

# Human genomics

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June 24, 2022

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# Part I

# Notes

# Chapter 1

## Introduction

### 1.1 Genetics and genomics

Genetics is the study related to specific genes and their variants with a known effect on the phenotype. Genomics is focused on the function and structure of the human genome, evolution and anything relating to the whole genome:

- Coding regions.
- Non-coding regions.
- Linear and non-linear structures.
- Cell physiology and pathology.

#### 1.1.1 Genetics

Genetics is the study of heredity, or how the characteristics of living organisms are transmitted from one generation to the next via DNA. It dates back to Augustinian friar and scientist Gregor Mendel. It involves the study of a specific and limited number of genes or their part that have a known function.

#### 1.1.2 Genomics

Genomics is the study of the entirety of an organism's genes, the genome. Using high-performance computing and mathematics techniques known as bioinformatics, genomics researchers analyse enormous amounts of DNA-sequence data to find variations that affect health, disease or drug response. When dealing with the human genome that means searching through about 3 billion units of DNA across 23000 genes.

#### 1.1.3 Differences between genetics and genomics

The main difference between genomics and genetics is that genetics scrutinizes the functioning and composition of the single gene, where genomics addresses all genes and their relationships in order to identify their combined influence on the growth and development of the organism.

## 1.2. BASIS OF HUMAN GENOMICS

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### 1.1.4 The role of computational biology

Computational Biology encompasses a wide range of numerical methods to analyse and integrate large scale data towards the understanding of molecular, cellular and structural biology. Possible studies of computational biology are:

- Semi-quantitative simulations of metabolic pathways.
- Characterization of 3D chromatic structure,
- 3D protein-protein interaction.
- Discovery and characterization of disease related variants.

The main subjects involved are:

- |             |               |                     |
|-------------|---------------|---------------------|
| • Biology.  | • Statistics. | • Computer science. |
| • Genetics. | • Calculus.   | • Bioinformatics.   |

The focus of this work is on how to mine raw data, mainly from sequencing, how to exploit it for quality control (QC) and how to interpret the obtained results in the context of human diseases, especially in cancer.

## 1.2 Basis of human genomics

The genetic make-up is different in all humans as it is responsible for our diversity. SNPs (single nucleotide polymorphisms) and CNVs (copy number variants) contribute to make us different. The majority of the external phenotypes come from genetic variance that are inherited or, in minor measure, acquired.

### 1.2.1 Single nucleotide polymorphisms

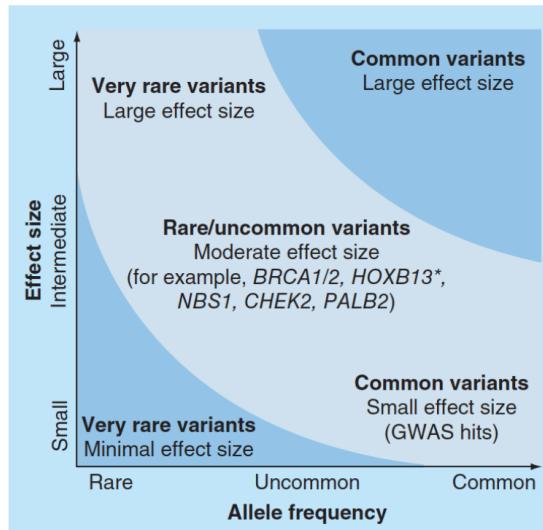
Single nucleotide polymorphisms or SNPs are changes of one nucleotide in the sequence of a gene. They constitute 1% of the difference between two unrelated individuals' genomes and they can be used as quality control assets.

### 1.2.2 Copy number variants

Copy number variants or CNVs are the differences in number of alleles for a gene present in one individual. They contribute much more than SNPs in the difference between unrelated individuals, but they're less known as inherited variants, as they are harder to quantify and detect. CNVs are distinguished as gain-CNV (where the number of alleles is greater than two) and loss-CNV (where the number of alleles is 2, 1, or 0). It is of note that if both parents have only one copy of a gene their child can have none. CNVs span  $\gg 1\%$  difference between two unrelated individual genomes.

### 1.2.3 Inherited variants

Inherited variants can be characterized by penetrance and allele frequency.



**Figure 1.1:** R. Eeles, Future Sci. OA (2016) 2(1), FSO87. Review on prostate cancer.

**Common SNPs:**  $\frac{1}{4}$  of individual is homozygous for A1,  $\frac{1}{4}$  is homozygous for A2,  $\frac{1}{2}$  is heterozygous. The minor allele frequency is around 30-50% with low penetrance.

**Rare SNPs:** typically have very large size effect: if they are related to very specific traits they will have high penetrance. They constitute deleterious variants.

### 1.2.3.1 Penetrance

Penetrance is the proportion of individuals carrying an allele (or genotype) that also expresses the trait (or phenotype) associated with it.

### 1.2.3.2 Allele frequency

Allele frequency is the ratio between the number of times the allele of interest is observed in a population over the total number of copies of all the alleles at that particular genetic locus in the population:

$$AF = \frac{\# \text{allele of interest}}{\# \text{copies of all the alleles at the genetic locus}}$$

Recent studies have shown that genetic variance contributes to predisposition to certain diseases. It is also emerging that rare pathogenic variants tend to have an high penetrance. This means that if a variant is pathogenic and rare, it's probable that all patients affected by the disease carry that particular mutation. This is represented by the top part of the diagram shown in figure 1.1. On the other hand, common variants could be associated to predisposition or susceptibility to the disease with low penetrance. In the middle on the diagram well-known variants correlated to cancer are found. The majority of these have a moderate size effect: not everyone who has the variation develops the disease.

