSCP), Mutant enriched PCR, Non-enriched PCR (NE-PCR), Cycleave assays, Multiplexed PCR SERS Surface enhanced Raman spectroscopy, PNA-aPCR-Liquid chip (PAPL) method

Introduction

Revealing the sequence of human DNA with over 3 billion base pairs, the Human Genome Project (HGP) revolutionized the science and increased the understanding of the DNA variants (mutations or polymorphisms) in biomedical researches [1–3]. They have also become crucial in oncology research, particularly when recognized as cancer hallmarks [4, 5]. Either they were therapy response predictors or associated with overall survival, the most important was their correlation with patients' quality of life [6, 7].

Furthermore, molecularly guided therapy needs precise molecular tests. The sequencing technologies and their advances, accompanied with PCR approaches, enabled these tremendous discoveries, followed by an acceleration of researches toward personalized medicine and a new, 'digital' era in biology [3, 8, 9]. However, there are still many challenging issues for obtaining appropriate quality DNA or RNA sample from biological material [10, 11], or to identify the harmful change in the genetic material in the multistep process of tumor progression, with diagnostic or prognostic potential. So, the modern methods have been developing over the years through different regions becoming more sensitive, specific, but cost-effective indeed.

NSCLC is the predominant form for approximately 80% of all lung cancers (LC), reported as one of the leading causes of death worldwide [12]. The variants of the *EGFR* gene as well as the signaling pathway of its altered protein in the neoplastic cells became very important in oncology, particularly in NSCLC research. The EGFR is a transmembrane protein whose signaling network is involved in healthy cellular development, growth, and differentiation. In neoplastic cells, its protein usually over-expressed [13, 14], and its intracellular, altered kinase domain is shifting the signals towards cancerogenesis [15]. Those alterations refer to the variety of mutations and usually affecting 18–21 exons of the *EGFR* gene, for instance, in-frame deletions, in-frame insertions/duplications, and point mutations [16–18]. Approximately 90% of them account for two oncogenic-driver mutations E746-A750 deletion in exon 19, and L858R point mutation in exon 21 [19–21]. They significantly correlated with clinical response to TK inhibitors-gefitinib (Iressa®; AstraZeneca, London, UK) and erlotinib (Tarceva®; Roche, Basel, Switzerland). Besides the high mutation rate, the *EGFR* gene is usually amplified [17, 18], on the other hand, it is highly polymorphic [22–24]. The most studied were single nucleotide polymorphisms (SNPs) able to alter TKI therapy response, and this refers particularly to -216G>T (rs712829) [25], CA repeat (rs11568315) [26, 27], and D994D (rs2293347) [7, 28]. The importance of this specific *EGFR* genetic background in advance-staged NSCLC patients in the context of either the mutations or the SNPs, is in their association with progression-free survival (PFS) and overall survival (OS) [19, 29–31]. That is to say, the influence and the rising knowledge of *EGFR* variants in oncologic research have been evolving over the years all around the world by conventional or recently developed methods applied in basic and clinical research of NSCLC patients.

A decade ago, the performed search recognized the diversity, frequency, and significance of both screening and target methods for analyzing *EGFR* variants [32]. It also emphasized their advantages or disadvantages for routine laboratory manipulations in clinical or basic investigations. In this

review, we assumed current perspectives and novelties of diverse methods in the last 10 years through different regions thus investigating *EGFR* variants of NSCLC patients.

