Specific Aims

This proposal aims to identify unique mutational signatures, evolutionary patterns, and predictive biomarkers for treatment response and resistance in Black Americans with Non-Small Cell Lung Cancer (NSCLC) living in dense urban areas. Metastasis, the spread of cancer cells from the primary tumor to spatially separated sites, is the leading cause of cancer-related death. Understanding the evolutionary dynamics that lead to metastasis is vital for developing interventions to prevent and treat advanced stages of cancer. While significant progress has been made in understanding the early stages of tumor evolution, the genetic and evolutionary underpinnings of metastatic cancer remain less explored. This knowledge gap is particularly evident in the historically understudied Black American population.

By focusing on Black Americans, we seek to uncover molecular mechanisms of cancer that may be distinct from those observed in White individuals living in similar urban environments but not exposed to the same environmental and socioeconomic disadvantages. Black Americans often face higher exposure to air pollution and other environmental carcinogens due to systemic inequities in housing and urban planning. These factors, combined with unique genetic predispositions, may drive different mutational processes and cancer evolution trajectories.

This research is crucial for understanding the underlying mechanisms of NSCLC in Black Americans, which can lead to more personalized and effective treatment strategies. Furthermore, the findings from this study could inform public health policies aimed at reducing environmental risks and promoting equitable housing and environmental laws. Through innovative methodologies, we aim to narrow the gap in cancer research and enhance clinical outcomes for this underrepresented group.

This proposal builds on the pioneering work of leveraging whole genome sequencing to infer the evolutionary history of cancer. Advanced computational tools, such as the Gain Route Identification and Timing in Cancer (GRITIC) method, have been developed to time complex copy number gains and map the clonal evolution of tumors. These tools have provided critical insights into the subclonal architecture and evolutionary trajectories of various cancers, revealing pervasive intra-tumor heterogeneity and ordered paths of primary tumor evolution.

This project aims to revolutionize our understanding of NSCLC progression through cutting-edge advancements in modeling cancer evolution. By fulfilling these aims, the initiative is poised to bridge critical knowledge gaps in oncology, providing new analytical tools that map the evolution of cancer with unmatched precision. These advancements are expected to enhance the timing and specificity of treatment interventions and to lay the groundwork for tailored therapeutic strategies that are finely adjusted to the genetic and environmental profiles of individual patients, with special attention to the unique needs of Black communities. Ultimately, this research will be used to significantly improve early cancer detection, thereby transforming patient care and outcomes.

Aim 1: Preprocess raw data from the Black Americans with NSCLC cohort *Hypothesis: Comprehensive data preprocessing will transform the raw data from the Black Americans with NSCLC cohort into a high-quality dataset suitable for detailed mutational and evolutionary analysis.*

- Perform data cleaning and preprocessing by removing low-quality sequences, aligning the reads to the reference genome, and filtering data for further analysis with `GRITIC`
- Normalize the data to account for batch effects and other technical variances
- Detect and classify mutations (single nucleotide variants, insertions, deletions, etc.) using established bioinformatics pipelines to prepare the dataset for subsequent evolutionary analysis.

Aim 2: Conduct evolutionary analysis to establish a baseline for subsequent comparison *Hypothesis: Applying advanced computational tools will reveal unique cancer evolutionary patterns, precise mutation timings, and distinct subclonal populations specific to the Black Americans with NSCLC cohort, providing a critical baseline for future studies.*

- Apply `GRITIC` and complementary tools from Gerstung et al., such as `cancerTiming`, `MutationTimeR`, and `PhylogicNDT`, to map the clonal and subclonal evolution of tumors in this dataset
- Identify key mutation timings and evolutionary patterns and use as a baseline for further comparison and benchmarking

Aim 3: Develop innovative methods to map the paths of mutational processes throughout clonal evolution with improved mutation-time resolution *Hypothesis: Improving mutation-time resolution of GRITIC will refine the accuracy of mapping mutational processes and timing driver mutations and enhance our understanding of cancer evolution.*

- Establish high-resolution mutation-time techniques to precisely time mutational signature activities
- Utilize the high-resolution timing data as inputs to improve the accuracy of timing key driver mutations
- Develop accessible open-source software for use by the scientific community

