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Development of accurate detection methods for hotspot mutations in non-small cell lung carcinoma

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Abstract

Non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) are two major types of lung cancer and are responsible for the highest mortality rates. The rate of NSCLC is approximately 80% to 85% of lung cancers. Hotspots in cancer can be defined as sites in DNA and proteins that are more likely to be mutated. Hotspot mutations are also related to the treatment of the corresponding cancer as the development of targeted agents acts as a major driving force. In this paper, the comprehensive synthesis of results is shown from studies on hotspot mutations in NSCLC. The findings indicate the specific location of the hotspot mutation with the corresponding sequencing method in diagnostic yield and several treatments.

Keywords: Non-small cell lung carcinoma; Small cell lung carcinoma; Hotspots mutations; Human epidermal growth factor receptor; Kirsten rat sarcoma mutations; Anaplastic lymphoma kinase

1. Introduction

Cancer is characterized by abnormal cell proliferation, and these proliferated cells can invade other parts of the body. Among all types of cancers, lung cancer has been reported as one of the leading causes of cancer-related mortality worldwide [1-4]. The two major types of lung cancer are Non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC), with NSCLC accounting for approximately 80-85% of lung cancers [1-4], and are responsible for the highest mortality rates associated with lung cancer.

Oncogenic drivers, known as “hotspots”, can be defined as specific mutated sites in DNA and proteins that are more susceptible to mutations. Identifying hotspot mutations are crucial to the treatment of corresponding cancers as the development of targeted agents acts as the major driving force of specific therapies. Therefore, identifying the precise location of hotspot mutations with their corresponding sequencing methods for diagnosing NSCLC has become a vital step towards the specific personalized treatment of lung cancer [1-4].

Over the past few years, targeted therapies aimed at specific hotspot mutations have become prominent for treating NSCLC worldwide. Key mutations include epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), ROS1, and KRAS. With the power of several next generation sequencing (NGS) methods, the accuracy and precision of diagnosis has been enhanced remarkably [1-4].

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2. Specific Location of the Hotspot Mutation

2.1. EGFR Mutations

The human epidermal growth factor receptor (EGFR) is a member of the ERBB tyrosine kinase family, which consists of 4 distinct receptors. [5-12]. As the expression of EGFR increases, its kinase activation signal is upregulated, which in turn promotes proliferation, neogenesis, angiogenesis, and migration of various types of cells, especially epithelial cells. Therefore, EGFR mutations have become a focal point in research related to NSCLC, accounting for 19% of overall observed NSCLC hotspot mutations. Based on a study in 2017, EGFR is the predominant oncogenic driver in NSCLC within Asia-Pacific and Russia, with an incidence rate of 49.3% [5-12]. The three most common EGFR mutations, exon 19 in-frame deletions (45%), and exon 21 L858R mutations, along with other exon 21 mutations, account for approximately 40-45%. These mutations make up 85% of observed EGFR mutations in NSCLC and 90% of all clinically significant mutations associated with EGFR sensitivity [5-12] (Harrison et al. 2020). In addition to the two classic mutations above, rare mutations such as exon 18 G719X, exon 21 L861Q, exon 20 S768I, and compound mutations are observed, but occur with low frequency. Given that patients with this subtype of NSCLC have the optimal response to TKI therapy, EGFR mutation screening is recommended in the clinical care of NSCLC patients [5-12].

