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Editorial

Advancements in next-generation sequencing for diagnosis and treatment of non-small-cell lung cancer

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In recent years, lung cancer has been the most commonly diagnosed cancer globally; 1.6 million people died of lung cancer in 2012 globally, making lung cancer the leading cause of cancer-related deaths.[1,2] Lung cancer can be mainly histologically classified into two types: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), accounting for approximately 85% and 15% of cases, respectively.[3] NSCLC can be further classified as squamous cell carcinoma, adenocarcinoma, and large-cell lung carcinoma, among which lung adenocarcinoma is the most common primary malignant tumor. Unfortunately, most NSCLC cases are diagnosed at a late stage when the survival rate is low; the 5-year survival rate is approximately 16%.[1,2]

Researchers have shown that lung cancer is a heterogeneous disease and that genome alteration plays a significant role in disease occurrence. In recent years, there have been large technological advances in genomic testing. Next-generation sequencing (NGS) is gradually replacing traditional capillary-based single-gene sequencing, such as a first-generation technique known as Sanger sequencing. Compared with traditional methods, NGS can comprehensively sequence complete genomes, exomes, and transcriptomes and identify novel chromosomal rearrangements and copy number alterations in some platforms; NGS also enables massively parallel sequencing with lower cost and higher throughput,[4] which helps to overcome the tumor heterogeneity and complexity. These advancements are important for understanding the occurrence of tumors such as lung cancer as well as for diagnosis and therapy (particularly the molecularly targeted therapies) of tumors. Additionally, NGS also plays a significant role in monitoring the curative effect of agents.

In this review, we summarize the current progress in the application of NGS for diagnosis and treatment of NSCLC.

Introduction of NGS

NGS typically refers to the second- and third-generation sequencing technologies which are more efficient and show higher throughput than first-generation sequencing technologies such as Sanger sequencing. Although the total cost of NGS is higher, it can provide more gene information for patients using

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much less tumor material, and costs are expected to decrease with the rapid development of NGS technology. Depending on the target sample resource and coverage, NGS includes, but is not limited to, whole-genome sequencing (WGS), whole-exome sequencing (WES), whole-transcriptome sequencing (RNA-seq), and targeted sequencing (both DNA and RNA).[5] WGS, WES, and RNA-seq are mainly applied to obtain large amounts of biological information through large-scale sequencing and to identify genetic alterations such as gene mutation and amplification. Because of the difficulty in managing large masses of data from NGS and the influences of the patients' surrounding environment on the results, further studies are needed to optimize this technique. However, the complexity of data analysis may be reduced by simultaneously testing samples from normal surrounding tissues and the blood. In contrast, targeted sequencing mainly identifies and confirms genome alterations related to cancer genes through deep sequencing genomic regions of interest. This may increase the sensitivity of detection and significantly reduce the cost and time of sequencing as well as complexity of data analysis, making this method a feasible alternative to currently used sequencing methods. Commonly used platforms of NGS include Roche-454, Illumina-Solexa, and ABI-SOLiD.[6] Different methods are applied in different platforms. For example, HiSeq 2000 Illumina-Solexa is mainly used for WGS, WES, and RNA-seq, while Myseq Illumina-Solexa is mainly used for WES and targeted sequencing.[7] The sequencing process can be summarized in three main phases, starting with sample preparation, followed by the sequencing step. The final step is the analysis of bioinformatic data, which is considered as the most critical phase. NGS has been used in lung cancer to identify promising biomarker candidates for early diagnosis, detect causative mutations in clinical cases, and guide targeted therapy decisions.[5,8−11]

Applications of NGS in clinical diagnosis and treatment

The mortality rate of lung cancer tends to be high for a few reasons. First, both SCLC and NSCLC are typically diagnosed at a late stage when treatments are not as effective as at an early stage.[12] Second, even if lung cancer is diagnosed early, the success rate of treatments is lower than for other types of cancer such as breast cancer.[13] Third, and most critically, the mutation burden of patients with smoking history is higher than that of patients with age-related cancers.[14] The third reason plays a crucial role in the pathogenesis of lung cancer because as somatic gene mutations accumulate, the number of driver genes also increases, affecting the initiation and progression of lung cancer. This leads to the low possibility of confirming one targeted gene with corresponding agents appropriate for that specific mutation in most cancer patients. Clinically, feasible therapeutic regimens are limited for tumor patients with several driver genes.