### 1.2.3.3 Differences in Genetic Make-Up, an example

One example of how the genetic make-up plays a role in disease is the ADME genes. ADME stands for *Absorption, distribution, metabolism and elimination*. It is a set of genetic variants that is

## 1.2. BASIS OF HUMAN GENOMICS

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able to change the ability of the organism to react to certain compounds causing pharmacokinetic variability and influencing the patient's treatment response. Both common and rare variants are involved. Figure 1.2 represent a subset of them.

**Table 1.** Comparison between pharmacogenomics approaches.

PGx Approach	GWAS	SNPs Panel	Candidate SNP
Sample size	Tailored for large populations	Tailored for small populations	Tailored for small populations
Number of investigated markers	Larger numbers	1–2 thousand	Smaller number
Hypothesis	Hypothesis-free and hypothesis generating	Hypothesis-free and hypothesis generating/PK and PD coverage	Selected on a priori knowledge
Study Design	Exploratory	Confirmatory/Exploratory	Confirmatory
Limitations	False Negative/control for multiple testing	Coverage of limited genes	False positive/non-replication of results/low genetic coverage

PGx: pharmacogenomics; GWAS: genome-wide association study; SNP: single nucleotide polymorphism.

**Figure 1.2:** From review: *Pharmacogenomic Profiling of ADME Gene Variants: Current Challenges and Validation Perspectives*

A therapeutic approach that considers these variations could be very useful in precision medicine.

### 1.2.4 Somatic Variants

Somatic variants are **not** inherited from parents and are not transmitted to offspring. Most of them, but not all, are harmless. They can also be present in only a subset of the cells in an individual.

#### 1.2.4.1 Classification

Somatic variants can be classified as:

- Single Nucleotide Variants (SNV). SNVs are a single point mutation restricted to a certain population of cells in an individual.
- Indels few nucleotides deletions.
- Rearrangements, like gene translocation, chromosome breakage and chromothripsis (which falls in the subcategory of chromosomal rearrangements).
- Somatic Copy Number Aberrations (SCNA).

#### 1.2.4.2 Types of acquired DNA aberrations

**1.2.4.2.1 Translocation** Translocation happens when a sequence is moved from one genetic locus to another. It can be:

- Balanced: two sequences exchange locus and the overall quantity of DNA is maintained.
- Unbalanced: only one sequence move, generating an insertion.

**1.2.4.2.2 Inversion** Inversion happens when a sequence inverts its orientation. This aberration involves only one chromosome. The sequence of the inversion doesn't change, and the event will only be detected at its head and tail.

### 1.3. EXPERIMENTAL TECHNIQUES TO DETECT VARIANTS/ABERRATIONS

**1.2.4.2.3 Copy number changes** Copy number changes are events in which the quantity of DNA changes. They could involve one or more chromosomes. They can be:

- Duplication: a sequence doubles its copy number.
- Deletion: a sequence is lost.

**1.2.4.2.4 Chromoplexy** Chromoplexy derives from the Greek *pleko*, meaning to weave, or to braid. It describes a class of complex somatic DNA rearrangements whereby abundant DNA deletions and intra- and inter-chromosomal translocations that have originated in an interdependent way occur within a single cell cycle.

**Chromothripsis** Chromothripsis derives from the Greek *thripsy*, meaning shattering into pieces. It describes a clustered chromosomal rearrangement in confined genomic regions that results from a single catastrophic event, usually limited to one chromosome.

**Kataegis** Kataegis derives from the Greek kataigis, meaning thunder. It describes a phenomenon that is characterized by large clusters of mutations (hypermutation) in the genome of cancer cells. An APOBEC family enzyme might be responsible for the kataegis process.

## 1.3 Experimental techniques to detect variants/aberrations

### 1.3.1 Karyotyping

Karyotyping is the process of pairing and ordering all the chromosome of an organism, providing a genome-wide snapshot of an individual's chromosomes. This experiment was used to try and detect genomic aberration, but it proved inadequate because its resolution wasn't enough. In particular it missed all sequence specific variants, breakpoints that could not be detected until the development of NGS.

### 1.3.2 Sequence capture for cancer genomics

In the paper summarized in 12 it is described a typical sequence capture for cancer genomics.

#### 1.3.2.1 Reference

After sequencing there is a need to align the reads to a reference genome. This is especially needed when studying somatic changes. The best reference for this kind of studies is the individual's own genome, usually retrieved from white blood samples. Both cancer and normal DNA can be aligned to detect if an aberration is cancer specific or is present in both normal and cancer DNA. The **match normal** tool is used to distinguish SNV from rare SNPs, somatic and germline indels, but also to make sure that copy number variations are somatic. Baits are nowadays used in the sequencing step, in order to sequence only the exome or specific part of the genome, as to make the sequencing process more cheap.