Research Strategy

Significance

Lung Cancer and Environmental Exposures Lung cancer remains the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for 85% of lung cancer cases in the US.[1] Akin to tobacco smoking, exposure to the complex mixture of air pollution, particularly fine particulate matter ($PM_{2.5}$) and nitric oxide (NO), poses a major risk factor for developing lung cancer. In heavily polluted cities like Los Angeles, exposure to these pollutants significantly increases the risk of developing lung cancer.[2,3] In 2014, the Nurse's Health Study found that living within 200 meters of a highway and a $10\ \mu g/m^3$ increase in $PM_{2.5}$ levels were associated with an increased risk of lung cancer (HR = 1.57; 95% CI: 1.26, 1.77).[4] Furthermore, a 2019 meta-analysis estimated that a $10\ \mu g/m^3$ increase in $PM_{2.5}$ exposure in Europe and North America increased lung cancer risk by 25%.[5]

Despite the clear evidence linking air pollution exposure to elevated lung cancer risk, the precise molecular mechanisms by which these complex pollutant mixtures initiate and promote NSCLC remain poorly understood, representing a critical knowledge gap. This study will investigate lung cancer in African Americans/Blacks (Black Americans), an understudied group that exhibits a high prevalence of aggressive, early-onset tumors that are often driven by distinct molecular profiles like EGFR mutations.[6] Elucidating the environmental drivers and biological pathways of lung carcinogenesis in this subgroup could reveal novel diagnostic approaches.

Addressing Lung Cancer Inequities in Black Americans Although Black Americans have lower smoking rates compared to non-Hispanic Whites, they experience significantly higher lung cancer incidence and mortality rates, especially among men.[7–9] This disparity is striking, as Black Americans tend to initiate smoking later in life and consume fewer cigarettes compared to their White counterparts.[8,10] Black women, despite smoking fewer cigarettes, have the same or higher incidence of lung cancer as White women.

Current lung cancer screening guidelines based on pack-years and age[11] fail to adequately identify Black Americans at risk. Black Americans are diagnosed with lung cancer at a significantly younger age than Whites, often before reaching the screening threshold of 30 pack-years or age 55.[12] The molecular drivers underlying these aggressive, early-onset lung cancers in the Black population remain unclear. However, disparities in environmental exposures, particularly air pollution, are suspected to play a role.[6] Evidence shows that Black Americans are consistently exposed to significantly higher levels of $PM_{2.5}$ and NO compared to non-Hispanic Whites. This study will utilize a multi-regional cohort of non-smokers, former smokers, and smokers, to identify the molecular connections between air pollutants and lung cancer in Black Americans.

Furthermore, existing studies do not account for how social determinants of health in Black Americans may modulate susceptibility to cancers.[9] Addressing this gap is crucial for accurately assessing risk and developing prevention strategies in diverse populations.

Characterization of Environmental Exposure Outdoor air pollution, including $PM_{2.5}$, is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC).[13] Past studies demonstrate a clear link between residing near major roadways and an elevated risk of developing lung cancer.[13] Exhaust from combustion engines releases a mixture of carcinogenic compounds into the atmosphere near major roadways. These pollutants include polycyclic aromatic hydrocarbons (PAHs), nitrogen oxides, and toxic heavy metals such as arsenic, nickel, and lead.[14] Previous studies have attempted to map air pollution levels using census tract data. However, these methods only detect a limited subset of pollutants, failing to capture the full complexity of environmental pollutants. Moreover, existing research does not account for how rising global temperatures associated with climate change may alter the chemical composition and carcinogenic potency of air pollution over time. Another major shortcoming is the lack of integration of social determinants of health, such as obesity, diabetes, and chronic inflammatory conditions, which may exacerbate susceptibility to cancer.

Harnessing Advanced Computational Models for Precise Mutation Timing and Cancer Progression Analysis Gerstung *et al.* apply a suite of sophisticated computational tools including `cancerTiming`, `MutationTimeR`, `PhylogicNDT SinglePatientTiming`, and `PhylogicNDT LeagueModel`, to analyze cancer progression and mutation timing. Another recent advancement in genomic analysis is `GRITIC`, detailed in Baker *et al.* This method utilizes advanced computational techniques to precisely time copy number gains in clonal populations. The strength of `GRITIC` lies in its ability to accurately determine the sequential timing of these copy number gains.

