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Julianna Mastroianni
jmastroianni@my.uri.edu

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Diagnostic Value of KRAS Mutation in Non-small Cell Lung Carcinoma

Julianna Mastroianni
Honors Project

Abstract

The kirsten rat sarcoma viral oncogene (KRAS) is found to be one of the most common mutated genes in non-small cell lung cancer (NSCLC). Decades have passed and researchers are still faced with difficulties understanding how the KRAS oncogene works and ways that it can be inhibited to provide NSCLC patients with a better prognosis. The three most common molecular methods for detecting the presence of KRAS are circulating free DNA (cfDNA), Sanger capillary sequencing and next generation sequencing (NGS). The specificity and sensitivity for detecting KRAS mutants has markedly improved and continues to advance by reducing cost and amount of specimen needed. In many cases of NSCLC, epidermal growth factor receptor (EGFR) mutations are also found as frequently mutated. The discovery of an EGFR mutation is typically reflexed with KRAS testing since KRAS is found downstream of EGFR and tends to reduce the success of tyrosine kinase inhibitors used to treat EGFR mutated tumors. The trouble with KRAS is the insensitivity of upstream EGFR inhibition and the continuation of pathway signaling of BRAF/MEK/ERK leading to uncontrollable proliferation of the tumor cells. This literature review provides an overview of how KRAS is detected, how it effects downstream pathways and the future possibilities for treatment and more sensitive methodologies.

Abbreviations

KRAS: Kirsten rat sarcoma viral oncogene
NSCLC: Non-small Cell Lung Cancer
cfDNA: circulating free DNA
NGS: Next generation sequencing
EGFR: Epidermal growth factor receptor
MEK: Methyl Ethyl Ketone
BRAF: B-Raf proto-oncogene
ERK: Extracellular Receptor Kinase
PNA: Peptide nucleic acid

PCR: Polymerase chain reaction
TKI: Tyrosine kinase inhibitor
DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
GEF: Guanine nucleotide exchange factors
GAP: GTPase activating protein
SOS: Son of sevenless
P13K: Phosphatidylinositol-3-Kinase
AKT: Protein Kinase B
mTOR: Mammalian Target of Rapamycin (a protein)
FFPE: Formalin fixed paraffin embedded
ddPCR: Droplet digital PCR

Objectives

1. To establish the common mutations found in NSCLC.
2. To identify different methodologies used to detect the KRAS mutations in NSCLC patients.
3. To compare KRAS mutated tumors with EGFR mutated tumors and their effects on prognosis for NSCLC patients.
4. To identify new treatment options undergoing extensive research that inhibit downstream pathways to KRAS to stop proliferation.
5. To determine the future methods for detecting KRAS and how they will improve sensitivity and specificity.

Introduction

The most frequently mutated oncogene found in non-small cell lung carcinoma NSCLC is the kirsten rat sarcoma viral oncogene (KRAS). KRAS mutation remains one of the most untargetable mutations for NSCLC. Typically, KRAS occurs as a missense mutation (Kerr & Martins, 2017, p.30). According to the National Human Genome Research Institute, a missense mutation is when the change of a single base pair causes the substitution of a different amino acid in the resulting protein (National Human Genome Research Institute). The KRAS mutation

is most commonly found on codons 12, 13 and 61(Kerr & Martins, 2017, p. 30). This mutant tends to alter protein conformation, resulting in accumulation of active GTP bound KRAS. If there is an accumulation of bound GTP, it may trigger signaling transduction pathways resulting in changes in cell proliferation, survival and metabolism (Kerr & Martins, 2017, p. 30). The KRAS mutation is found in one-third of NSCLC and is also commonly seen in colorectal cancer (Kerr & Martins, 2017, p.30). Detecting KRAS in colorectal cancer cases has a direct correlation to prognosis, unlike lung cancer where the prognostic value is still unclear and is found to be deemed undruggable in lung cancer cases. This is one the main reasons why KRAS mutations are being studied to help determine what can be targeted for drug therapy (Kerr & Martins, 2017, p.36).