The methodology of literature search

The search of three electronic databases, ISI Web of Science, Pub Med, and Scopus were performed on April 2nd, 2020 for eligible papers considering methods for detection of *EGFR* variants in NSCLC patients. The search interval comprised papers published from 2010 until 2020 with the following searched terms for Pub Med database, with appropriate modifications for ISI Web of Science and Scopus: (receptor, epidermal growth factor [MeSH Terms]) OR EGFR) AND gene [MeSH Terms]) OR polymorphism, genetic [MeSH Terms]) AND carcinoma, non-small-cell lung [MeSH Terms]) OR NSCLC [All Fields]) AND methods [MeSH Terms]) AND humans [MeSH Terms] AND ("last 10 years" [PDat] AND Humans [Mesh]). If the studies until April 2nd, 2020 were written in English or Russian, they were included in the search, while the reviews, meta-analyses, editorials, case reports were actually excluded from this search. Available studies were collected, merged and then all duplicates were removed. According to the inclusion and exclusion criteria, titles and abstracts screened considering methods for detection *EGFR* variants (mutations or polymorphisms) in NSCLC patients to assess final full-text eligible studies. Those studies were afterwards analyzed as full-text articles in clinical research according to the year of publication or region (geographical). If the study team was from different countries or even continents, those papers summarized as 'consortium'. Russia and Turkey merged as Euro-Asia while America analyzed alone or usually as a part of the consortium of different countries.

All results are shown for the last 10 years, although the ones concerning 2020 (with particularly 3 months results) are not included in further discussion. Because of the redundancy of results, similar methods merged to simplify interpretation. Descriptive statistics performed using a pivot table in Microsoft Excel (2007).

In this literature search, PCR methods are summarized based on the standardized abbreviations in guidelines reported in 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) [33]. Concerning this, real-time PCR is interpreted as quantitative PCR (qPCR) and reverse transcription PCR, as RT-PCR (not 'Real-Time' PCR, as reported in earlier papers), but qRT PCR as Real-Time Quantitative Reverse Transcription PCR [34], indeed. The abbreviated terms such as qPCR, 'HRMA', 'ARMS', 'dPCR', and 'SPM' include appropriate methods (see "Abbreviations").

Results

The eligibility triage of available studies started with 5647 and ended with 987 full-text articles to be analyzed as 'clinical' research (Supplementary material 1). Clinical research results showed that immunostaining, hybridization techniques, and the proteomic used in the 2010s had a downward trend over the years. Moreover, among all searched methods, PCR and sequencing dominate in the applications with similar trends. Although the year 2013 had the highest usage score for both methods, NGS had an uptrend with an obvious increase in 2019 (Fig. 1). Recently developed methods were expectedly represented in a lower percentage but prevalently applied in 2014 and 2015 (Fig. 1). The percent of PCR methods followed by sequencing methods (as well as NGS) was highest in Asia, Europe, America, and a consortium of different countries (Fig. 2).

The diversity of PCR methods were detected and included the following: classical PCR, HRMA, ARMS, qPCR, qRT PCR, RT PCR, digital PCR, or specific PCR. Figures 3 and 4 present the classical, endpoint PCR implemented the most in 2013, and Asia, of course, but with a downward trend over the years. It is important to emphasize that qPCR, ARMS, and digital PCR had an uptrend over the years, while others such as HRMA, qRT PCR, RT PCR, or specific PCR methods, have had a downtrend in recent years (Fig. 3). All PCR methods are much more used in Asia than in the rest of the regions, indeed (Fig. 4). In Europe and a consortium of countries, qPCR and conventional PCR, as well as digital PCR are involved in a higher percentage than other PCR methods (Fig. 4).

Discussion

This review has analyzed screening or target methods in clinical research for the detection of the *EGFR* variants in NSCLC patients in the last decade and through different regions, indeed. These issues are leaning to our previous work [32] and showing that the immunostaining techniques have been used in the early 2000s, usually in combination with some other techniques but with a dropdown trend. Contrary to PCR-like or the sequencing techniques, whose usage increased over the years, these trends continued within here. Considering that the studies of the *EGFR* variants revealed the potential variances in genetic patterns in NSCLC of various populations [22, 35, 36], this review extended the analysis of new methods applications in the regions worldwide. Anyhow, to explain the complexity of interactions between the variants of the