However, `GRITIC` and similar methods face limitations, such as the computational intensity of Bayesian inferences and Markov Chain Monte Carlo (MCMC) approaches, which restrict their application in large-scale or real-time scenarios. Additionally, they often require assumptions about mutation rates and copy number states that may not hold in all scenarios. Currently, `GRITIC` requires DNA segments to have at least 20 single nucleotide variants (SNVs), and does not extend to systems with high copy number ($\geq\ 9$).[15]

Our proposed research aims to build upon and enhance the current GRITIC methodology by improving the precision of mutation signature timing. We will develop improved mutational signature activity timing approaches with higher resolution, enabling more precise tracking of mutagenic exposures over clonal evolutionary time. These refined results will serve as priors for developing more accurate driver mutation timing approaches. By integrating temporally varying distributions of mutation signatures, we will significantly enhance the precision of mutation timing and the temporal ordering of key driver mutations.

Additionally, we are committed to creating open-source, user-friendly software tools to disseminate these advanced methodologies to the broader research community. By making these tools accessible, we aim to promote widespread adoption and foster collaborative efforts to advance cancer research. Through these improvements, our research will overcome the current limitations of GRITIC and similar methods, providing more precise and scalable approaches to studying cancer evolution and the impact of environmental factors on mutational processes.

Potential for Transformative Impact This study will employ advanced geospatial methods to quantify individual exposures to air pollutants in Black communities in LA, Chicago, New Orleans, Charlestown SC, Richmond VA, and Rochester NY. Crucially, it will integrate this environmental exposure data with social determinants of health and biological factors that modulate disease susceptibility in these communities. Black populations in LA have historically faced disproportionately higher exposure to air pollution due to factors like redlining, the placing of industrial facilities near their neighborhoods, and a lack of green spaces. Despite having some of the lowest rates of smoking in the US, LA suffers from some of the worst highway-generated air pollution. By precisely characterizing these elevated exposures and combining them with data on obesity, diabetes, chronic inflammation, and other risk factors prevalent in Black communities, the goal is to develop a comprehensive analysis that elucidates how environmental drivers interact synergistically with social and biological parameters to initiate and promote aggressive, early-onset NSCLC in this population.

This multidisciplinary approach, which combines external exposure assessments with internal susceptibility factors, is poised to provide novel mechanistic insights into the environmental carcinogenesis pathways that contribute to the excessive lung cancer burden observed in Black communities. By correlating precise air pollution exposure data with epidemiological cohorts and molecular tumor profiling from Black NSCLC patients, the research will forge a comprehensive model of how environmental toxins catalyze lung carcinogenesis amidst the backdrop of social and biological vulnerabilities in this underserved population. We anticipate that this innovative approach will provide new insights into the role of air pollution in the development of NSCLC among Black Americans. This will help us develop early detection strategies. This is increasingly vital as, despite overall declining lung cancer rates, the incidence of NSCLC among women of color is rising in LA and similar urban areas.

Innovation

1. Focus on an Understudied Population: Targeting the Black Americans with NSCLC cohort addresses a significant gap in cancer research. Understanding the distinct mutational signatures and evolutionary patterns in this population can lead to more personalized and effective treatment strategies, ultimately improving clinical outcomes and addressing health disparities.

2. Comprehensive Evolutionary Analysis: The use of advanced computational tools, including GRITIC and complementary methods from Gerstung *et al.*, to map the clonal and subclonal evolution of tumors is a significant innovation. This approach will provide a detailed understanding of the evolutionary dynamics and precise mutation timings specific to the Black Americans with NSCLC cohort.

3. Improved Precision Mutation Timing: Another major innovation is the development of methods for mapping the trajectories of mutational processes with improved resolution of mutation timing. By establishing high-resolution techniques to precisely time mutational signature activities and refining the accuracy of driver mutation timing, this aim will significantly enhance our understanding of cancer evolution.

4. Correlate Environmental Toxin to Higher Rates of NSCLC: The study will identify molecular mechanisms correlated with exposure to environmental toxins, which is a highly impactful aspect of this research. Understanding these correlations can lead to insights into how environmental factors contribute to cancer development and progression in Black Americans with NSCLC.