2.2. KRAS Mutations

The Kirsten rat sarcoma viral oncogene homolog (KRAS) is located on the chromosome 12p11.1-11.2, comprises six exons, and was first detected in the NSCLC gene in 1984. The KRAS encodes a membrane-bound protein that is situated on the intracellular surface of the cell membrane, and also has been found on the EGFR signaling pathway, which is integral for the occurrence and development of tumors. The KRAS gene is the determinant in the transduction proteins that regulate the growth, proliferation, angiogenesis, and other processes of tumor cells that require intracellular proteins for signal transduction [13-28]. KRAS mutations result in the encoding of abnormal proteins that stimulate and promote the growth and metastasis of malignant tumor cells without being affected by upstream EGFR signaling. KRAS mutations are one of the most prevalent hotspot mutations in NSCLC, with an incidence rate of approximately 30%. The most common KRAS mutations include substitutions in exon 12 or 13 (G12C, G12D and G12V), with G12C mutation being the most common, especially for smokers, followed by G12V mutations. Recent data suggest that NSCLC with KRAS mutations may be a molecularly diverse entity that can coexist with EGFR mutations or EML4-ALK translocations [13-28].

2.3. ALK Mutations

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase abnormally expressed in several tumors. As a member of the insulin receptor superfamily, ALK consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular casein amino acid kinase domain. The ALK protein regulates cell proliferation and apoptosis by activating downstream signaling pathways including STAT3 and MAPK, RAS/ERK, PI3K/AKT and many others[13-28].

Echinoderm microtubule-associated protein 4 (EML4) is part of the echinoderm microtubule-associated protein-like family, consisting of an N-terminal basic region, a HELP domain, and a WD repeat region. Due to a small inversion of the short arm of chromosome 2, the EML4 N-terminal basic region, HELP domain, and part of the WD repeat region are fused with the tyrosine kinase domain of ALK, resulting in the formation the EML4-ALK fusion gene. Notably, all regions of the EML4 in the fusion gene exhibit oncogenic activity, with the basic region demonstrating the highest oncogenic activity [13-28].

This oncogenic activity is highly dependent on the dimerization of fusion partners EML4 and ALK to activate the tyrosine kinase. Chromosomal rearrangements at the ALK gene locus on chromosome 2 accounts for 3%-5% of NSCLC tumors. The most recurrent ALK-related mutation in NSCLC refers to the formation of the EML4-ALK oncogene, where the 5' end of the EML4 gene is fused to the 3’end of the ALK gene [13-28].

2.4. BRAF Mutations

The BRAF gene was initially identified and cloned in human Ewing sarcoma by Ikawa et al. in 1988 [29]. It is an active DNA sequence capable of transfecting NIH3T3 cells due to its high homology with both CRAF and ARAF. Located on chromosome 7q34, the BRAF gene encodes a serine/threonine protein kinase and is a member of the RAF family. BRAF is mainly expressed in neural and testicular tissue, whereas CRAF is widely distributed throughout the human body [13-28].

RAF acts as a downstream signaling mediator of KRAS and can activate the mitogen-activated protein kinase (MAPK) pathway. Activating mutations in BRAF are found in 1% to 3% of NSCLC cases and are often associated with a history of smoking. These mutations can occur at the V600 site of exon 15 (as is the case in melanoma) or outside this region; they are typically detected using PCR sequencing or NGS methods [30].

In recent years, studies have indicated that BRAF protein can act independently of RAS protein. BRAF gene mutations are independent and mutually exclusive from EGFR and KRAS mutations, which means they do not occur at the same time. BRAF and KRAS gene mutations may occur at similar stages of carcinogenesis, after initiation but before malignant transformation, suggesting a similar role in carcinogenesis [30]. Biologically, BRAF mutations can enhance the kinase activity and structurally activate MAPK2 and MAPK3. Unlike EGFR and KRAS mutations, which are more prevalent in non-smoking lung cancer patients, BRAF mutations are predominantly observed in NSCLC patients with a history of smoking [30].

