RESEARCH ARTICLE

Contribution of Next Sequencing Generation in Lung Cancer and Its Prognostic Implication

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ABSTRACT
Lung cancer is one of the most commonly diagnosed cancers worldwide. It is the leading cause of cancer-related deaths in both men and women.
In 2020, there were an estimated 2.2 million new cases of lung cancer and 1.8 million deaths due to the disease.
Historically, lung cancer has been more common in men, but the gap has been closing.
Smoking tobacco is the leading cause of lung cancer. Survival rates for lung cancer vary greatly depending on the stage at diagnosis and other factors. Overall, the prognosis for lung cancer is often poor, with a relatively low five-year survival rate compared to some other cancers.
In this work we aim to show new paths in the diagnosis of lung cancer, through the study of several mutations and proteins, mostly detected by Next-generation sequencing (NGS) which has significantly transformed our understanding of cancer, by providing high-throughput and cost-effective methods for analyzing genomic information. In the context of lung cancer, NGS has played a crucial role in advancing our knowledge of the disease, improving diagnosis and treatment, and guiding personalized medicine approaches. key points highlighting the importance of next-generation sequencing in lung cancer:
Comprehensive Genomic Profiling
Identification of Driver Mutations
Stratification of Patients
Predicting Treatment Response
Monitoring Disease Progression
Clinical Trials and Drug Development
Early Detection and Prognosis
A large meta-analysis has been done, as well as a detailed study of 86 patients diagnosed with lung cancer in the ANALIZA laboratory. In this sense the most frequently implicated mutations in this tumor have been analyzed, ALK, ROS1 and EGFR, the positions they occupy in the genes, in addition to the programmed death ligand 1 (PD-L1), an immune control protein, which is expressed in activated immune cells and in tumor cells, and how its identification allows us to direct treatment in a more optimal way.
In summary, next-generation sequencing has revolutionized the field of lung cancer research and clinical practice. By providing detailed insights into the genomic landscape of tumors, NGS facilitates personalized treatment approaches, early detection, and ongoing monitoring, ultimately leading to improved patient outcomes.
Keywords: biomarkers, mutations, lung cancer, next-generation sequencing NGS

Introduction:

EPIDEMIOLOGY

The Global Cancer Observatory estimated more than 2.2 million incident cases and approximately 1.8 million deaths worldwide by 2020, with Europe being the continent where the highest mortality rates can be seen, reaching 22.6/1000,000,000 inhabitants, along with North America and China, which have similar figures [1].

However, in the last ten years there have been notable advances in treatment and diagnosis, together with the cessation of tobacco consumption, which has led to a decrease in mortality, specifically 48% in men and 23% in women. [1]

Globally, lung cancer deaths in men are decreasing at an average of 2.9% per year, while in women, there is a slight increase in incidence. An estimated 1,796,144 people died from the disease in 2020. [2]

On the other hand, according to the Spanish Medical Oncology Society, a total of 31,282 people were diagnosed with this pathology, leaving it in 3rd position, behind colon and rectum cancer and breast cancer [3].

The National Institute of Statistics reports 22,438 deaths, including trachea and bronchial cancer, making it the leading cause of death from cancer [4].

HISTOLOGICAL VARIETIES

There are 2 histological varieties: non-small cell lung cancer (NSCLC), which is further subdivided into adenocarcinoma, characterized by mucin production and being the most frequent subtype in our environment in both men and women; squamous, which comprises 20%; large cell carcinoma (3%), and neuroendocrine carcinoma (1-2%). On the other hand, we have small cell lung cancer (SCLC), whose clinical course is the most aggressive. The mechanism associated with these factors is due to the induction of oxidative damage with consequent DNA deletion and alteration of somatic genes.

Some studies place asbestos exposure and asbestosis as an additive in terms of increased lung cancer mortality in non-smokers, as well as its synergistic effect in smokers [5].

GENETIC FACTS

To our knowledge there is not enough evidence to support the genetic involvement. However, first-degree relatives have an increased risk of developing it.

A meta-analysis of 28 case-control and 17 observational cohort studies of people with a positive family history found an RR of 1.84 (95 % CI 1.64-2.05) for developing lung cancer [6].

Other studies have identified polymorphisms in several enzymes, such as cytochrome p450 enzymes and DNA repair genes, as well as germline mutations in EGFR [6].