However, gene alterations of lung cancer can be diagnosed early in biopsy tissues and driver genes with targeted agents can be identified with NGS, which are very helpful for patients. NGS may be useful for monitoring the effect of treatment and patient conditions by continuously sequencing driver genes to identify novel gene mutations in patients.

Below, we describe the application of NGS in the diagnosis and therapies of NSCLC.

Applications in diagnosis

Detecting driver genes of NSCLC and its advantages

The gene alterations conferring a selective advantage to tumor cells of NSCLC include epidermal growth factor receptor (*EGFR*) mutation, Kirsten-rous avian sarcoma (*KRAS*) mutation, anaplastic lymphoma kinase (*ALK*) rearrangement, human epidermal growth factor receptor-2 (*HER2*) mutation, v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutation, receptor tyrosine kinases rearrangement, reactive oxygen species 1 (*ROS1*) rearrangement, phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*) mutation, and mesenchymal-epithelial transition (*MET*) amplification,[15] which play crucial roles in the neoplasia and proliferation of tumor cells. These gene mutations or amplification may be useful for predicting ideal targets of specific agents.

Formalin-fixed paraffin-embedded (FFPE) tissue is considered a gold standard in cancer diagnosis; however, the possible substandard quality of FFPE samples and the temporal and spatial heterogeneity of samples make the use of traditional diagnostic methods challenging. Lim et al[16] performed comprehensive genomic profiling (CGP) of advanced lung adenocarcinoma cases using an NGS-based clinical cancer gene assay. FFPE tissues from 51 lung adenocarcinoma patients whose tumors were previously tested negative for *EGFR*/*KRAS*/*ALK* by conventional methods were collected; CGP was performed via hybridization capture of 4557 exons from 287 cancer-related genes and 47 introns from 19 genes frequently rearranged in cancer. They found that 58% of wild-type patients as determined by standard testing for *EGFR*/*KRAS*/*ALK* showed genomic

alterations identifiable by CGP, suggesting the possible benefits of targeted therapy. When standard molecular testing for NSCLC is negative, CGP can be used to determine targeted therapy. Hagemann et al[17] and Moskalev et al[18] conducted relevant studies that also support this conclusion. Recent years have demonstrated the very similar genetic characteristics between circulating tumor DNA (ctDNA) and DNA in tumor tissues, with a consistency of 60−95%, highlighting the importance of gene detection based on ctDNA.[19]

NGS is a useful and efficient method for gene detection and can provide more comprehensive information with much less tumor material than traditional methods such as Sanger and polymerase chain reaction (PCR), as well as assess gene mutations including driver genes from critical samples and ctDNA. This enables the identification of patients who may be sensitive to different targeted agents before treatment to help doctors design appropriate therapies. Moreover, the test results can be available in a relatively short time and guide the diagnosis and targeted treatment of lung cancer. Gao et al[20] evaluated the feasibility of the NextDaySeq-Lung panel, an NGS-based assay for mutation analysis of key driver genes in lung cancer, in a clinical setting. In total, 138 FFPE samples of NSCLC were examined in parallel with assays developed for NGS, quantitative PCR (qPCR), and Sanger sequencing (Sanger) platforms to detect somatic mutations in *EGFR*, *KRAS*, *PIK3CA*, and *BRAF*. In this study, the assays with the three platforms were compared and analyzed. The results showed that compared with Sanger, the NGS and qPCR assays exhibited significantly higher sensitivity, as Sanger failed to detect variants with mutation rates lower than 15%. Additionally, NGS and qPCR assays showed similar analytical sensitivity, specificity, and high concordance. The NGS assay exhibited further advantages over qPCR in providing accurate information regarding allele sequence and mutation frequency, as well as detecting non-hotspot mutations. The authors showed that the NGS assay has significant technical advantages over Sanger and qPCR assays and has good potential as a molecular diagnostic assay in the clinical setting.