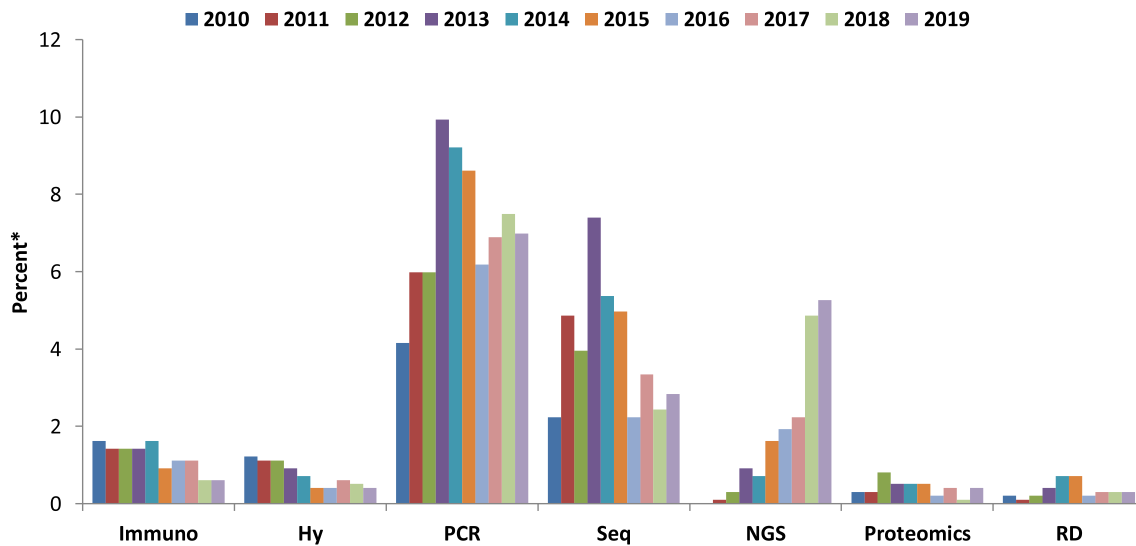

Fig. 1 Percent of methods in clinical research over the years for *EGFR* variants in NSCLC patients. *Counted in the relation to total number of 987 full text articles for clinical research. Immuno-Immunostaining techniques; *Hy* hybridization techniques; *PCR* polymerase chain reaction; Seq-Direct sequencing; *NGS* next generation sequencing; Proteomics- Mass spectrometry, ELISA, DHPLC, and HPLC; *RD* recently developed