There is no Mendelian inheritance pattern in this type of neoplasm, but a relationship with CYP1A1 polymorphisms, capable of inducing the P-450 enzyme, has been demonstrated.

MOLECULAR MARKERS

The molecular basis of lung cancer is the gradual accumulation of genetic and epigenetic changes in the cell nucleus.

The proto-oncogenes stand out, especially the MYC family, RAS, HER, which are the most frequently altered, as well as TP53, RB and CDKN2A, suppressor genes known as anti-oncogenes and the LOH chromosomal alteration, located on the short arm of chromosome 3 [7].

For lung adenocarcinoma, the most relevant are the activating mutations of the epidermal growth factor receptor (EGFR) and KRAS [8].

In the former, they are found in about 15%, being present in a higher proportion in non-smokers (43% compared to 11% in smokers) [7].

EGFR is a gene located on the short arm of chromosome 7 and is responsible for encoding a transmembrane protein with abundant extracellular content, which acts as a bridge between this domain and the extracellular tyrosine kinase, hence the targeted therapy [1].

It plays a key role in angiogenesis, inhibition of apoptosis, metastatic progression and chemoresistance [1].

In advanced stages, it predicts a more favourable prognosis due to increased sensitivity to EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib, genitinib and afanitinib [1].

As for the ALK mutation, it is located on the long arm of chromosome 2 and is composed of 30 exons. Like the EGFR mutation, it belongs to a family of receptor proteins called RTKs (receptor tyrosine kinases).

It is activated by ligand binding to the extracellular domain, which triggers signalling pathways within the cell, MAPK, PI3K/mTOR, JAK-STAT and SHH, which promotes cell proliferation[1].

Apart from these, there is the ROS1 gene, located on chromosome 6q22, which consists of an N-terminal extracellular domain, a transmembrane region, a C-terminal intracellular domain and a carboxy-terminal tail. Despite sharing similar structural and functional characteristics to the ALK protein, it has a unique cellular domain containing 6 repeat motifs of similar composition to the extracellular matrix, giving it the characteristics of an adhesion molecule. Thus, when certain gene fusions occur and its activity is altered, oncogenic pathway cascades are activated: PI3K-Akt, mTOR, RAS-MAPK/ERK, VAV3, SHP-1 and SHP-2, which results in failures in cell differentiation, proliferation, growth and survival [1].

KRAS is located on the short arm of chromosome 12 and encodes a guanosine triphosphatase, involved in cell signaling. It regulates cell proliferation, maturation and differentiation.

This mutation occurs in one third of SCLC cases and is the second most common genetic mutation after p53 mutations.

On the other hand, KRAS mutations occur more frequently in long-term smokers, which confers a worse prognosis, in addition to the lack of targeted therapies.

Other driver mutations occur with a frequency of less than 1-4%, including ALK, ROS1, RET, HER2 and BRAF gene rearrangements [9].

The ALK gene rearrangement, however, is extremely important in the clinic because this mutation generates a fusion product, especially EML4, which is able to predict sensitivity to ALK tyrosine kinase inhibitors such as crizotinib and ceritinib.

NGS, a high-throughput DNA sequencing (next generation sequencing) methodology, is currently used to detect these genetic alterations [10,11].

PD-L1 (Programmed Death-Ligand 1) is a protein that plays a significant role in the immune system and is often associated with lung cancer and cancer immunotherapy. PD-L1 is a protein expressed on the surface of certain cells, including cancer cells and immune cells. Its primary role is to regulate the immune response by binding to its receptor, PD-1 (Programmed Death-1), on T cells. This binding inhibits the activity of T cells, preventing them from attacking cells expressing PD-L1, including cancer cells which can exploit the PD-L1/PD-1 pathway to evade the immune system. When cancer cells express PD-L1 and engage with PD-1 on T cells, it can suppress the T cell's ability to attack the cancer cells. This mechanism allows cancer to escape immune detection and continue growing [12,13].

Regarding the clinical use PD-1/PD-L1 inhibitors are commonly used in the treatment of non-small cell lung cancer (NSCLC), which is the most common type of lung cancer. They have shown significant efficacy, particularly in patients with high PD-L1 expression or advanced-stage disease.

Material and Methods

Meta-analysis: Most relevant literature was reviewed regarding the genes of interest, using the NCBI Pubmed database, in which massive sequencing techniques (NGS) have been used for their identification.