5. Development of Open-Source Tools: Creating accessible, user-friendly software tools that will be disseminated to the research community ensures that the advanced methodologies developed in this study can be widely adopted and applied in other cancer research contexts. This commitment to open science and collaboration is crucial to community-wide innovation by promoting broader impact and advancements in the field.

Through these innovative approaches, this research will bridge critical knowledge gaps in oncology, particularly regarding

the unique needs of Black communities. The insights gained from this study are expected to lead to more personalized and effective treatment strategies, improved early cancer detection, and ultimately, better patient care and outcomes.

Approach

Aim 1: Preprocess raw data from the Black Americans with NSCLC cohort

Introduction Aim 1 focuses on preprocessing the raw genomic data collected from Black Americans with NSCLC. This process is essential for ensuring the accuracy and reliability of subsequent analyses. Given the complexity and volume of genomic data, meticulous preprocessing is required to remove biases, correct errors, and prepare the data for detailed mutation and evolutionary analysis.

Whole genome sequencing (WGS) is a comprehensive method for analyzing the entire genomic DNA of an organism. This technique allows for the identification of genetic variations, including single nucleotide variants (SNVs), insertions and/or deletions (indels), and structural variations across the entire genome. Next-generation sequencing (NGS) refers to a collection of modern sequencing technologies that enable the rapid sequencing of large amounts of DNA. These technologies have revolutionized genomic research by providing high-throughput, accurate, and cost-effective sequencing solutions.[16] NGS allows for easy implementation of WGS, providing a powerful framework for uncovering the genetic underpinnings of diseases such as NSCLC.

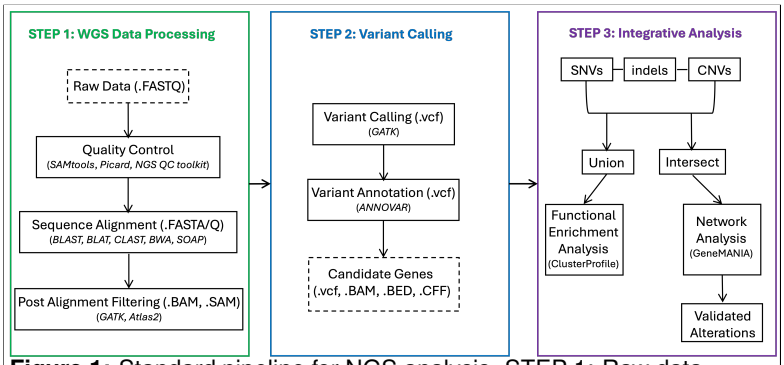

Figure 1: Standard pipeline for NGS analysis. STEP 1: Raw data (FASTQ) are aligned to the reference data (FASTA). Resulting alignments are stored in binary alignment map (BAM) file format. STEP 2: Sequence variants are identified and annotated using tools such as GATK and ANNOVAR. Candidate genes are stored in various file formats, *e.g.* .vcf, .BAM, .BED, .CFF. STEP 3: Candidate genes with SNVs, indels, and CNVs are analyzed and validated using tools such as ClusterProfile and GeneMANIA.

Given the nature of genomic data, which often contains significant amounts of noise due to sequencing processes, a series of rigorous data cleaning protocols is commonly implemented, (Fig. 1). This includes removing low-quality sequences to prevent erroneous interpretations, aligning the raw reads to a reference genome to identify the genomic coordinates of the sequences, removing duplicate reads to avoid overrepresentation, and correcting sequencing errors to improve data accuracy. Additionally, it is crucial to account for mutations present in healthy cells to differentiate between somatic mutations and germline variants, thus focusing on cancer-specific changes.[16,17]

Once the data has been cleaned, the next step is normalization. Genomic data can be affected by various technical variances and batch effects that arise during sample collection, sequencing, and processing. These variances can obscure true biological signals if not properly addressed.[18] The normalization process involves adjusting for systematic biases and standardizing the data to ensure that it is comparable across different samples and experimental conditions. This is particularly important when dealing with large datasets collected from diverse sources, as it enhances the comparability of the data and ensures that any observed differences are due to biological variations rather than technical artifacts.