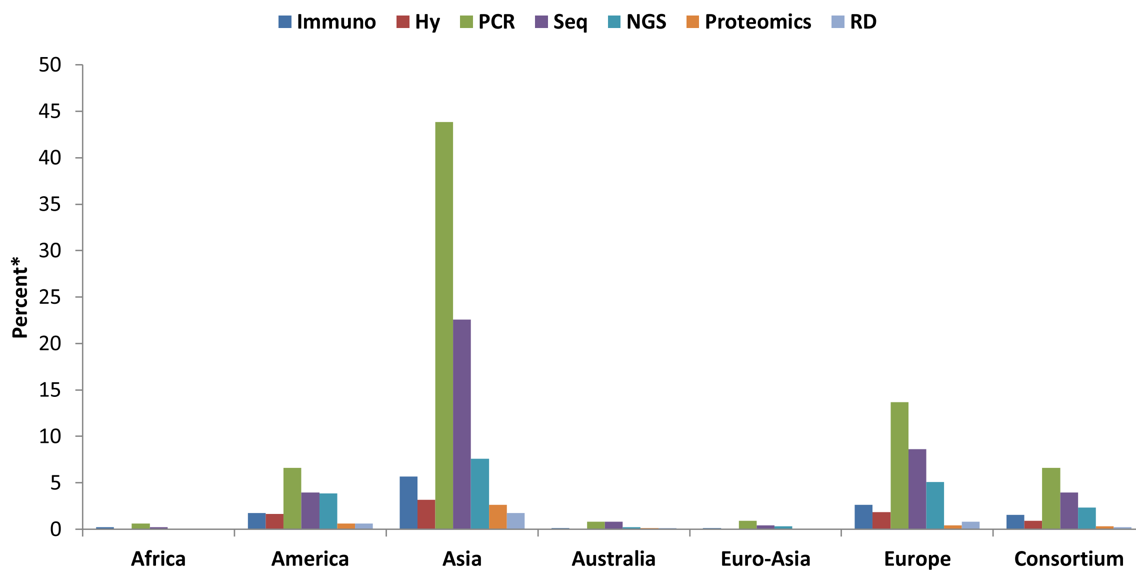

Fig. 2 Percent of methods in clinical research through regions for *EGFR* variants in NSCLC patients. *Counted in the relation to total number of 987 full text articles for clinical research. Immuno-Immunostaining techniques; *Hy* hybridization techniques; *PCR* polymerase chain reaction; Seq-Direct sequencing; *NGS* next generation sequencing; Proteomics- Mass spectrometry, ELISA, DHPLC, and HPLC; *RD* recently developed

EGFR and NSCLC patients, it was clear that a comprehensive approach is necessary-from the conventional ones to the novel, high-through output methods.

The diagnostic potential of two oncogene-driver mutations, the deletion in exon 19 and point mutation in exon 21 was evident and the methods of choice for their identification were direct DNA sequencing and PCR, but with limited clinical application. Immunostaining techniques are convenient screening methods for routine practice in clinical laboratories since they could recognize these hot-spot mutations with mutation-specific rabbit monoclonal antibodies, and also they are cost-effective, rapid, with high specificity, and sensitivity [37, 38]. Despite all this, they could detect the well-known only, not the novel *EGFR* alterations, significant for the introduction of the new therapeutics. In further, their effectiveness is concerning since the PCR or

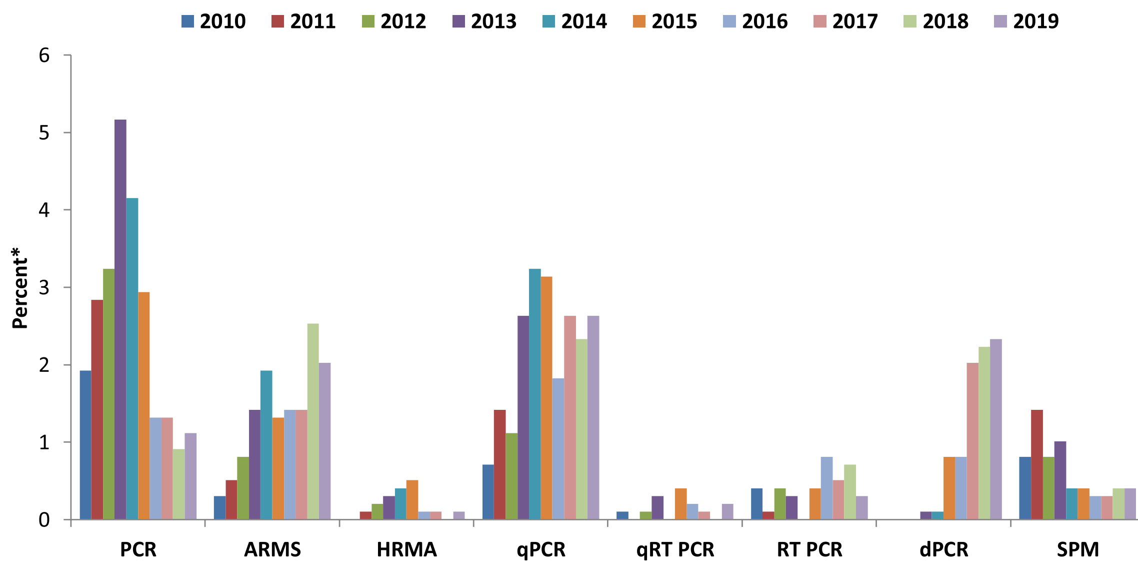

Fig. 3 Percent of various PCR methods over the years in clinical research for *EGFR* variants in NSCLC patients. *Counted in the relation to total number of 987 full text articles for clinical research. *PCR* polymerase chain reaction; *HRMA* high-resolution melting analysis; *ARMS* amplification refractory mutation system; *qPCR* quantitative PCR, *qRT-PCR* real-time quantitative reverse transcription PCR; *RT-PCR* reverse transcription PCR; *dPCR* digital PCR; *SPM* Specific PCR methods