In the initial search, 558 articles were found ("lung cancer NGS"), of which we discarded 213 articles quoting the last two years.

In the next selection, a total of 7 articles were kept, out of the remaining 345, discarding those that referred to a specific mutation, specific treatment or lacked relevance to the study.

Table 1. Sample Size of Each Article.

Article	1	2	3	4	5	6	7
N° cases	102	390	533	1200	1141	9239	273

Case study: In our reference laboratory, an initial anonymized selection of 300 cases diagnosed with lung cancer and with genetic mutations that have already been studied was carried out, obtaining 86 case studies.

Subsequently, the results obtained are compared with those shown in the medical literature.

For our research, we used the Idylla kit, a molecular diagnosis kit based on automatic real-time polymerase chain reaction (PCR), with the aim of diagnosing specific mutations in different cancers, in order to carry out targeted therapy against them.

With regard to lung cancer, the EGFR genes (which detects 51 mutations in exons 18, 19, 20 and 21);

the ALK gene (17 fusions in EML4, KIF5B, HIP1, KLC1, TPR and TFG); and ROS1 (13 fusions in EML4, KIF5B, HIP1, KLC1, TPR and TFG); ROS1 (13 fusions in CD74, SDC4, SLC34A2, EZR, TPM3, GOPC and LRIG3); RET (7 fusions in KIF5B and CCDC6) and finally, METex14(transcript detection in exon 13). [14] On the other hand, we have the OncoKitDx (NGS) panel, responsible for detecting specificmutations in solid tumours.

This procedure consists of extracting DNA from the formalin-fixed, paraffin-embedded tissue block submitted for subsequent analysis of mutations in the gene, as well as analysis of the rearrangements of the ALK and ROS1 genes by means of a massive targeted sequencing study with the Action OncoKitDx panel and the NextSeq 550 platform (Illumina).

Subsequently, the sample is classified and the percentage of tumour cellularity of the sample studied is counted. The Action OncoKitDx panel allows sequencing of the complete exonic regions of the EGFR gene. The analysis of ALK gene rearrangements is performed using probes that cover the intronic regions in which breakpoints have most frequently been identified: intron 19 of the ALKgene and introns 31, 32, 33, 34 and 35 of the ROS1 gene [15]. In this way, the panel allows the detection of any of the possible rearrangements that occur between the regions adjacent to the indicated breakpoints of the ALK and ROS1 genes, regardless of the gene with which they are fused.

After DNA extraction from the submitted sample, enzymatic fragmentation and subsequent enrichment of the regions of interest by hybridisation with capture probes is performed on the NextSeq 550 platform by reversible cyclic termination synthesis, which allows the detection of mutations and rearrangements in the selected genes.

Bioinformatics analysis of the results is performed using the Data Genomics platform. The sequences obtained are aligned with the reference sequence and filtered according to quality criteria to identify variants. Both the panel and the analysis programme have in vitro diagnostictagging.

In addition, it is able to detect point mutations in the tumour with an allele frequency of 5%. However, at least 30% tumour cellularity is required to reach this detection limit, which can be compromised if the sequencing read depth is less than 200 reads.

The use of mass sequencing technology for comprehensive molecular annotation of tumours complies with the recommendations of the European Society of Medical Oncology (ESMO). [11] In biomedical research we frequently encounter qualitative data or variables, through which a group of individuals are classified into two or more mutually exclusive categories. Proportions are a common way of expressing frequencies when the variable under study has two possible responses, such as whether or not an event of interest occurs (illness, death, cure, etc.) [16,17].

When the aim is to compare two or more groups of subjects with respect to a categorical variable, the results are usually presented as double-entry tables called contingency tables [18,19].

Thus, the simplest situation of comparison between two qualitative variables is one in which both have only two possible response options (that is, dichotomous variables). In this situation the contingency table is reduced to a two-by-two table [20,21].