There are five clinically relevant KRAS mutations that reflect the most common KRAS variants according to codons associated with NSCLC for testing in the laboratory. These mutations are G12C, G12D, G12V, G13D, and Q61H (Sherwood, Brown, Rettin, Schreieck, Clark, CLaes, Agrawal, Chaston, Kong, Choppa, Nygren, Deras, & Kohlmann, 2017, p. 2). The G12C mutation is an amino acid substitution at position 12 from a glycine to a cysteine (My Cancer Genome, 2018). The G12D mutation is an amino acid substitution at position 12 from a glycine to an aspartic acid (My Cancer Genome, 2018). The G12 V is an amino acid substitution at position 12 from a glycine to a valine (My Cancer Genome, 2018). The G13D mutation is an amino acid substitution at position 13 from a glycine to an aspartic acid (My Cancer Genome, 2018). Lastly, the Q61H mutation is an amino acid substitution at position 61 from a glutamine to a histidine (My Cancer Genome, 2018). The most commonly studied cell lines for KRAS mutated genes are MIA PACA-2, PANC-1, MDA-MB231, SW620 and NCI-H460 (Sherwood, et

al, 2017, p. 2). The most commonly seen mutation in lung cancer patients that have a history of smoking in the past or currently are smokers is G12C, which is a transversion mutation that involves the substitution of a purine nucleotide to a pyrimidine (Tomasini, Walia, Labbe, Jao, & Leighl, 2016, p.1451). The most common mutation seen in lung cancer patients that are non-smokers is G12D, which is a transition mutation that involves the substitution of a purine to purine or pyrimidine to pyrimidine (Tomasini, Walia, Labbe, Jao, & Leighl, 2016, p.1451).

Testing for KRAS

Traditionally, KRAS mutations have been detected in the molecular laboratory by an invasive surgical resection of a tissue sample of the lung that is diseased or thought to be diseased. After surgical resection, the sample would then be preserved by formalin fixation and the tissue section would be embedded in a paraffin block. This method is indeed invasive and more costly to the patient. Advancements for detecting mutations have been made and are less invasive. One method in particular is referred to as the "liquid biopsy" (Garzón, villatoro, Teixidó, Mayo, Martinez, Llanos Gil, Viteri, Morales-Espinosa, & Rosell, 2016, p. 513). This is a non-invasive, simple blood draw that allows for the serum or plasma from the blood collection to be used for genetic analyses. The component in the serum or plasma that can be used for genetic testing is referred to as circulating free DNA (cfDNA) (Garzón, villatoro, Teixidó, Mayo, Martinez, Llanos Gil, Viteri, Morales-Espinosa, & Rosell, 2016, p. 513). The cfDNA found in the blood presents the same mutations found in the primary tumor mass (Garzón, villatoro, Teixidó, Mayo, Martinez, Llanos Gil, Viteri, Morales-Espinosa, & Rosell, 2016, p. 513). The cfDNA presents the same mutations found in the tumor due to cellular necrosis and apoptosis

which releases tumoral DNA into the bloodstream (Garzón, villatoro, Teixidó, Mayo, Martinez, Llanos Gil, Viteri, Morales-Espinosa, & Rosell, 2016, p. 513). The presence of cfDNA offers an alternative, rapid, and reproducible option for KRAS mutation testing. The cfDNA was analyzed using Real-Time PNA PCR. This method uses a peptide nucleic acid (PNA) which inhibits the amplification of the wild type allele during PCR amplification. (Garzón, villatoro, Teixidó, Mayo, Martinez, Llanos Gil, Viteri, Morales-Espinosa, & Rosell, 2016, p. 513). This allows for the technologist to interpret the results by only seeing mutation bands and not the wild type. Utilization of PNA-mediated real-time PCR clamping method allowed for seven mutation in the KRAS gene to be detected with one-step. The PNA probes and DNA signals were used conjunctively in the assay, where the PNA probe was complementary to the wild type DNA sequence and suppressed the wild type amplification while enhancing possible mutant sequences that were competitively binding with the DNA primer. A positive signal was indicated by a SYBR Green fluorescent dye (Lee, Lee, Han, Kwon, Han, & Choi, 2014, p. 101).