2.5. ROS1 Mutations

ROS1 is classified within the insulin receptor family of class II receptor tyrosine kinases (RTKs). In 1982, ROS1 was identified as a proto-oncogene with unique carcinogenic effects from UR2 avian sarcoma viruses [13-26]. The ROS1 gene is located on the q21 region of chromosome 6 in humans with cDNA full-length of 4 exons, encoding a protein of 2347 amino acids, and has a molecular weight of 259 kDa. According to recent studies, at least 9 different types of NSCLC have been identified ROS1 fusion genes, including FIG-ROS1 and CD74-ROS1, first discovered in glioblastoma, as well as SLC34A2-ROS1, TPM3-ROS1, SDC4-ROS1, EZR-ROS1, LRIG3-ROS1, KDELR 2-ROS1, and CCDC6-ROS1 [13-26]. Recent studies have shown that ROS1 RTK gene rearrangements exist in various malignant tumors, including NSCLC. As a newly discovered subtype of NSCLC, ROS1 gene rearrangement accounts for approximately 1%-2% of NSCLC. This subtype predominantly affects young, non-smoking patients with lung adenocarcinoma, mirroring the clinical characteristics with those of ALK-rearranged NSCLC [13-26].

3. Diagnostic Sequencing Method

3.1. FISH (Fluorescence in Situ Hybridization)

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique that uses fluorescent labels instead of isotope labels to precisely locate the positioning of specific nucleic acid sequences. It has been widely used in clinical pathology and plays a vital role in accurately diagnosing various types of diseases including cancer, guided clinical targeted therapy, and evaluating patient prognosis [31-32].

FISH is used as the preferred detection method by some due to its short experimental cycle, rapid results, high specificity, and relatively low cost. Moreover, FISH detection has been used as a detection method to verify the immunohistochemistry results of these genes. Some domestic and international guidelines recommend FISH testing as a testing method [31-32]. Through FISH analysis, hotspot mutations in NSCLC which include point mutations and structural rearrangements in proto-oncogenes, such as: EGFR, and BRAF can be easily identified. However, detecting rearrangement in genes such as ALK, ROS1, RET and NTRK can be challenging as these genes are often accompanied by multiple partner genes, and FISH is unable to identify specific partner genes, only the changes in these genes. This leads to some difficulties detecting ALK and ROSI for NSCLC [31-32].

3.2. IHC(Immunohistochemistry)

Immunohistochemistry (IHC) was previously regarded as a sensitive and specific alternative to FISH for evaluating ALK-positive NSCLC, being able to deliver reliable results within one day. However, IHC is still evolving in terms of detecting other targetable genomic alterations [33-35]. For NSCLC, IHC is the only conclusive method for assessing PD-L1 protein staining as PD-L1 protein expression does not appear to be linked to known genomic alterations in the PD-L1 gene. In addition to tumor genotyping, tumor PD-L1 protein expression should also be detected to determine first-line treatment options for NSCLC in addition to chemotherapy. However, IHC lacks a standard global protocol, and the cost of this technology is relatively expensive in comparison to FISH, primarily due to its precise equipment. Additionally, it is difficult to obtain quantitative results with high specificity [33-35].

3.3. Next-generation Sequencing (NGS)

Next generation sequencing (NGS) is a comprehensive technology and can be classified into DNA-based and RNA-based analyses. This new technology overcomes many of the limitations of Sanger sequencing, direct sequencing, and allele-specific testing, and is rapidly being adopted by more centers [36-40]. NGS utilizes a highly parallel approach, which

relies heavily on automation, data storage, and computational processing power. This enables the quantitative analysis of rare alleles and the simultaneous assessment of multiple genes or even entire genomes [36-40]. NGS remains sensitive even in samples with few tumor cells, providing advantages over direct sequencing and the ability to identify novel abnormalities that cannot be detected by allele-specific testing. It is sensitive enough to identify many molecular rearrangements in blood samples [36-40]. Furthermore, NGS can often detect intronic changes that were previously only detected by FISH.

However, NGS still has its limitations, primarily related to the amount of data generated, which can necessitate substantial data storage capabilities as well as a skilled bioinformatics team. The U.S. FDA has approved certain ctDNA tests (DNA-based NGS) for identifying EGFR mutation-positive patients, with one of the tests using NGS being capable of identifying abnormalities in 55 genes. As more data becomes available, the use of liquid biopsies to evaluate other molecular abnormalities may become increasingly widespread [36-40].