Because of the dramatically different responses to the same targeted agent among patients with different gene mutations, it is of great importance to identify the genotype of patients before designing treatment regimens to avoid the high costs and side effects associated with futile treatment or revision of treatment programs following the outcomes of gene sequencing. To accomplish this, NGS demonstrates advantages over traditional sequencing methods for higher throughput and shorter testing time, enabling the sequencing of several hot spots simultaneously in a short time. Traditional methods can only detect one hot spot at one time; therefore, NGS reduces costs and generates useful gene information in lung cancer to help clinicians in the diagnosis. NGS methods meet the obvious need for improved, fast, and highly efficient molecular pathology approaches.

NGS samples

NGS is a high-throughput technique used to obtain accurate sequencing results with less DNA from limited biopsy tissues when the amount of material is too limited for traditional testing. The samples must be kept under conditions that maintain the integrity of nucleic acids.[21] Tissue samples are often FFPE, and DNA extracted from these tissues can still be used for gene sequencing; however, many factors of FFPE processing such as storage time may disturb DNA integrity.[22] Additionally, biopsy is a painful procedure for the patients and can be technically challenging for operators to perform based on tumor locations.[23] Also, heterogeneity exists between primary and secondary tumors or even inside a single mass, causing different responses to treatment targeted at one single point. Different samples from multiple areas can be sequenced by NGS to partially overcome the tumor heterogeneity and guide combination therapy targeted at different points.[23−25] However, it is not feasible to monitor the effect of treatment by continuous biopsies over the long term. One promising alternative to taking biopsies and sequencing tumors is to sequence ctDNA via non-invasive blood draws,[26,27] which may make cancer detection a standard laboratory assay. This non-invasive test can be conducted easily and repeatedly, and meanwhile, ctDNA may potentially reflect all heterogeneous genetic mutation profiles which may not be realized in conventional tissue biopsy. Given that, ctDNA can provide new insight into diagnosis of NSCLC. In addition, Velizheva et al[28] emphasized the suitability of non-formalin cytology specimens for simultaneous NGS testing of lung adenocarcinoma using amplicon resequencing panels and showed that NGS allowed for the input of cytological smears equal to concurrent histology or cell blocks, which were shown to be accurate for the detection of therapeutically actionable somatic mutations and gene rearrangements.

Applications in treatment

Predicting response to individualized treatment

When prescribing personalized medicine to NSCLC patients, clinicians should conduct genotype analysis,

and NGS is an appropriate approach. NGS can provide doctors with applicable information regarding driver genes for targeted treatment to determine the efficacy of drugs and prognosis of patients. The applications of NGS in lung cancer mainly depend on the spots with targeted agents detected using this method. For instance, numerous *EGFR*-tyrosine kinase inhibitors (TKIs) are targeted at patients with *EGFR* mutations, including the first-generation drugs gefitinib, erlotinib, and icotinib, second-generation afatinib and neratinib, and third-generation drug AZD9291. Patients may benefit from *EGFR*-TKIs if they are *EGFR*-positive according to NGS. For advanced patients with *EGFR* mutation-positive lung cancer, the objective response rate and progression-free survival (PFS) period were obviously better following *EGFR*-TKI therapy than after platinum-doublet chemotherapy.[29,30] However, *KRAS* mutation is a negative predictive factor of *EGFR*-TKIs and mainly causes primary tolerance[31]; thus, patients with *KRAS* mutations should not be treated with *EGFR*-TKIs.[32] Marchetti et al[33] were the first to describe a correlation between clinical response and a semi-quantitative index (reflects the trend in the proportion of mutated versus wild-type copies of the *EGFR* gene) of mutated *EGFR* levels in the first days of treatment. Serial ctDNA specimens were prospectively collected from 20 NSCLC patients harboring activating *EGFR* mutations during *EGFR*-TKI treatment. Two different quantification approaches showed that the PCR test designed for detecting *EGFR* mutations was extremely sensitive. However, because PCR-based assays use primers with known mutations to amplify mutated *EGFR* sequences, this approach will miss uncommon genetic alterations that can be detected by NGS in one run.