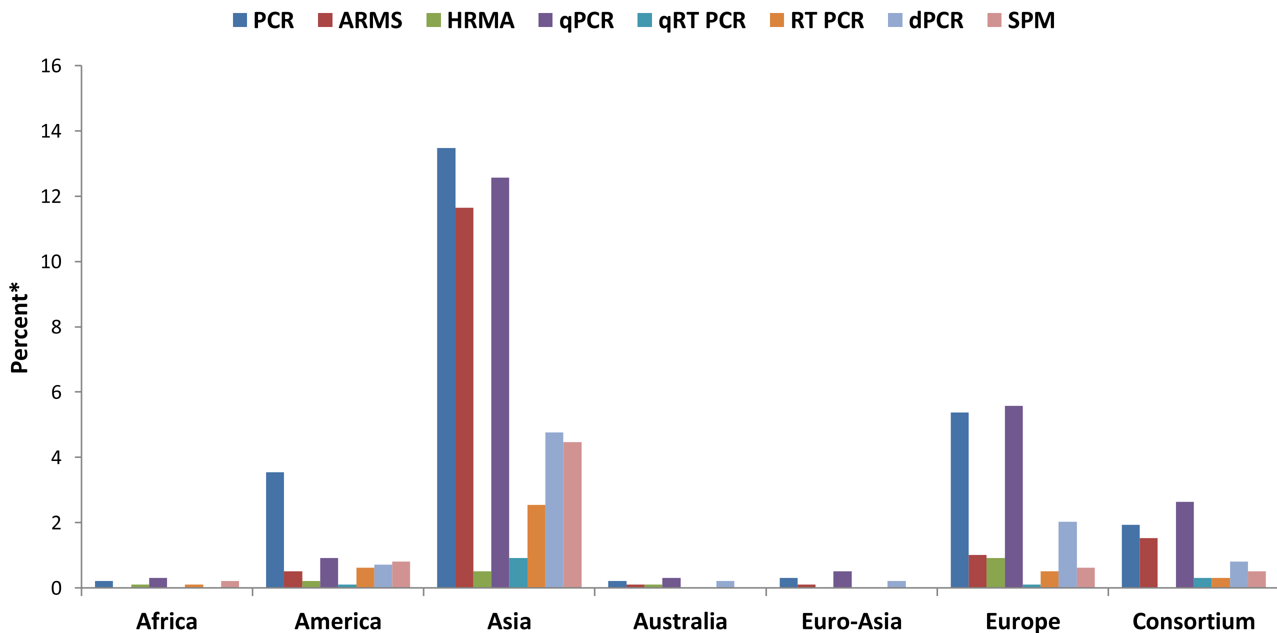

Fig. 4 Percent of various PCR methods through the region in clinical research for *EGFR* variants in NSCLC patients. *Counted in the relation to total number of 987 full text articles for clinical research. *PCR* polymerase chain reaction; *HRMA* high-resolution melting analysis; *ARMS* amplification refractory mutation system; *qPCR* quantitative PCR; *qRT-PCR* real-time quantitative reverse transcription PCR; *RT-PCR* reverse transcription PCR; *dPCR* digital PCR; *SPM* specific PCR methods

sequencing methods, usually applied as the competitive methods, confirmed some of the *EGFR* mutations missed by immunostaining techniques [37]. All mentioned could refer to the decrease in applications in recent years in clinical research (Fig. 1).