In November 2023, the "Chinese Expert Consensus on Clinical Practice for Detection of Fusion Genes in Non-Small Cell Lung Cancer Based on RNA-based NGS" was officially published in the "Chinese Journal of Lung Cancer". One point in this consensus is that fusion genes detected by RNA-based NGS can guide targeted therapies for fusion variants in NSCLC. Additionally, current RNA-based NGS can detect driver gene fusions such as ALK, RET, ROS1, NTRK, NRG1 and MET. This promotes the combined application of DNA-based and RNA-based NGS for one-time simultaneous testing in the clinical diagnosis and treatment of NSCLC, enabling patients to benefit greatly from fusion gene testing [36-40].

3.4. Treatments of NSCLC

Chemotherapy can be a treatment option for stage 4 NSCLC if there are no genetic mutations involved, especially for patients at the last two phases [41- 47]. The combination of cisplatin or carboplatin with gemcitabine is a common first-line empirical treatment for advanced NSCLC. A major advancement in the treatment of advanced NSCLC has been the development of apoptosis-inhibitory drugs that target small tyrosine kinase molecules, such as EGFR gene mutations, ALK fusion genes, or ROS1 gene rearrangement. These drugs are known as tyrosine kinase inhibitors (TKIs), and they embody the personalized precision treatment of tumors. For this group of patients, molecular targeted therapy should be considered the first-line treatment. Unfortunately, after a year of TKIs, most patients would choose to undergo chemotherapy or other treatments. [41- 47]

In order to improve the efficacy of treatment, the combined application of chemotherapy and molecular targeted drugs should be considered. However, the effectiveness of chemotherapy in the treatment of advanced NSCLC is limited, with the possibility for significant adverse reactions. Its status has been actively challenged by emerging treatments such as molecular targeted therapy, anti-angiogenic therapy, and immunotherapy [41- 47].

In the past decade, molecular targeted therapy has dominated the advancements of NSCLC treatment. There are several combinations of targeted drugs corresponding to the above mutations [41- 47]. One example would be the EGFR-TKIs (e.g., Gefitinib, Erlotinib), which have significant clinical effects. Pemetrexed or Docetaxel combined with standard care can be applied for patients with ALK-EML4 mutation. However, KRAS mutations are associated with a poor prognosis after wild-type EGFR is treated with EGFR-TKI, but the relevant data is still controversial. Despite current research progress, the evidence that KRAS can be used as a target gene for therapy is still inconclusive. Due to the high cost of molecular targeted therapies, there is a pressing demand for the development of new NSCLC treatments.

Nowadays, immunotherapy has achieved promising results in phase I/phase II clinical trials for NSCLC, offering improved tumor response rate, reduced toxic side effects, and good patient tolerance [41-47]. This will provide a brighter prospect for NSCLC treatment. Key immune checkpoint inhibitors include Anti-CTLA4 and Anti-PD1/PD-L1 antibodies. Based on that, Nivolumab was invented as the fully humanized immunoglobulin targeting PD-1, and was the first of these checkpoint inhibitors to be approved by the FDA for the treatment of advanced NSCLC. In contrast, anti-CTLA-4 antibodies such as ipilimumab and tremelimumab have not demonstrated clinically meaningful efficacy as monotherapy in NSCLC [41-47]. However, there are still limitations of immunotherapy, including determining the optimal timing for taking immunological drugs, duration of treatment, deciding between monotherapy or combination therapy, identifying the most beneficial patient groups, drug resistance mechanisms, and strategies to overcome resistance

4. Conclusion

The presentation of hotspot mutations plays a crucial role in the diagnosis and treatment of NSCLC and various other cancers. Among those sequencing methods, NGS stands out for its great potential. As the market of immunotherapy of

NSCLC expands, it is poised to become a global trend in the field of prescience medicine. Therefore, addressing the limitations of immunotherapy can provide a new avenue for the development of treatment of NSCLC.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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