As another example, *ALK* fusion can be detected by NGS. Crizotinib, a dual inhibitor of *c-MET*/*ALK*, shows high potential for the treatment of patients harboring *ALK* fusions. PROFILE 1001[34] initially demonstrated the effect and tolerance of crizotinib in *ALK*-positive NSCLC patients and PROFILE 1005[35] further confirmed the safety and effectiveness of crizotinib. Further comparison of crizotinib and docetaxel/carboplatin to determine the efficacy of second-line therapy for advanced *ALK*-positive NSCLC patients was conducted in the PROFILE 1007 trial.[36] The results showed that PFS of the crizotinib group was significantly improved compared to the chemotherapy group (7.7 *vs*. 3 months, $P < 0.0001$). The results of PROFILE 1014 demonstrated that crizotinib was better than chemotherapy for the treatment of *ALK*-positive NSCLC patients with non-squamous cell carcinoma.[37]

In addition, NGS can provide a comprehensive mutational assessment including uncommon mutations such as *PIK3CA* and *ROS1*, which play a pivotal role in the proliferation of neoplastic cells and represent ideal targets of specifically designed agents. Other gene alterations with the potential to be designed as drug targets might also be identified by NGS.

Drug resistance

Currently, therapies for advanced NSCLC patients are extremely complex and resistance in some patients to various therapeutic drugs is one of the most important reasons for treatment failure. Primary drug resistance refers to the presence of gene alterations that cause resistance to specific agents before treatment, while secondary resistance can be caused by the mutation and amplification of resistance genes induced by the medicine; in secondary resistance, initially useful agents may decrease in effectiveness or become ineffective during therapy. A few studies showed that NGS can be used to detect resistance genes in patients with tolerance to guide treatment choices.[7] Additionally, NGS technology has the potential to predict resistance at the genetic level before imaging progress or clinical progress to enable earlier effective treatment of patients.[38]

For example, the T790M mutation causes *EGFR* mutation-positive patients with lung adenocarcinoma to be resistant to *EGFR*-TKIs such as gefitinib, which is relatively common in the clinic. Recurrence and progression (PFS 9−10 months) will eventually occur in nearly all patients after they undergo initially effective treatment with *EGFR*-TKIs, which likely results from tolerance development.[39,40] Third-generation TKIs targeting T790M mutation, such as AZD9291 and CO-1686, show potential for treating resistant patients with an effective rate >50% and PFS >9 months in phase I and II trials.[40] Doctors can change the therapy according to the results of NGS after the development of resistance to TKIs in *EGFR*-positive patients or predict resistance before progression. Several studies have reported the reduction or even disappearance during TKI treatment of *EGFR*-activating mutations in the plasma, which reappeared together with the T790M resistance mutation. In these studies, the T790M mutation in the plasma was detected 15−344 days before disease progression based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria.[41] Because of the advantages of using liquid biopsy, NGS makes frequent sampling possible, leading to early detection of resistance.