There are different statistical procedures for the analysis of contingency tables such as the test, Fisher's exact test, McNemar's test or Cochran's Q test, among others. This article will present the calculation and interpretation of the test as a standard method of analysis in the case of independent groups [20,21,22,23]

The test allows us to determine whether two qualitative variables are associated or not. If at the end of the study we conclude that the variables are not related, we can say with a certain level of confidence, previously set, that both are independent. [24]

To calculate it, it is necessary to calculate the expected frequencies (those that should have been observed if the independence hypothesis were true), and compare them with the frequencies observed in reality. In general, for an r x k table (r rows and k columns), the value of the statistic is calculated as follows[25].

$$\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{k} \frac{\left(O_{ij} - E_{ij}\right)^2}{E_{ij}}$$

Thus, the statistic measures the difference between the value that should result if the two variables were independent and the one that has been observed in reality. The greater this difference (and, therefore, the value of the statistic), the greater the relationship between both variables. The fact that the differences between the observed and expected values are squared at (f::1) makes any

difference positive. The test is thus a non-directed test (bilateral approach test), which tells us whether or not there is a relationship between two factors but not in what sense such an association occurs [26,27]

Results

The results of our cases in comparison with the genes analyzed in the different studies [28].

Table 2. Meta-analysis Results.

studies	Sample size	ALK Mutation	ROS1 Mutation	EGFR Mutation	KRAS Mutation	PD-L1 Mutation
1	102	3%	-	11%	28%	12%
2	390	53,6%	53,8%	67,8%	64,2%	-
3	533	89,9%	89,9%	85,4%	-	25%
4	1200	7,8%	-	50,1%	-	-
5	1141	53,6%	53,3%	67,8%	64,2%	-
6	9239	64,7%	35,6%	78,9%	-	46,9%
7	273	-	-	63%	5,5%	24,9%

In a study conducted in La Rioja, where 102 patients were analyzed, it was observed that 11% presented EGFR mutation, 3% ALK rearrangement, 28% K-RAS mutation and 12% expressed PD-L1 [29].

Work 2, successfully analyzed all driver genes (EGFR, ALK, ROS1 and BRAF) in 644 relevant articles. Among them, mutations included ALK (53.6%), ROS1 (53.8%), BRAF (53.9%), KRAS (64.2%), EGFR (67.8%), MET (58.6%,) and RET in 54.6% of the samples. [30]

In work 3, a total of 533 patients were enrolled, where the median age was 72 years (range 25-94 years) (range 25-94 years), with 64.7% being male (345 patients), 46% of whom were diagnosed at stage IV. PD-L1 status was assessed in 497 patients, with 25% (133 patients) having PD-L1 expression above 50%. Furthermore, 394 (74.1%) had a history of smoking, compared to 25.9% (138 patients) who had never smoked. [31]

Furthermore, in this study, the success rate for genetic alteration testing for the 4 driver genes (EGFR, ALK, ROS1 and BRAF), which was the primary target, was 80.1%. Alteration rates for each gene separately were as follows: 85.4% for EGFR mutations, 89.9% for the ALK and ROS1 fusion gene and 85% for the BRAF mutation.

In the 4th paper, a total of 1200 patients were included with a median age of 60 years. Here, the frequencies of EGFR genomic alterations and ALK rearrangement were identified as 50.1% and 7.8%, respectively. In addition, 56 different uncommon EGFR mutations were identified. In patients younger than 40 years, the percentage of ALK positivity was as high as 28.2% and 3.2% harbored multiple rearrangements of this gene [32]

In paper #5, data was extracted from 38 studies that met inclusion criteria. Here, the calculated rates were 53.6% for ALK, 53.9% for BRAF, 64.2% for KRAS, 67.8% for EGFR and 53.3% for ROS1. In addition, an additional category was added for those genes that had not been specified, resulting in a total of 55.7% [33]

In paper 6, tests for tumor markers were positive for 78.9% of the EGFR gene, 64.7% of ALK and 35.6% of ROS1. On the other hand, PD-L1 determination was performed worldwide in the same period and was positive in 46.9% (16).

In paper no. 7, 68 cases (24.9%) had positive PD-L1 expression. The data showed that the highest rate was for the EGFR gene, with 63% (172), followed by TP53 with 32 patients (11.7%) and KRAS with 15 patients (5.5) [34].

From our collected data it was found 40 mutations in 39 of the 86 cases studied, which represents 46.5% of subjects with at least one genetic alteration.
It should be noted that only one patient had a simultaneous mutation for PDL-1 together with EGFR and only one other patient had mutations for the ROS1 and EGFR genes together.

On the other hand, 1 case was obtained that presented a mutation for ALK and for EGFR.
The highest incidence was the PDL-1 mutation, which was positive in 34 cases (29.24% or 87.1% if only cases with genetic alterations are taken into account).