The next method that will be discussed is Sanger capillary sequencing. Sanger capillary sequencing has held the title of being the gold standard for DNA sequencing throughout the years. Although this method has been widely used it does have a downfall. The low sensitivity of this method does not allow mutations with allelic frequencies less than around 20% to be detected, which may lead to false negative results (Sherwood, et. al, 2017, p. 2). The testing was performed by direct sequencing of PCR products amplified by genomic DNA and then electrophoresed on agarose gels. The results were noted as positive if a mutation was detected in both the forward and reverse DNA strand (Lee, et. al, 2014, p. 101) In the study of "Key differences between 13 KRAS mutation detection technologies and their relevance for clinical

practice", sanger capillary sequencing produced weak PCR products and only produced peaks that were observable below the detection level. In this study this method did not identify any of the KRAS mutations of any allele frequency tested (Sherwood, et. al, 2017, p. 8). In the study "*KRAS* Mutation Detection in Non-small Cell Lung Cancer Using a Peptide Nucleic Acid-Mediated Polymerase Chain Reaction Clamping Method and Comparative Validation with Next-Generation Sequencing", 15.7% (21 out of 134) of the specimens tested for KRAS mutation using sanger sequencing were positive, but when compared with another method an additional two more patients were positive that were not detected by sanger (Lee, et. al, 2014, p. 102). However, this difference was not considered significant.

The next method that will be discussed for detecting KRAS mutation is Next Generation Sequencing (NGS). This method has been on the rise in many pathology laboratories throughout the world. It provides technologists an insight such as de novo detection of variants and detection of actionable targets (Sherwood, et. al, 2017, p. 2). Another advantage of NGS is that the KRAS mutation is typically available in parallel with many other genes allowing for more tests to be performed on the limited amount of tumor material received (Sherwood, et. al, 2017, p. 11). Although this method is very attractive regarding its detectability of genes, some downfalls are the increased sequencing costs for this technology and the requirement of more manual steps and interpretation of other assays (Sherwood, et. al, 2017, p. 11). In the Sherwood, et. al. study, two different principles of NGS were evaluated. The two different principles were hybridization capture and amplicon-based sequencing. The amplicon-based assay identified all five KRAS mutations and detected the mutation at all levels of allele frequency leading towards 0.5% as the lowest level (Sherwood, et. al, 2017, p. 6-8). With NGS as a direct sequencing

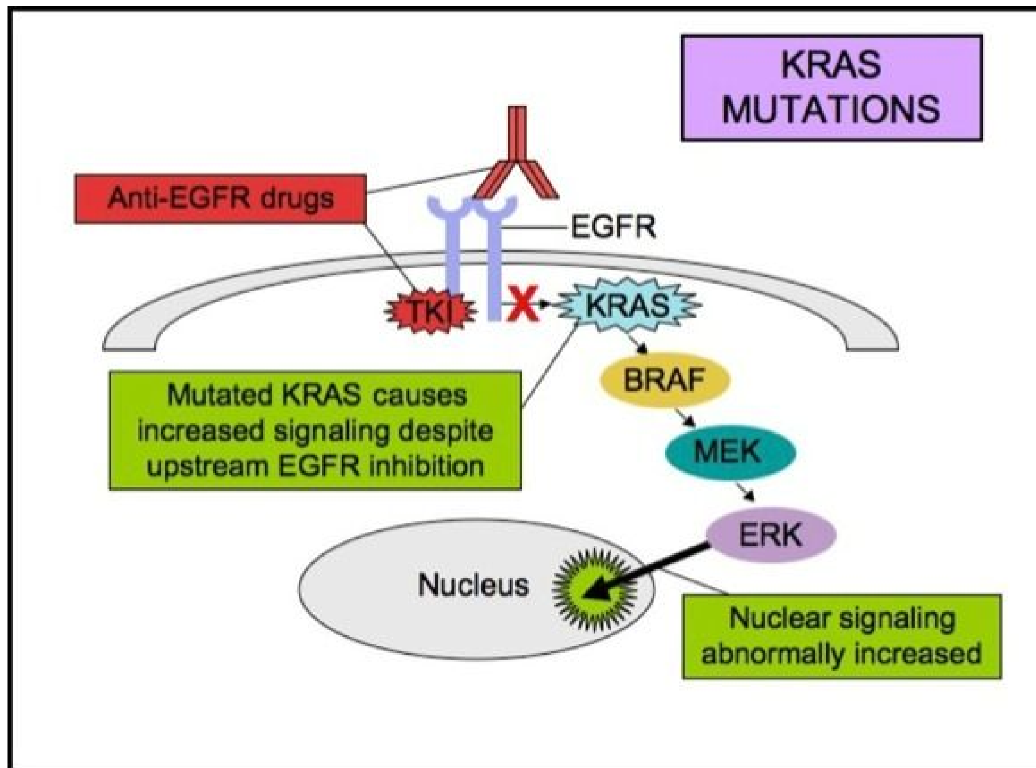
approach, it can be helpful to a molecular technologist by providing a way to test a wide spectrum of rare and commonly occuring mutations in individuals all in one test sample (Ramteke, Patel, Godbole, Vyas, Karve, Choughule, Prabhash, & Dutt, 2016, p. 6).