Starting from the 2010s, over the years, the similarities in usage decrease was evidenced not only for immunostaining techniques in clinical researches but also for hybridization and proteomic techniques (Fig. 1). Previous reports showed the improved response rate, time-to-progression, and

survival in NSCLC patients associated significantly with the high *EGFR* gene copy number or high polysomy evaluated by fluorescence in-situ hybridization (FISH) [39, 40]. Therefore, at that time, the FISH was a method of choice, and the predictive role of *EGFR* gene amplification suggested for the clinical benefit either with EGFR monoclonal antibodies or TKI therapy [40, 41]. But the others found FISH inconsistency with the outcome and favored *EGFR* mutation detection with sequence analysis for predicting response and prolonged progression-free survival in NSCLC patients [42]. Also, the technical robustness of hybridization techniques in the clinical application [39, 41] might be a reason for usage decrease over the years, found in this review (Fig. 1).

DNA sequencing, usually combined with PCR-based methods had important diagnostic or prognostic potential [42]. Among the whole set of methods analyzed here over the years, direct sequencing and PCR dominate in the applications (Fig. 1). This is the most frequent combination of methods applied prevalently in Asia, but also in the rest of the examined regions with a similar trend (Fig. 2). The direct or Sanger sequencing method was the first-generation-sequencing method with only several genes sequenced at a time [43, 44]. In the years that followed, the fast progress of the sequencing techniques revealed the second generation in 2005 [45], the third-generation in 2010 [44, 46, 47], and even the fourth-generation of sequencing methods appeared in 2012 [48]. Contrary to all the advances in sequencing technologies, the Sanger sequencing remains a gold standard for the confirmation of the results, despite the robustness and all the other constraints [49]. Even with the appearance of novel generations in the sequencing of DNA or RNA in the early 2010s, the prevalent application in that period for *EGFR* variants in NSCLC patients was direct sequencing and PCR with the highest score in 2013 (Fig. 1). One can notice their slight descending trend till 2019, but with the evident rising of the next generation sequencing (NGS) applications (Fig. 1).

To elucidate the importance of the next generation sequencing herein results were separated from direct sequencing and showed an uptrend over the years with the highest percentage in the application in 2019 (Fig. 1). Namely, the sequencing methods evolved in terms of lowering the costs, reducing time, and increasing the length read, enabling the sequencing of the whole-genome, the whole-exome, but also the whole-transcriptome [48, 50, 51]. The second-generation or the NGS performed as massively parallel, simultaneous sequencing reactions [45], thus referring to Roche 454, Illumina Solexa, and ABI-SOLiD technologies [48]. The main advantage of the NGS is the ability to reveal the novel variations that cannot detect with genotyping, particularly the SNPs that could affect the activity of anticancer therapeutics [7, 25–28, 52]. The NGS relay on PCR that could introduce some errors, but it generally improved higher sensitivity. In this review, various applications of the NGS identified, i.e., SEQUENOM MassARRAYiPLEX assay is applied frequently for the *EGFR* SNP detection [53, 54]. Another NGS method is CAPP-Seq-cancer personalized profiling by deep sequencing performed in circulating tumor DNA obtained from patients with NSCLC who developed T790M mutation connected with acquired resistance of EGFR-TKIs, and recurrence of the lung cancer [55]. The technological advancements enabled the variety of platforms as the third or even fourth generation sequencing technologies, reported as accurate, faster, without previous PCR amplification step, with the lower amount of starting sample, and lower costs than the NGS [44, 46–48, 52, 56]. Anyhow, these methods are still time-consuming for the clinical setting, labor-intensive, they require bioinformatics expertise in management and extraction of clinically relevant data, and due to equipment costs, almost not applicable in low- to middle-income counties [1, 44, 51, 57].