The final preprocessing step involves the detection and classification of mutations. This step is crucial for understanding the genetic underpinnings of NSCLC and involves identifying SNVs *i.e.*, point mutations that can significantly impact gene function, as well as identifying indels that result in structural genomic changes. Additionally, it is essential to identify and analyze copy number variants (CNVs), which are large regions of the genome that have been duplicated or deleted. CNVs can have significant implications for gene expression and tumor progression. Using established bioinformatics pipelines to classify and annotate these genetic variations provides insights into their potential impact and relevance to cancer progression[16]. By accurately detecting and classifying these mutations and CNVs, we can create a comprehensive genetic profile of NSCLC in Black Americans, which serves as a foundation for further evolutionary analyses.

Rationale Aim 1 focuses on preprocessing the raw genomic data from Black Americans diagnosed with NSCLC, a demographic often underrepresented in cancer research. This step is crucial for establishing a reliable baseline for subsequent analyses. By implementing rigorous data cleaning and preprocessing protocols, we aim to remove low-quality sequences, align the reads to the reference genome, and filter data for further analysis using `GRITIC`. The resulting high-quality dataset will be essential for accurately mapping mutational signatures and evolutionary patterns specific to this population.

1.1. Perform data cleaning and preprocessing by removing low-quality sequences, aligning the reads to the reference genome, and filtering data for further analysis with `GRITIC` In collaboration with Dr. Loretta Erhunmwunsee, we

will collect extensive WGS data from Black Americans with NSCLC. The data will undergo common cleaning and preprocessing steps to remove biases and errors. This includes aligning sequences to reference genomes, removing duplicate reads, correcting sequencing errors, and accounting for mutations in healthy cells. We will implement stringent quality control measures to validate the data's integrity and completeness, ensuring it meets the analytical requirements of the computational methods to be applied. This step is expected to be time-consuming, given the complexity of preprocessing genomic data, however, we do not anticipate any challenges.

1.2. Normalize the data to account for batch effects and other technical variances In this phase, we will normalize the dataset to account for batch effects and other technical variances. Normalization is crucial to ensure that the data is comparable across different samples and experiments. This involves adjusting for systematic biases that may arise during data collection and sequencing processes. We will employ well-established statistical techniques to standardize the data,[18] making it suitable for downstream analyses such as mutation detection and evolutionary trajectory modeling.

1.3. Detect and classify mutations (single nucleotide variants, insertions, deletions, etc.) using established bioinformatics pipelines to prepare the dataset for subsequent evolutionary analysis. After normalizing the data, we will detect and classify mutations, SNVs, indels, and CNVs using established bioinformatics pipelines.[16] These pipelines will enable us to systematically identify and annotate genetic variations within the dataset. The accurate detection and classification of mutations are critical for subsequent evolutionary analyses, as they provide the foundation for understanding the genetic dynamics and progression of NSCLC in this population, and are required preprocessing steps for future analysis with `GRITIC` and tools from Gerstung *et al.*

Challenges & Alternative Approaches While we do not anticipate issues with these preprocessing steps, we expect it to take a significant amount of time due to its non-trivial nature. The computational demands of analyzing large WGS data will be managed by leveraging high-performance computing (HPC) resources. We will also ensure seamless integration of results from different computational methods by using standardized data formats and developing custom scripts for data merging and analysis where needed.

Aim 2: Conduct evolutionary analysis to establish a baseline for subsequent comparison

Introduction Aim 2 focuses on conducting an evolutionary analysis of cancer in the Black Americans with NSCLC cohort to establish a baseline for subsequent comparison. This involves applying computational tools to map the clonal and subclonal evolution of tumors in the dataset and identifying key mutation timings and evolutionary patterns. Key to this aim is the use of GRITIC and complementary methods developed by Gerstung *et al.*, such as `cancerTiming`, `MutationTimeR`, and `PhylogicNDT`. This baseline will be used for further comparison and benchmarking in Aim 3.