### 1.3.2.2 Deepness

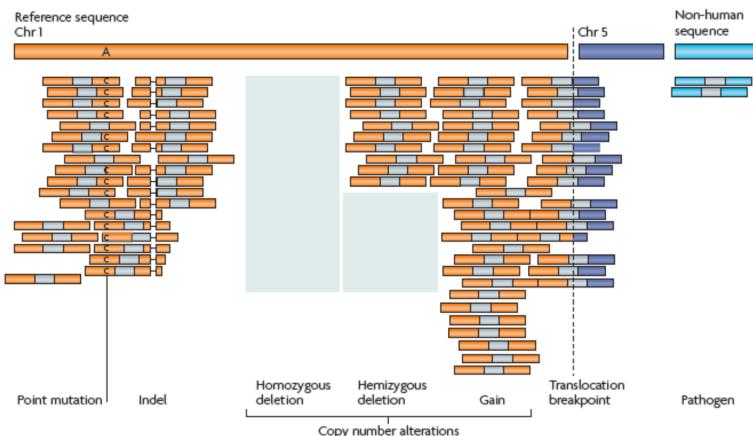
Another fundamental parameter in sequencing is the deepness. A more deep sequencing is needed to find subclonal event that could increase cancers' fitness, like the ability to escape the immune system. It is necessary because not all cells present all the mutations characterizing the subclonal event and because the purity of a tumour sample is not always optimal.

### 1.3.2.3 Single End (SE) and Paired End (PE) reads

**1.3.2.3.1 Single end sequencing** Single-read sequencing involves sequencing DNA from only one end. This solution delivers large volumes of high-quality data, rapidly and economically.

**1.3.2.3.2 Paired end sequencing** Paired-end sequencing allow to sequence both ends of a fragment and generate high-quality alignable data. It facilitates the detection of genomic rearrangements and repetitive sequence elements, as well as gene fusions and novel transcripts while providing double the coverage as a single-end protocol. It gives important information of the relative position of a molecule with respect to the reference, and is a necessary choice when structural aberration need to be assessed. This protocol is however twice as expensive as the single-end one.

**1.3.2.3.3 Ability of paired end sequencing to detect genomic aberrations** Figure 1.3 gives a nice graphical overview of genomic aberrations detectable by NGS, especially using PE sequencing. In particular the traslocation breakpoint in figure 1.3 would not be detectable without paired end sequencing. The most important parameter when studying deletions and insertion is to have enough coverage to perform significant downstream analysis, besides structural informations.



**Figure 1.3:** Advances in understanding cancer genomes through second-generation sequencing, Meyerson et al., Nature Reviews Genetics 2010

# Chapter 2

## Coverage

### 2.1 Computing coverage

The three key concepts needed when performing genomics analysis are the local and global coverage and allelic fraction.

#### 2.1.1 Local coverage

The local coverage  $cov$  at position (base)  $i$  is the number of reads that span  $p_i$ .

$$cov = \#r_i : r_i \in p_i$$

#### 2.1.2 Allelic fraction

The allelic fraction  $AF$  at position  $i$  is the proportion of reads that supports the reference (or alternative) base in  $p_i$  over the total number of reads that span  $p_i$ .

$$AF = \frac{\#r_{alternative_i} (reference_i)}{\#r_i} : r_i \in p_i$$

#### 2.1.3 NGS global coverage

The Lander-Waterman equation is used to compute NGS global coverage. The equation is:

$$C = \frac{L * N}{G}$$

Where  $C$  is the global coverage,  $G$  is the haploid human genome length,  $L$  the read length and  $N$  the number of mapped reads.

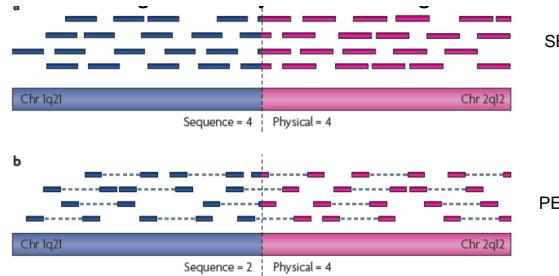
### 2.2 Mapping in NGS

When mapping NGS reads to a reference genome the number of correctly mapped reads is always lower than expected. This is due to sequencing or aligning errors or due to major translocations that will impair a good mapping of the reads. When considering which part of the reference genome is

### 2.3. TUNING COVERAGE

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covered by the mapped reads, a distinction between physical and sequence coverage has to be made. A schematic representation of the problem is displayed in figure 2.1.



**Figure 2.1:** Schematic difference between sequence (on the left) and physical coverage (on the right).

#### 2.2.1 Sequence coverage

Sequence coverage is the amount of oversampling (how many times a base is sequenced). It is needed to detect nucleotide alterations with high sensitivity. To do so for the 3 billion bases of the human genome a 30-fold coverage on average ( $30X$ ) is usually required. This means that 90 million of bases need to be mapped for each sample.

#### 2.2.2 Physical coverage

The expected distance between the paired reads is used to uniquely place the reads on the genome. Physical coverage refers to the average number of times a base is read or spanned by paired end reads. Unexpected read pairs are used to detect structural anomalies. So, while sequence coverage refers to a single base resolution, physical coverage refers to structural coverage of the genome.

### 2.3 Tuning coverage

In some experiments setups there's the need to carefully control the amount of intended coverage.

#### 2.3.1 SNP detection

SNPs are by definition present in all of the cells of an individual, so only enough redundancy or local coverage to distinguish the reference from the alternative base is needed. Ideally for heterozygous SNPs half of the reads will support the reference base while the other half the alternative one. Typically to perform this kind of detections only 10 to  $15X$  coverage is needed.

#### 2.3.2 Subclonal events

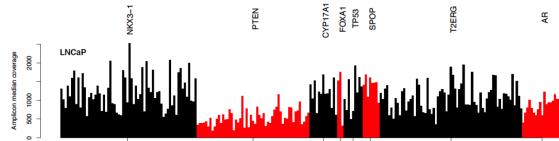
Subclonal events from a tumour or hematopoietic sample are not harboured by all the cells in an individual, so the coverage must be increased with respect to the one needed for SNPs detection. The same approach must be taken to detect homozygous mutations and low abundant events, like low-expression transcripts and to reduce the effect of weak binding in chIP-seq.