The statistical analisis showed that $\chi^2 = 1,11$ thus χ^2 calculated is lower than χ^2 theorical, which means it is non-significant thus there are no difference.

Discussion:

The usefulness of the NGS technique has been demonstrated, for the identification of a large number of possible genetic mutations, which, when it comes to determining treatment, has a high prognostic value.

This study was based on cases provided by the international laboratory Analiza, extracted from their anonymous database. The account of mutations found represents 46.5% of subjects with at least one genetic alteration.

Our results indicate the most frequently mutated gene is PD-L1 with 29.4%, coinciding with studies 3 and 7, with 25% and 24.9%, respectively. Meanwhile, studies 1 and 6 showed mutations in 12% and 46.9%, and in the rest of the studies this gene was not studied.

The ALK gene, demonstrates higher values, such as 89.9% in study 3 and 64.7% in study 6. Mutations for the ROS1 gene were analyzed in studies 2,3,5 and 6, obtaining a very heterogeneous percentage, with very altered values, in relation to our results (1.1%).

As for the EGFR gene, in 4 of the 7 works, it is placed as the most frequently mutated gene, the only one studied in all the jobs, with similar values. These results differ significantly from ours, as this gene was only found to be mutated in 1.1%, as was the ROS1 gene.

Finally, the KRAS gene was studied in articles 1,2,5 and 7 with 28%, 64.2%, 64.2% and 5.5%, respectively, the latter coinciding with our results, where we found mutations in 4.3%.

Among the genetic mutations studied, EGFR has been found to have a better prognosis than PDL-1, although there are currently therapies directed against both that have considerably improved the prognosis, osimertinib and atezolizumab, nivolumab and pembrolizumab respectively.

These differences in prognosis, apart from targeted therapies, are based on the fact that the majority of patients diagnosed with the PD-L1 mutation were smokers, so they will more often suffer from microcytic cancer, which is usually more aggressive and diagnosed at more advanced stages.

According to a 2020 study, the prevalence of the EGFR mutation differs according to race, histological type and demographic and risk factors. Furthermore, it can be seen that the majority of lung cancers with this mutation are adenocarcinomas, especially in East Asia, where this mutation is present in 78% of adenocarcinomas, compared to 10-16% in other ethnicities, or 12% in Mediterranean populations [1]

ALK alterations act as oncogenic drivers in about 1-10% of NSCLC cases, although this figure varies according to the population, being more prevalent in Asia, the Pacific and Maori ethnicity.

It is more frequent between the ages of 30-50 years, being younger than the average age of patients suffering from this type of cancer. This alteration tends to provoke a more aggressive behavior in the tumor, and is diagnosed at older ages compared to patients who are free of it. However, they respond better to treatment with tyrosine kinase inhibitors (TKIs) directed against ALK, as well as against EGFR+.

According to studies, the frequency with which ROS1 occurs in NSCLC varies between 0.9% and 2.6%, with similar rates in Asia, Europe and North America, and with a global prevalence estimate of 1.9%.

Study 4 notes that 83% turned out to be adenocarcinomas and using genomic profiling, the following conclusion could be reached "73.9% of NSCLC patients harbored at least one actionable alteration recommended by the National Comprehensive Cancer Network".

It's important to note that while PD-1/PD-L1 inhibitors have provided significant benefits to many lung cancer patients, not all patients respond to these drugs, and there can be side effects associated with their use. The choice of treatment, including immunotherapy, is based on several factors, including the stage and type of lung cancer, PD-L1 expression levels, and the patient's overall health [35].

Finally, we have included KRAS gene mutations, as a meta-analysis of 28 studies showed that tumors with this mutation have a worse prognosis and no or reduced response to EGFR tyrosine kinase inhibitors.

Notably, KRAS mutations are more common in Western populations than in Asian or Australian populations (23-33% versus 2-15%, respectively).

Conclusion:

It can be seen that the results obtained with the cases provided by our reference laboratory are in line with the articles reviewed. Except for the results of EGFR mutations, which samples from our database are significantly lower than expected.

In order to increase the representativeness of the results, we shall continue working to increase the sample size.

We must insist on the early diagnosis of neoplasia, as nowadays there are targeted therapies against the several mutations described, basically thanks to the analysis through NGS, which improve the prognosis of the disease, while highlighting the need to individualize the molecular diagnosis of each patient, to provide personalized treatments and achieve higher rates of curation.

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