Another set of methods reported lower costs, simplicity but also high specificity and sensitivity, under this review termed recently developed (RD) methods. It involved certain novelties, applied prevalently in 2014 and 2015, but in lower percent than PCR or sequencing methods (Figs. 1, 2). Still, their involvement is not in the terms of single methods usage, but usually with confirmation of sequencing methods. For their broad application, further clinical investigations are recommended. The several following methods reported for high sensitivity, but they are referred usually to as common, activating, or acquired *EGFR* mutations, not to the novel *EGFR* variants in NSCLC patients. For example, gold nanoparticle (AuNP)–based platform [58, 59], or Mach–Zehnder Interferometer sensor and isothermal solid-phase DNA amplification technique (MZI-IDA sensor system) could detect even 1% of the mutant allele and could be applied without thermal cycling [60]. Sensitive and noninvasive methods for *EGFR* mutation detection were microfluidic paper-based electrochemical DNA biosensor (-PEDB) that used saliva of the patient [61], and the liquid-chip array for fast *EGFR* mutation detection in plasma [62]. A few of them were non-molecularly based methods applied to detect the interaction of EGFR in protein complexes like proximity ligation assay (PLA) in the terms of downstream EGFR signaling [63]. The mammalian-membrane two-hybrid assay (MaMTH) able to detect changes in those proteins caused by *EGFR* mutations [64], or PNA microarray-based fluorometric assay, detected common *EGFR* mutations in a highly sensitive and high specific manner [65]. Fluorescence resonance energy transfer-based preferential homoduplex formation assay (F-PHFA) is presented as an assay with a short turnaround time and relatively low cost. It could be implemented with real-time PCR equipment for the detection of the *EGFR* mutations in NSCLC patients derived from cell free-DNA samples [66].

Recently, two scientists Emmanuelle Charpentier and Jennifer A. Doudna have shared the Nobel Prize, for the discovery of DNA editing with clustered regularly interspaced short palindromic repeats (CRISP/Cas9) [67]. Thus, among all of these recently developed methods, the most promising is the CRISP/Cas9 reported as cost-effective, easily applicable, and suitable for clinic researches, for example in detection of the mutation with low allele frequency in cell-free DNA [68, 69].

Among all the NSCLC patients in Asia, a subset with good response to TKI had specific phenotype: Asian ancestry, women, adenocarcinoma histology, and non-smokers [16, 19, 22]. All the applied methods screened with this review were predominant in Asia (Figs. 2, 4). That corresponds with the evidenced larger fraction of mutant NSCLC cases there [22, 36, 70], and the technological progress related to economic development might be reflected in this application [71, 72]. The application of methods in all examined regions was in the following order: PCR, direct sequencing, and NGS, prevalently in Asia, followed by Europe and America, or consortium of different countries (Fig. 2). But to notice, the percent accounted for the final sum of eligible full-text articles, not for the number of patients included in the studies.

Oncological societies, the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) gave very detailed recommendations for usage of adequate assays in the specific molecular guidelines. Usually, they refer to the sequencing and PCR-based methods for advanced NSCLC [73–75]. In 2016, Food and Drug Administration (FDA) approved the Cobas® EGFR Mutation Test v2 (P120019/S007), based on the qPCR technology, as a diagnostic test in helping NSCLC stratification for treatment decisions [76], but the system and reagents might not be cost-effective for whole regions applications [65].

The sensitivity of methods for *EGFR* mutation detection in NSCLC patients in the context of molecular diagnostics is indispensable but affected by several limitations. Obtaining a good quality and high quantity of the DNA sample is a challenging issue from formalin-fixed paraffin-embedded (FFPE) tissues that might be contaminated with wild-type DNA [76]. Also, a high mutation detection limit of direct DNA sequencing (approximately 25%) and long turnaround time could affect further the clinical-decision making. To overcome this, highly sensitive tests have been developed and usually referred to PCR-based methods like amplification refractory mutation system (ARMS), with high specificity and sensitivity up to 1% [77], or even higher sensitivity of 0.12–2.73% reported with digital droplet PCR (ddPCR) [78]. However, in clinical researches, conventional PCR is still mostly utilized, followed by quantitative PCR, ARMS, and ddPCR (Fig. 3). But in the time of use context-the qPCR, dPCR, or even ARMS increased in usage from 2010 to 2019 (Fig. 3) contrary to the traditional PCR that has had a slight decrease in recent years, probably due to lower sensitivity than the methods mentioned above. Others applied in smaller percent with inconsistency in trends like HRMA, qRT PCR, RT PCR, or specific PCR methods (Fig. 3).