## 2.3. TUNING COVERAGE

### 2.3.3 Amplicon-based approaches

Amplicon sequencing is a highly targeted approach that enables researchers to analyse genetic variation in specific genomic regions. The ultra-deep sequencing of PCR products or amplicons allows for efficient variant identification and characterization. This method uses oligonucleotide probes designed to target and capture regions of interest, which are amplified by PCR and sequenced by NGS.

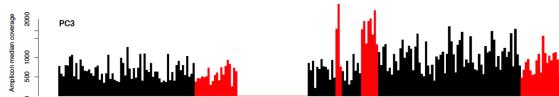
#### 2.3.3.1 Heterozygous deletion of PTEN



**Figure 2.2:** Example of local coverage of 10 genes for cell line LNCaP. On the x axis genomic location, while on the y axis the amount of amplicons sequenced.

In figure 2.2 a barplot representing the local coverage (y axis) in the gene locations (x axis) can be observed. The coverage is on average about  $600x$  and it's not evenly distributed, as it typically is for this kind of experiment. Averaging the coverage for each gene it is clear how for PTEN it is significantly less. Since some coverage is still present for that gene the most probable event to have caused this type of event is a heterozygous deletion.

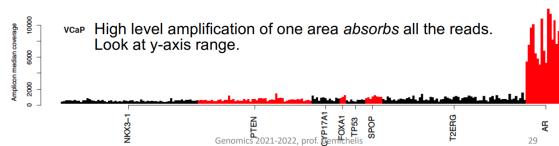
#### 2.3.3.2 Homozygous deletion of PTEN



**Figure 2.3:** Example of local coverage of 10 genes for cell line PC3. On the x axis genomic location, while on the y axis the amount of amplicons sequenced.

In the plot 2.3 from a different cell line a clear monoallelic deletion and a partial biallelic deletion of PTEN can be observed.

#### 2.3.3.3 Amplification of AR



**Figure 2.4:** Example of local coverage of 10 genes for cell line VCaP. On the x axis genomic location, while on the y axis the amount of amplicons sequenced.

## 2.4. DATABASES FOR NGS ANALYSIS

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In figure 2.4 a massive amplification of the antigen receptor typical of prostate cancer can be seen. This massive amplification is probably due to a mistake of the assay. Amplification on the AR does not allow for the analysis and discovery of other copy number variations because all of the reads will be mapped to the AR site.

### 2.3.4 NGS-based approaches

NGS-based approach are based on the sequencing of all of the sample genome, skipping the probing and amplification passages.

#### 2.3.4.1 Advantages over amplicon-based approaches

With this type of approaches it is easy to increase the experimental coverage at later point in time. It is enough to perform another run of sequencing on the same sample or library and then combine the output from different runs to increase coverage. This isn't possible with array-based technologies.

#### 2.3.4.2 Problems of NGS-based approaches

There are limiting factors to be considered when performing NGS DNA sequencing experiments. In particular:

- Repeated regions are difficult to sequence and map correctly.
- Structural information are difficult to obtain.

This problems could be mitigated by increasing the read length, but this would make the sequencing process more error prone. There is a need to make a trade-off between read length and single-base accuracy.

## 2.4 Databases for NGS analysis

Two very known databases for NGS analysis are:

- Genome Reference Consortium.
- USCS Genome Browser.

### 2.4.1 Genome reference consortium

The genome reference Consortium assembles a reference genome reflecting the most common sequences in population at each position while tracking information on polymorphisms.

### 2.4.2 USCS genome browser

The USCS Genome Browser allows to select a reference genome and query all of its known features.

# Chapter 3

## Genetic Fingerprinting

Genetic fingerprinting is a technique used to identify some characteristics of a genome (a pattern of variable elements), like SNPs or minisatellites, in order to uniquely characterize a genome.

Genetic fingerprinting can be used to compare a genome with a reference sample or to compare different genomes between each other, in order to determine their diversity or analogy.

DNA fingerprinting is applied in different fields:

- In forensics, for identification purposes;
- In lineage related tests, for cells or humans. Eg. paternity test, hereditary tests.
- For the certification of the origin of cells used in the laboratory, to make sure that the cells are the right ones and that there are no major genetic drifts. Needed when using certain cell lines, for publishing purposes.

### 3.0.1 Variants used for genetic testing

There are different variants that can be used for genetic fingerprinting, such as Single Nucleotide Polymorphisms (SNPs) or Inherited Copy Number Variations (CNVs). Remember: SNPs are substitutions of a single nucleotide at a specific position in the genome, whereas copy number variation is a phenomenon in which sections of the genome are repeated and the number of repeats in the genome varies between individuals.

Basically everything that is inherited and that is a polymorphism can be used in genetic testing, however some variants are more amenable than others. SNPs are the most *amenable* ones, since they are simple, abundant in the genome and easy to detect in sequencing data at any coverage depth. For these reasons, in this chapter we will focus on the development of SNP-based genetic tests.

### 3.1 SNPs features

#### 3.1.1 Hardy-Weinberg equilibrium and Minor Allele frequency

One property of SNPs which has to be taken into account when using them for genetic testing is the **Hardy-Weinberg equilibrium**. In population genetics, the Hardy-Weinberg equilibrium states that allele and genotype frequencies in a population will remain constant from generation to

### 3.1. SNPs FEATURES

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generation under neutral selection, so in the absence of other evolutionary influences, like genetic drift, mate choice, sexual selection, mutation and so on.