KRAS and EGFR In NSCLC

Along with KRAS being one of the most frequent mutations in NSCLC, alterations in epidermal growth factor receptor (EGFR) mutations are found in 10-30% of lung adenocarcinomas (Vijayalakshmi & Krishnamurthy, 2011, p. 179). According to the American Cancer Society, EGFR is a protein on the surface of cells and when functioning normally it helps cells grow and divide. In many NSCLC patients, an accumulation of EGFR leads to increased proliferation of oncogenic cells, leading to malignancy. The promising feature of having an EGFR mutation is that there are multiple different targeted therapies that can be used to try and stop proliferation. One of the most common therapies is the use of tyrosine kinase inhibitors (TKIs), which bind to the intracellular portion of EGFR and compete with ATP to dysregulate downstream signaling pathways to EGFR (Korpanty, Graham, Vincent, & Leighl, 2014, p. 1). The two most studied therapies are Gefitinib and erlotinib. These two therapies were designed and found to be helpful to patients before the EGFR mutation was known (Vincent, Kuruvilla, Leighl, & Kamel-Reid, 2012, p.35). After overwhelming positive results from patients with NSCLC treated with those therapies occurred, it was further studied that these patients that were reacting to the drugs harbored at least one of the EGFR mutations (Vincent, et. al, 2012, p.35).

KRAS proteins aid in the cell cycle and help produce and regenerate new cells (Tomasini, et. al, 2016, p. 1451). The RAS proteins originate in the cytosol and are recognized

by farnesyl transferases which results in the incorporation of the proteins into the inner cell membrane (Tomasini, et. al, 2016, p. 1451). EGFR is found upstream and can act as an extracellular stimuli which activates RAS proteins and can lead to the downstream activation of BRAF, MEK, and ERK (Tomasini, et. al, 2016, p. 1451). KRAS and EGFR mutations are typically mutually exclusive, meaning that it is very rare to find both mutations present in a patient with NSCLC (Affiliated Pathologists Medical Group, 2016). When a patient presents with NSCLC it is typical that EGFR mutation analysis is performed by PCR and if that is negative it is usually reflexed with KRAS PCR analysis to determine if anti-EGFR therapy will benefit the patient (Affiliated Pathologists Medical Group, 2016). The reason for KRAS being reflexed after EGFR testing is due to the fact that the KRAS protein is always on the "on" position, signaling pathways that are downstream and leading to uncontrollable growth of cancer cells and not being able to be inhibited by targeted therapy of other proteins (Shtivelman, 2017). The image below depicts how the use of anti-EGFR drugs do not prevent mutant KRAS from signaling its downstream pathways, causing cell proliferation to continue at an increased rate.



(Image credit: Affiliated Pathologists Medical Group)

Treatment/Targeted Therapies

Currently, there are no targeted therapies that have been proven to be successful for KRAS inhibition. Researchers are extensively searching for ways to inhibit KRAS signaling since it is such a desirable target due to its high mutation frequency in patients with NSCLC. Positive results for inhibiting KRAS directly has not yet been established but is in progress. However, there are other non-direct inhibition methods that are being studied for KRAS mutation therapy. One method that is currently being studied is decreasing KRAS protein synthesis by downregulation of KRAS gene expression (Tomasini, et. al, 2016, p. 1452). This method, although progressing slowly due to limited efficient delivery and sensitivity, includes the use of antisense oligonucleotides that can be administered via plasmid or viral vectors

intravenously, that target specific RNA sequences blocking the translation of mRNA to RAS protein (Tomasini, et. al, 2016, p. 1452-1453). Another potential way to inhibit KRAS is the inhibition of GEF or activation of GAP proteins. This method is being studied because GEF promotes the active state of KRAS and GAP promotes the inactive state of KRAS (Tomasini, et. al, 2016, p. 1453). Competitive binding peptides have been developed to bind at the same location as SOS (a Ras-GEF protein) and small molecules have been developed to enhance GAP activity in KRAS mutant tumors to try and inactivate KRAS, but is still under major development and needs to be introduced to clinical trials (Tomasini, et. al, 2016, p. 1453).