Adaptability is probably the most important issue connected with the advantages of the PCR-based methods that explain their numerous applications in the detection of the *EGFR* variants of NSCLC patients evidenced by this review. The progression was from endpoint PCR to more sophisticated methods [79–82]. Several novel PCR platforms detected here reported a higher sensitivity than conventional sequencing but referred to common mutations. The PCR-invader method [80], or the PNA clamping method might detect even 1% of mutant alleles [83, 84], and i.e. nanofluidic digital PCR arrays, able to detect and quantify *EGFR* mutations with a detection limit down to (0.02–9.26%) in a low amount of lung cancer tissue sample [81]. Some of the laboratory-developed tests, often affordable, and in here termed-the specific PCR methods, with relative simplicity in a real-life clinical setting were applied in the early 2010s mostly in Asia (Figs. 3, 4). Although they have significant application potential, usually referred to as highly sensitive, but also without clear and transparent procedures, for broader usage they need to be officially validated. It includes restriction fragment length polymorphism PCR (RFLP-PCR) [25], PCR invader method, and Cycleave assays [85], single-strand conformation polymorphism (PCR-SSCP) [86], mutant enriched PCR [87], and others.

The invasiveness of the surgical resections in usually inoperable advance-staged NSCLC patients accompanied with other comorbidities is replaced with the small biopsies which were proposed by the World Health Organization in 2015 [88]. The tendency of *EGFR* mutation detection is towards replacement with non-invasive, cell-free DNA-based assays with high sensitivity and specificity. Besides, the tissue-based assays usually require re-biopsy and have certain constraints due to the small quantity of sample, tumor heterogeneity, which might contain a low amount of the mutated cells, and high background of the wild-type DNA [89–91]. One of that highly precise and reproducible approaches is the digital PCR (dPCR) based on the water-in-oil emulsion, where simultaneously PCR reactions amplified in more than a million nanoliter droplets. It showed good performances even with poor quality of the DNA sample degraded by FFPE lung tumor tissue [78], but in further the good correlation for *EGFR* mutation detection in tissue and plasma samples [92]. Despite the evident increase in usage of dPCR in recent years (Fig. 3), it is predominantly applied in Asia (Fig. 4). However, the NGS methods and the dPCR have high costs

[93] that probably interfere with wide clinical applications of these approaches. Furthermore, the cost-effectiveness of qPCR, classical PCR, as well as ARMS, makes them mostly applied in Asia, Europe, or in the rest of the examined regions for *EGFR* variants detection (Fig. 4).

Conclusions

With this review, the focus was on methods for all reported and clinically relevant *EGFR* variants connected to response to therapy, progression-free survival, or overall survival of NSCLC patients through different regions of the world. Immunostaining techniques were applied earlier but with a decrease in usage in clinical research similar to the hybridization and proteomic techniques. The PCR and sequencing methods remain a golden standard in clinics, with evident influence and increase in usage of the NGS approach. A variety of sequencing and PCR platforms exist nowadays, reporting high sensitivity, and specificity for the *EGFR* variant detection, short turnaround time, high reproducibility, or even simplicity in procedures, but the costs usually restrict their broader application. Among recently developed methods, the most promising are the CRISP/Cas9 and the ddPCR. Concerning the key issues from this review, all the tested methods are prevalently applied in Asia. A precise, rapid, uniform, and widely applicable test with diagnostic or prognostic purposes is a big challenge for the future, with the improvement of NSCLC patients' quality of life as the main and urgent aim.

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Author contributions Conceptualization, editing and supervision: VJ. Formal analysis, investigation and writing: JO. Search the database of papers: JT.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Consent to participate The work was done according to all ethical principles and criteria of good scientific practice and data used from official databases that were processed statistically. The study does not contain personal data about patients nor does it include animal studies.

Consent to publish All authors agree with the publication of the research.

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