In the simplest case of a single locus with two alleles denoted  $A$  and  $a$  with frequencies  $f(A) = p$  and  $f(a) = q$ , respectively, the expected genotype frequencies under random mating are  $f(AA) = p^2$  for the AA homozygotes,  $f(aa) = q^2$  for the aa homozygotes, and  $f(Aa) = 2pq$  for the heterozygotes. In the absence of selection, allele frequencies  $p$  and  $q$  are constant between generations, so equilibrium is reached. The general equation that the allele frequencies need to fit in to be considered in equilibrium is:

$$(P + Q)^2 = 1, P^2 + 2PQ + Q^2 = 1. \quad (3.1)$$

SNPs that respect this equilibrium are also the most studied, thus more informative.

#### 3.1.2 Minor Allele Frequency

When performing genetic fingerprinting, the aim is to maximize the probability to have different genotypes in unrelated individuals.

For this reason, the more advantageous SNPs will be the ones in which the allelic frequency of the variants is the highest possible. Highest variability in the population allows to distinguish better more individuals.

Number-wise, a frequency of  $\frac{1}{3}$  for each SNP would maximize the variability, but those SNPs wouldn't be in HW equilibrium and we might have missed calls.

Therefore, the optimal SNPs to detect individuals' differences and similarities are those with genotype frequencies:  $P_{AA} = 0.25$ ,  $P_{BB} = 0.25$ ,  $P_{AB} = 0.5$ . 50% of individuals for that SNP will have a heterozygous genotype, 25% a homozygous genotype for the reference allele, 25% for the alternative allele.

This is equivalent to say that best SNPs will be the ones with **MAF** = 0.5. Minor allele frequency (MAF) is the frequency at which the second most common allele occurs in a given population.

#### 3.1.3 Projects regarding SNPs

Some useful projects developed over the years are:

- **dbSNPs:** it is a database of small scale nucleotide variants. The database includes both common and rare single base nucleotide variation (SNV), short ( $\leq 50\text{bp}$ ) deletion/insertion polymorphisms, and other classes of small genetic variations. <https://www.ncbi.nlm.nih.gov/snp/>
- **HapMap3:** is the third phase of the HapMap project whose aim is to develop a haplotype map of the human genome to describe the common patterns of human genetic variation in order to allow researchers to find genes and genetic variations that affect health, disease and individual responses to medications and environmental factors. The HapMap is a catalog of common genetic variants that occur in human beings. It describes what these variants are, where they occur in our DNA, and how they are distributed among people within populations and among populations in different parts of the world. <https://www.sanger.ac.uk/resources/downloads/human/hapmap3.html>

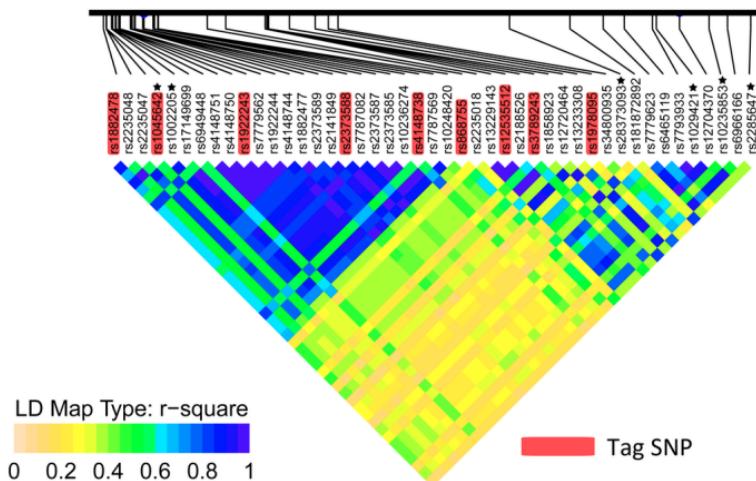
### 3.1. SNPs FEATURES

#### 3.1.4 Haplotype Blocks

Another important feature to consider for SNPs selection are **Haplotype blocks**. Haplotype blocks are blocks along the genome that tend to be inherited as segments (no recombination inside a haplotype block). In these sizable regions there is little evidence for historical recombination and only a few common haplotypes are observed.

So for example, if there are 10 SNPs in a block of 1 MB, the genotype of one specific SNP in that block gives an indication the genotype of the other SNPs in the same block, since they are inherited together. Hence, if there is a haplotype block, there is no point in sequencing all SNPs in that block, it is sufficient to select some specific SNPs. Also, when running a fingerprint assay, there is no point in using all SNPs in a haplotype block since they won't bring additional information independently.

SNPs in the same HB are said to be in **Linkage Disequilibrium (LD)**. Linkage disequilibrium measures the non-random associations between alleles or polymorphisms at different loci. A higher LD indicates a SNPs with a stronger tendency to co-segregate. Haplotype Blocks are therefore commonly represented with *linkage disequilibrium plots* 3.1. In these plots, SNPs are represented in a way that does not respect the genomic distance, but the order along the genome (position of each SNP relative the others).



**Figure 3.1:** LD plot of SNPs with top-ranked bayes factors in CHB (Han Chinese in Beijing) of 1000 Genome Phase I. The colors indicate the strength of pairwise LD according to  $r^2$  metrics. The SNPs marked with asterisks represent independent strong associations. Tag SNPs are here shadowed in pink.

The colors indicate the strength of pairwise linkage disequilibrium (LD) according to  $r^2$  metrics. In fact, not all of the SNPs are informative to distinguish between individuals. In 3.1 **Tag SNPs** are shadowed in pink. A Tag SNP is representative of a region with high linkage disequilibrium and represents a group of SNPs (called haplotype).