SNVs are point mutations where a single nucleotide in the genome is altered. Copy number gains occur when a segment of the genome is duplicated, resulting in multiple copies of that segment (Fig. 2). This can happen due to errors during DNA replication or as a result of genomic instability, which is a common feature in cancer cells. If an SNV occurs before a copy number gain, it will be present in all copies of that segment. If it occurs after, it will only be in some copies. As illustrated in Figure 2, SNVs accumulate over time during tumor development. The ratio of SNVs on single copies to multiple copies can indicate the relative timing of copy number gains. The earlier a copy number gain occurs, the greater the ratio of SNVs on single copies to multiple copies, reflecting the accumulation of mutations before and after the gain. Understanding the timing of these copy number gains relative to the accumulation of SNVs provides insights into the sequence of genetic events that drive tumor development.

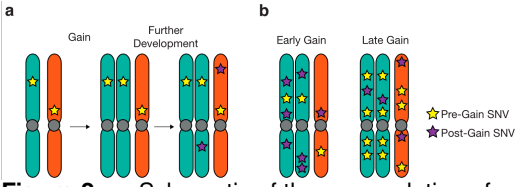

Figure 2: a. Schematic of the accumulation of SNVs during tumor development. SNVs on the gained allele are copied over to the new allele. b. Illustration of the concept that the earlier a copy number gain occurs, the greater the ratio of SNVs on single copies to multiple copies. Figure courtesy of Baker *et al.*

`GRITIC` is an advanced method designed to accurately infer the timing of sequential copy number gains in tumor genomes. It utilizes a sophisticated Markov Chain Monte Carlo (MCMC) algorithm to model the temporal order of copy number alterations, providing precise estimates of the timing of these events relative to other genetic changes. This method is particularly powerful for understanding the evolutionary dynamics of cancer, as it allows researchers to reconstruct the sequence of genetic events that drive tumor progression.

`GRITIC` was developed with several key advantages that overcome the limitations of previous clonal copy number alteration (CNA) timing methods. Namelt it can i) recover complex gain states, ii) it can identify intermediate states to map the complete history of gains over time, and iii) it reduces dependence on the assumption of parsimony, which is frequently used in evolutionary reconstruction methods. `GRITIC` uses binary trees to enumerate all possible routes for gained segments on each chromosomal haplotype. It then leverages the multiplicity states of single nucleotide variants (SNVs) *i.e.*, the number of SNVs before and after a gain, and their ratios on each gained segment to infer the posterior probabilities of all potential routes.

This includes determining the relative timing of the corresponding gains and any whole-genome duplication (WGD) events if present. This inference is accomplished using a Bayesian Markov Chain Monte Carlo (MCMC) approach. The route with the highest posterior probability then represents the complete event history of copy number gains in a tumor sample.

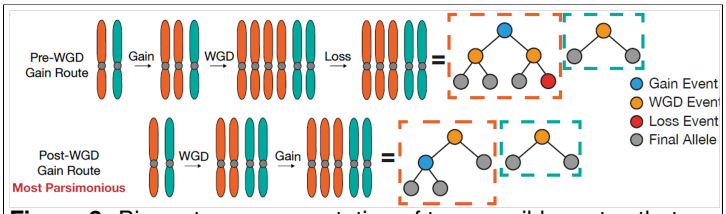

Figure 3: Binary tree representation of two possible routes that result in a 3+2 copy number state in a WGD tumor. The post-WGD route is the most parsimonious as it involves the fewest events. Figure courtesy of Baker *et al.*

Complementing `GRITIC` are several other methods developed by Gerstung *et al.* `cancerTiming` is a tool that estimates the timing of single nucleotide variants (SNVs) relative to copy number changes. By integrating SNV and copy number data, `cancerTiming` provides insights into the chronological sequence of mutational events. `MutationTimeR` is focuses on estimating the timing of mutations within individual cancer samples. It uses a Bayesian framework to integrate various data types, including SNVs and CNVs, to infer the relative timing of mutations. Following this analysis, `PhylogicNDT` is used for phylogenetic analysis and clonal decomposition of tumor genomes. `PhylogicNDT` can analyze multi-sample datasets to reconstruct the evolutionary history of a tumor, identifying distinct clonal populations and their genetic relationships.