Similarly, although the initial effect of crizotinib to *ALK*-positive patients is positive, most patients will have acquired resistance by 1 year after starting therapy.[42] Approximately 1/3 of patients receiving crizotinib show recurrence during treatment because of resistance mutations in *ALK* or amplification of the *ALK* fusion gene[43]; some patients exhibit activation of other *ALK*-related signaling pathways.[44] In addition, resistance to crizotinib can also be caused by amplification of the c-Kit gene and other potential bypass mechanisms of resistance, including activating mutation of *KRAS* and *EGFR*. All of these resistance mutations may be detectable with NGS in the clinic in one test.

The apparent advantage of NGS lies in its potential to identify previously unknown modes of resistance that cannot be easily detected by other methods. Jin et al[45] performed mutational profiling in a cohort of 83 NSCLC patients with TKI-sensitizing *EGFR* mutations at diagnosis and who had acquired resistance to three different first-generation *EGFR*-TKIs using targeted NGS of 416 cancer-related genes. They identified 322 genetic alterations with a median of 3 mutations per patient. A total of 61% of patients still exhibited TKI-sensitizing *EGFR* mutations, and 36% of patients acquired *EGFR*-T790M. In addition to other known resistance mechanisms, they identified *TET2* mutations in 12% of patients. Interestingly, they also observed *SOX2* amplification in *EGFR*-T790M-negative patients, which are restricted to icotinib treatment resistance, a drug widely used to treat Chinese NSCLC patients.

Limitations of NGS in clinic

Even if NGS technology shows high potential for the diagnosis and therapy of NSCLC, such as detecting gene mutations that can be treated with targeted agents and resistance genes when patients show resistance to some agents, there are also some limitations to NGS such as inconsistencies between NGS results and clinical observations. Moreover, additional studies are needed to evaluate the large number of mutations observed by NGS for development of effective therapeutic targets. The accurate analysis and reliability of the information achieved by NGS remains challenging and this method increases the difficulty of explaining the results because of tumor heterogeneity, which makes detection of low-level mutations difficult and is influenced by the surrounding environment.[7] Most studies only reported a series of gene mutations, but did not analyze the effects of these mutations on tumor invasion; thus, additional studies are needed.

Conclusion and future prospects

In summary, although there are still some limitations to NGS technology, its value has been demonstrated in clinical studies. NGS can not only improve the diagnosis of lung cancer in the clinic, but also provide genotyping of NSCLC (particularly lung adenocarcinoma) at the genetic level and confirm the presence of driver genes, providing useful information for individualized medication and targeted therapy in the clinic. Lung adenocarcinoma has an obvious advantage for personalized treatment because of the substantial effect of *EGFR*-TKIs and *ALK*-TKIs in patients with certain driver genes.

Additionally, this method can explain the resistance of patients to certain medicines after initially effective treatment through comprehensive sequencing. Gene mutations can be reassessed in patients before changing therapies, improving the prognosis of patients. Moreover, NGS may observe gene alterations before the clinical resistance of patients because alterations may be detected at the genetic level before the appearance of detectable changes caused by the alterations. These alterations could be found later by biopsies or rebiopsies in therapy monitoring. Sanger sequencing remains the gold standard for detecting a small number of biological markers with limited sensitivity (approximately 20%), as sequencing with specific primers is required to identify somatic mutations; NGS may be advantageous for early detection with higher sensitivity. Currently, NGS is mainly used in lung cancer for DNA sequencing, but may be applied in other aspects such as RNA sequencing and chromatin immunoprecipitation (ChIP) sequencing to sequence the small RNA and epigenetic genome to provide complementary information and enable cross-validation.

Furthermore, massive genomic information gathered by NGS provides a great opportunity to explore more gene mutations that may be promising therapeutic targets. Several driver genes will gradually accumulate over time or during drug treatment, causing changes in the biological behavior of tumor cells; NGS may be useful for detecting changes in driver genes as well as predicting the biological behavior of tumor cells and multiple resistance genes.[14]

NGS is expected to become widely used in the clinic as the technology improves, leading to the early

diagnosis of NSCLC and identification of a greater number of driver genes and potential drug targets.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article.

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