#### 3.1.5 Other SNPs features

Other SNP features to take into consideration when analyzing:

### **3.1. SNPs FEATURES**

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- Choose SNPs that are in areas that are not likely to undergo somatic aberrations. So exclude chromosomal locations which undergo frequent somatic aberrations, e.g. areas commonly deleted in tumor will produce LOH but probably also no calls, since there is no DNA.
- Choose SNPs equally represented/spread all around the genome (not in specific chromosome regions).
- Select autosomal only SNPs (not on chromosome X).
- Select SNPs in exons. If we were to run a targeted assay, this would cover more exons instead of introns. It will also be more probable to have signal from a non-DNA assay, for example if calling a genotype from RNA sequencing data (even though it is not always done).
- Exclude/include disease or drug response associated loci.
- Include/exclude loci with significantly different MAF in different ethnicity. If we include them we can also have a lineage type of tests in the same assay.

#### **3.1.6 Number of SNPs to select**

If we want to build a test to run genetic fingerprinting using SNPs, how many polymorphic loci (SNPs) should be tested?

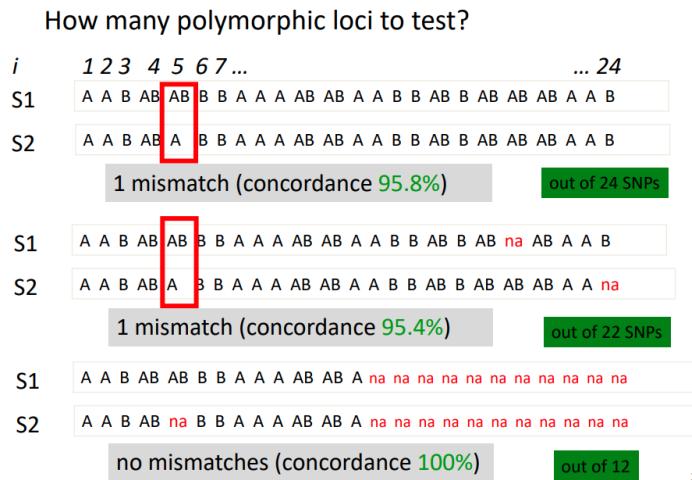
We want to make sure that the measure of the test will be able to differentiate unrelated individuals. But we must also remember that many variables must be taken into account, possible mismatches in particular. Those can be due to the sequencing process itself (experimental mismatches) but also to changes due to somatic events (biological mismatches). All these events can be used in the test with a different weight, based on how likely they are.

##### **3.1.6.1 Experimental mismatches : Genotype call error rate**

During sequencing, each machine will produce some errors, resulting in some loci for which no data will be available. If those loci include some SNPs of interest, then no call will be associated to that SNP. Experimental mismatches are related to the error rate of the technology used, they are platform dependent.

### 3.1. SNPs FEATURES

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**Figure 3.2**

**Some examples:** In each example in figure 3.2 there are two samples with the same number of potential SNPs: 24. To determine the difference/similarity of the two samples we can look at the genotype for each position and count mismatches.

Legend:

- 'A' stands for 'AA' (e.g. homozygous genotype for the reference allele); often referred to as Aa.
- 'B' stands for 'BB' (e.g. homozygous genotype for the alternative allele); often referred to as Bb.
- 'AB' stands for heterozygous.

Now, analyzing the three cases in figure 3.2:

- First Example: over the 24 loci, there is only one mismatch. This translates to a level of concordance of 95.8%. Those 2 individuals are highly related or DNA comes from the same samples.
- Second example: there is only one mismatch but there are some 'na', indicating that for some positions we don't have a call (not available data). Therefore, in this case the concordance is measured out of 22 SNPs and is equal 95.4%.
- Third example: here a lot of 'nas' are present, leading to have only 12 SNPs available. This brings to a concordance of 100%.

Different examples produced different levels of concordance. What do we trust the most? The first set of SNPs is the one that we trust the most, because it has the higher number of available SNPs. Wider number of SNPs provides the most reliable information.

#### 3.1.6.2 Biological mismatches

In the context of disease samples and tumors, many somatic events can happen, like deletions, gains of copies, homozygous deletions, ecc. Some common ones are:

### 3.2. GENETIC DISTANCE

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- **Loss Of Heterozygosity (LOH)**: event that results in loss one parental copy of a region which results in the genome having just one copy of that region. If that region contained a heterozygous locus (e.g. SNP), there will be loss of heterozygosity.

$$\text{probability of } AB \rightarrow A: P(AB, A) = P(AB) \cdot P(A|AB) \quad (3.2)$$

- Gain Of Heterozygosity (GOH): due to a mutation in a site often polymorphic through inheritance. These are pretty rare.

$$\text{probability of } A \rightarrow AB: P(A, AB) = P(A) \cdot P(AB|A) \quad (3.3)$$

- Double Mutation (DM): very rare.

$$P(A, B) = P(A) \cdot P(B|A) \quad (3.4)$$

Biological mismatches can be properly modeled in our assay. We can, in a data driven way, assess the error rate for the genotyping for some specific SNPs or run tests. We can also think in terms of SNP-specific or tissue-specific probabilities.

The joint probability of two events  $E_1$  and  $E_2$ ,  $P(E_1, E_2)$  or  $P(E_1 \wedge E_2)$  is  $P(E_1, E_2) = P(E_1) \cdot P(E_2|E_1)$  and  $P(E_2|E_1)$  is called the conditional probability of  $E_2$  given  $E_1$ .