Alternative routes to inhibit KRAS is by designing inhibitors of downstream pathways to KRAS. One possible target is PI3K and mTOR (Tomasini, et. al, 2016, p. 1453). According to Tomasini et al. (2016), "P13K is a cytoplasmic molecule downstream of KRAS and is part of the PI3K/AKT/mTOR pathway" (p.1453). The PI3K/AKT/mTOR pathway is an intracellular pathway that has a role in regulating the cell cycle. Targeting PI3K alone has been deemed insufficient for treating patients with NSCLC but targeting mTOR, a serine/threonine kinase downstream of PI3K, has shown better overall survival in phase II trials, making it a promising target in the treatment of NSCLC (Tomasini, et. al, 2016, p. 1453). On the other hand, inhibitors of the BRAF/MEK/ERK (MAP kinase) pathway are also being studied. Targeting either BRAF or MEK for inhibition of KRAS is similar to inhibiting components of the PI3K/AKT/mTOR pathway since they are also downstream of KRAS and promote cell cycle and proliferation (Tomasini, et. al, 2016, p. 1453-1454). Due to the fact that KRAS activates both pathways aforementioned, dual inhibition of both pathways to fully block KRAS signaling in the cell is

being further investigated in phase I trials as a potential treatment (Tomasini, et. al, 2016, p. 1455-1456).

The Future For KRAS

The methods discussed previously for detecting KRAS are the conventional ways for testing for one of the most common mutations found in NSCLC. Since this mutations is such a desirable target for treatment of many NSCLC patients, researchers are continuously searching for more ways to detect KRAS mutations and ways to inhibit its signaling pathways. The first method undergoing research that will be discussed is co-amplification of KRAS and EGFR (CRE). This method is a multiplex-PCR that links the co-amplified exons (KRAS exon 2 and EGFR exon 18-21) of KRAS and EGFR as a single linear fragment for direct sequencing, while maintaining a cost-effective method with reduced variability and turnaround time (Ramteke, et al, 2016, p. 4). CRE has the ability to co-amplify all five exons with a very limited amount of formalin fixed paraffin embedded (FFPE) tissue and can be used to analyze KRAS and EGFR clinically due to its production of a 915 base pair product, while also limiting the cost and turnaround time of determining the mutational status across the whole KRAS exon 2 and EGFR kinase domain (Ramteke, et al, 2016, p. 5-6).

The other method that is being utilized by researchers is droplet digital PCR (ddPCR), which can detect low frequency in nine different KRAS mutations in oncogenic NSCLC (Pender, Murillas, Rana, Cutts, Kelly, Fenwick, Kozarewa, Gonzalez de Castro, Bhosle, O'Brien, Turner, Popat & Downward, 2015, p.1-2). The methodology behind this assay is by the use of a digital PCR probe with a specific oligonucleotide for each mutation (G12C, G12D and G12V) and

DNA extraction from FFPE and PCR reaction mixture (Pender, et al, 2015, p. 2,4). This multiplex assay permits the identification of specific KRAS mutations observed in NSCLC with reproducibility and the capability of detecting rarer KRAS mutations at the G12/13 and Q61 codons while also demonstrating more sensitivity than Sanger and next generation sequencing with the assay linearity down to 0.03-0.045% (Pender, et al, 2015, p 13-14). Overall, this is another option that is under investigation for routine clinical use.

KRAS being the most common, yet most difficult to understand, mutation in NSCLC deems it a very attractive and desirable target for physicians to be able to detect and alter the course of treatment for patients. Currently, there is not a definitive answer on how to inhibit KRAS signaling pathways but methods are constantly undergoing research and clinical trials to try and pinpoint how this mutation can be silenced. According to Pasi Jänne, MD, PhD, from the Dana Farber Cancer Institute, the two approaches that are extensively being researched currently are targeting agents that are specifically blocking G12C mutations, which he is hopeful of going into a clinical trial soon and could be the first targeted agent for KRAS if it works, or by inhibiting other downstream proteins of KRAS since KRAS tends to send out signals to not stop growing, leading to proliferation of cancer cells (2017). The future is bright for detecting and targeting KRAS, and will benefit an enormous population of patients with NSCLC.

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