Rationale Understanding the evolutionary trajectories of tumors is crucial for identifying critical mutational events that drive cancer progression. By establishing a comprehensive baseline of tumor evolution, we can compare different methodological approaches and validate the model developed in Aim 3. This will provide insights into the effectiveness of different therapeutic strategies and enhance our understanding of tumor evolution in Black Americans with NSCLC.

2.1. Apply `GRITIC` and complementary tools from Gerstung et al., such as `cancerTiming`, `MutationTimeR`, and `PhylogicNDT`, to map the clonal and subclonal evolution of tumors in this dataset In this phase, we will apply the `GRITIC` and complementary tools from Gerstung *et al.*, such as `cancerTiming`, `MutationTimeR`, and `PhylogicNDT`, to map the clonal and subclonal evolution of tumors in this dataset. This step involves detailed computational analysis to infer the historical development of tumors, identifying key mutational events and their timings.

2.2. Identify key mutation timings and evolutionary patterns and use as a baseline for further comparison and benchmarking Next, we will identify key mutation timings and evolutionary patterns and use these as a baseline for further comparison and benchmarking. This involves synthesizing the results from the computational analyses to highlight critical mutational timings and evolutionary patterns. Detailed reports and visual representations, such as evolutionary trees and mutation timelines, will be prepared to document these baseline findings comprehensively.

Challenges & Alternative Approaches We anticipate several unique challenges in this aim, particularly regarding the accurate modeling of tumor evolution and the integration of multiple computational tools. High inter-individual variability in evolutionary patterns may complicate the identification of common mutational trajectories. In addition, the computational demand of tools like `GRITIC`, `cancerTiming`, `MutationTimeR`, and `PhylogicNDT` will require HPC resources. Furthermore, we will incorporate cross-validation techniques to validate our findings and mitigate the risk of overfitting.

Aim 3: Develop innovative methods to map the paths of mutational processes throughout clonal evolution with improved mutation-time resolution

Introduction Aim 3 focuses on developing innovative methods to map the paths of mutational processes throughout clonal evolution with improved mutation timing. This includes establishing improved precision mutation-time techniques, utilizing these techniques to improve the accuracy of timing key driver mutations, and developing accessible open-source software for the scientific community.

We have previously developed an approach to assess changes in putative mutation signature activity during the clonal development of a tumor. This involved separating clonal mutations into those occurring early in clonal development (present on multiple duplicated copies) and late (present on a single copy). Mutation signature spectra for single and double base substitutions and indels were derived, their weights estimated using non-negative least squares, and spectral changes between epochs were tested using a likelihood ratio test.

`GRITIC` calculates the proportion of SNVs at each clonal mutation multiplicity state within a gained segment to time individual copy number gains along the mutation timeline. It uses binary trees to represent possible routes for gained segments on each chromosomal haplotype, leveraging Bayesian MCMC approaches to infer posterior probabilities and timing.

Rationale Accurate timing of mutational events is crucial for understanding the clonal evolution of tumors and identifying critical mutational processes. Traditional methods often lack the resolution required to precisely time these events. By developing improved precision mutation-time techniques, we can achieve a more detailed understanding of the evolutionary history of tumors. This will enable us to identify key driver mutations with greater accuracy, providing insights into the

mechanisms driving tumor progression. Additionally, making these techniques available as open-source software will allow other researchers to apply these methods to their datasets, facilitating broader advancements in cancer research.

3.1. Establish high-resolution mutation-time techniques to precisely time mutational signature activities This sub-aim involves developing and validating computational methods that build on top of `GRITIC` to accurately time mutational events with high resolution. These techniques will leverage high-throughput sequencing data and sophisticated statistical models to improve the temporal resolution of mutation signature timing. We will time single base substitution signature exposure dynamics. Timed copy number gains within a segment will be used as boundaries for a maximum number of discrete time periods. The set of SNVs within each time period will be used to calculate mutation signature weights, as has been done previously for subclonal mutation signature timing. We will then fit the signature proportions to a final set of merged time periods such that the likelihood of SNV timing falling into each period is maximized and the Bayesian information criterion of merging time periods is minimized.