The main point is that all mismatches must be taken into consideration. For this, all implemented tests use more than the minimal number of SNPs that allow to identify individuals.

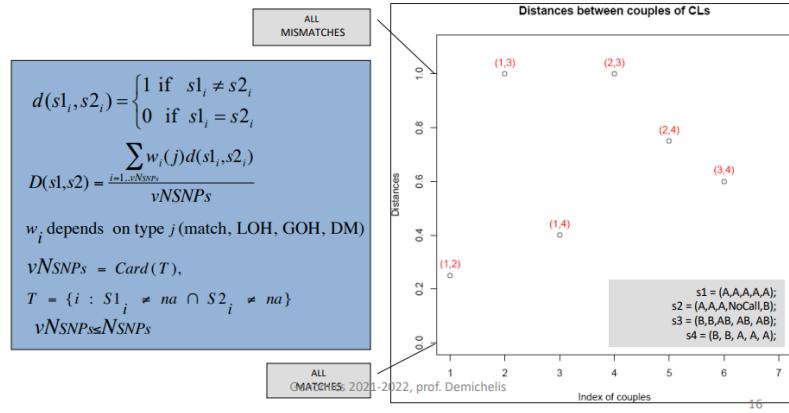
## 3.2 Genetic Distance

Having defined the number of SNPs to use, with maximum MAF and other amenable characteristics, the genetic test should provide a measure of some sort, which will be the output metric, associated with a probability of the measure to be correct.

As a simple measure, we can count the number of loci where two samples show different genotype and normalize on the total number of queried loci, defining a certain level of discordance (or concordance). The output value will be the 'genetic distance' between the two samples given the selected loci. The distance is proportional to the number of discordant calls.

In figure 3.3 we can see an example of a typical graph used to measure the genetic distance using SNP-based genetic testing. We have 4 samples with a set of 5 SNPs for each one. The distance is measured among all possible pairs, whose indexes are reported on the x-axis.

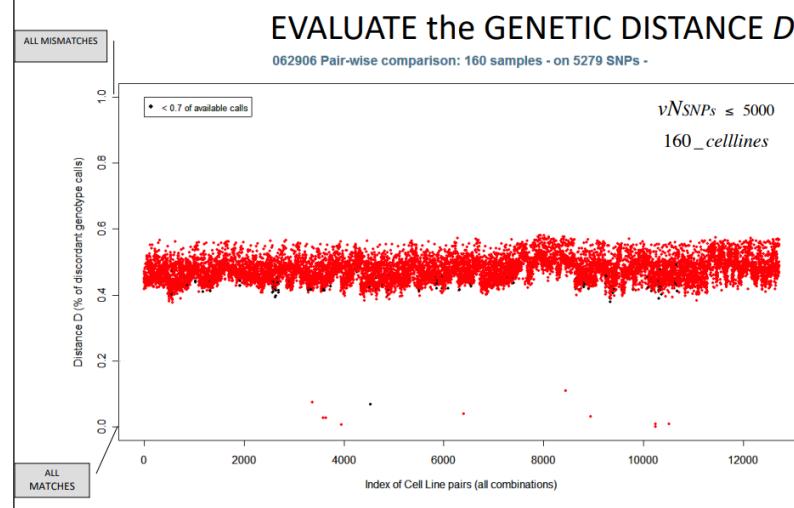
### 3.2. GENETIC DISTANCE



**Figure 3.3:** Genetic Distance graph with 4 samples. On the y axis we have the genotype distance, allegedly from 0 to 1. On the x-axis we have the index of all possible pairs in the dataset.

- s1 and s2 have 3 A in common, one locus has no call and another one produces a mismatch. 1 mismatch out of 4 produce a distance of 0.25.
- samples s1 and s2 have 5 mismatches out of 5, so a distance (or discordance) of 1.

If we put that into an equation will have that: for each position i (SNP) between sample 1 and 2 we can have 1 if the genotype is different, 0 if they are identical. Then we determine the distance D by summing up the different scores obtained for each SNP. We can associate different weights  $w_i$  to different mismatches or we can put all equal to one. Then we divide by the total number of SNPs for which we have available calls, vNSNPs, which will be lower or equal to the total number of SNPs, NSNPs.



**Figure 3.4:** Genetic Distance graph with 160 samples

This other example at figure 3.4 shows the distance, measured by genetic fingerprinting, of a collection of 160 samples of cell lines.

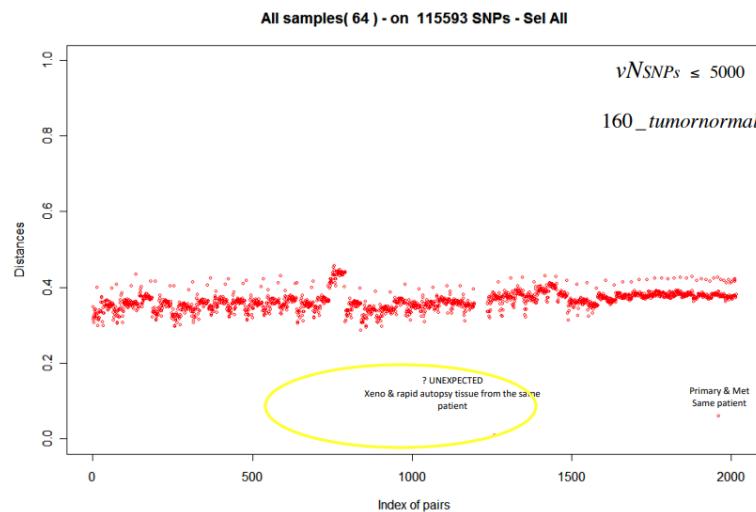
### 3.2. GENETIC DISTANCE

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The number of possible pairs corresponds to:  $160 \cdot 159 / 2$  (number found in the x-axis).