`GRITIC` evaluates each possible gain route R and represents the segment gain timings within a vector t, where t denotes the timings of segment gains. It also captures the relationship between these times and their corresponding clonal mutation multiplicity states M as the binary matrix A_M^R. The relationship between the proportions of mutations at each multiplicity state is $m = \frac{A_M^R t}{\sum A_M^R t}$. The timing of each SNV can be sampled from the posterior distribution, defined as:

$$P(t_{\text{SNV}}, M, t_{\text{seg}}, c, R \mid \theta_{\text{seg}}, r^A_{\text{SNV}}, r^T_{\text{SNV}}) \propto P(t_{\text{SNV}} \mid \delta_i) P(\delta_i \mid M, t_{\text{seg}}, \theta_{\text{seg}}) P(M, t_{\text{seg}}, c, R \mid \theta_{\text{seg}}, r^A_{\text{SNV}}, r^T_{\text{SNV}})$$

where t_{SNV} is the timing of the individual SNV, t_{seg} is the timing of the segment, c is the clonal share of SNVs, θ_{seg} represents the segment parameters, including SNV alternative read and total read counts (r^A_{SNV} and r^T_{SNV}), segment major copy number (n^A), and tumor purity (ρ), and δ_i represents the range of timepoints that bound the multiplicity state.

3.2. Utilize the high-resolution timing data as inputs to improve the accuracy of timing key driver mutations `GRITIC` incorporates the timing of a single WGD but does not handle a second WGD event. Based on the work of Baker *et al.*, we will update the `GRITIC` tool to include a second WGD event. This enhancement will improve the model's ability to accurately capture the complex evolutionary history of cancer genomes.

By timing individual SNVs and leveraging the capabilities of `GRITIC`, we can align mutation timing with clonal copy number gains. Inputs such as mutational signature timing and the proportions of mutations at each clonal mutation multiplicity state will be crucial. improved precision timing data from 3.1 will inform Dirichlet prior distributions, modeling mutation signature proportions over time to enhance accuracy (Fig. 4). Using temporally varying distributions of mutation signatures as priors will improve precision.

By jointly estimating mutation timing and time-series proportions of mutation signatures, we will integrate mutation signatures with gain route and timing, dramatically improving precision. This will enhance the temporal resolution of various driver mutations, including SNVs resulting in a gain or loss of function, benefiting individual tumor studies and enabling comprehensive analyses of cancer development pathways.

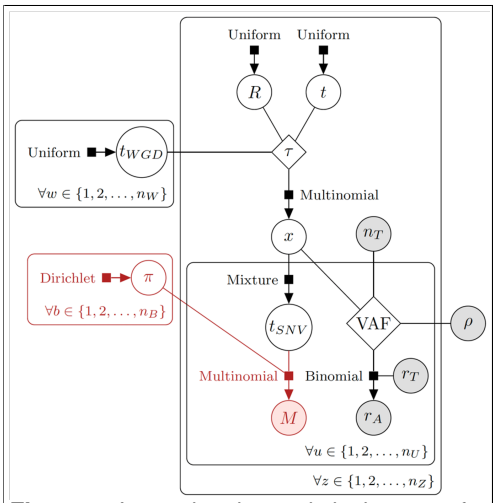

Figure 4: Improving the statistical power of GRITIC to determine the order of driver mutations. Mutation signature timing (red) will both be added to GRITIC to improve gain and mutation timing precision. In these probabilistic graphical models, data is represented by solid circles, probabilistic variables by empty circles, deterministic variables by diamonds and probability distributions by solid squares.

3.3. Develop accessible open-source software for use by the scientific community Combine improvments to `GRITIC` from aims 3.1 and 3.2 into an accessible open-source software for use by the scientific community. To ensure the broad application of our improved precision mutation timing techniques, we will create user-friendly software tools. These tools will be made available as open-source software, accompanied by detailed documentation and tutorials to facilitate their use by other researchers in the field.

Challenges & Alternative Approaches Developing improved precision mutation timing techniques poses several challenges, including the need for large and high-quality datasets, sophisticated computational models, and significant computational resources. To address these challenges, we will collaborate with other research institutions to access extensive genomic datasets and leverage HPC resources. Additionally, we will employ rigorous validation techniques to ensure the accuracy and robustness of our methods. If initial approaches prove inadequate, we will explore alternative statistical models and computational frameworks to achieve our objectives.

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