By applying this measure to a larger collection of samples like this one, with many SNPs, we expect to find an **average distance** among all possible pairs that very unlikely will be close to 1. The MAF of the SNPs is 0.5 but it will never happen that, with a high number of SNPs, the discordance will be 1. We will have an average distance that in this case around 0.5, since by chance we all share some genotypes on a large number of SNPs.

Here we find certain pairs with a very low distance, sometimes almost equal to zero (dots at the bottom). This is a surprising result because it shows that those pairs, which were suppose to be different cell lines, were actually not different cell lines (only less than 70% of SNPs have available calls).



**Figure 3.5:** Genetic Distance graph with tumor samples

In this last example at figure 3.5 genetic fingerprint was performed on a collection of 160 tumor samples, with a larger SNP array (more than 100.000 SNPs).

From the analysis, two samples with very low distance were observed. One of the two samples came from a Rapid Autopsy Program and the other one from a xenograft model.

RAP are programs for which patients at the end of their life agree to donate their tumor tissues which can be used for research. In these very complex but highly valuable programs, the material must be taken within two hours after death. Those sample are usually highly characterized but after a while the track of the patient's identity is lost. Here, what happened is that one man who donated tissue by this program was sequenced and for some of those metastasis models were generated and implanted into a mouse and a xenograft model was derived. Thanks to fingerprinting it was possible to determine the same origin between xenograft and patient.

The power of this technique is very high, it allows also to identify and remove things that we don't want in our study. E.g. if running a study (like a GWAS study) on a certain interesting geographic area, we will want to remove the members of the same family because that would skew the results. Genetic fingerprint can be used for this purpose.

## 3.2. GENETIC DISTANCE

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### 3.2.1 Some questions

**Q1:** Would the average of unrelated samples distance increase or decrease after selection of ideal SNPs?

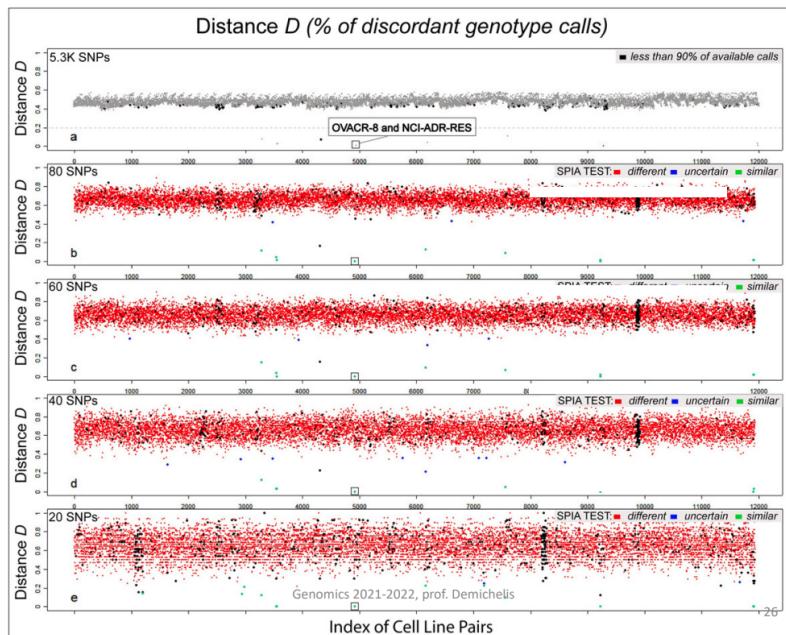
If we maximize the likelihood that SNPs have a different genotype among individuals and we use these to determine the measure, then the average distance of unrelated individuals will increase.

**Q2:** Is it likely to obtain a genotype distance  $D = 1$ ?

We get distance 1 only if we are looking at too few viable SNPs. Whereas with a well selected pool of SNPs, and a high enough number of SNPs, it is very unlikely that the distance is equal to 1.

### 3.2.2 Further considerations

How does the genetic distance among different samples change when varying the number of selected SNPs used to perform the test?



**Figure 3.6:** Genetic Distance graph at decreasing number of selected SNPs

The genetic distance among many samples, with an array of 5.3K SNPs, was measured, using a decreasing number of SNPs (from the initial total number of SNPs to decreasing numbers of highly selected SNPs) 3.6.

It is noticeable that, in the second plot where 80 SNPs matching the required characteristics were selected, the average Distance across all pairs is higher than in the previous example, in which all available SNPs were used ( 0.45 vs. 0.65). Also, the standard deviation of greater. Decreasing the number of SNPs to 60, then to 40 and 20 leads to have the same average distance between pairs, which settles around 0.66, but higher standard deviation.

In reality we always need enough SNPs, enough information, in order to prevent unexpected issues and to be sure that for any pairs of sample we have enough information to trust our measure.

### 3.3. BUILDING A SNP-BASED GENETIC TEST

## 3.3 Building a SNP-based genetic test

Building an identity test base on SNPs is a MULTI-STEP process, consisting in:

1. Definition of a genotype/genetic distance to compare samples;
2. Definition of SNPs requirements, based on the intention of the assay.
3. Selection of SNPs:
  - This can be done in a data-driven manner, through an iterative procedure of training and test on known sample set;
  - Or, performing the selection based upon MAF and Hardy-Weinberg equilibrium. For example, using HapMap data.
4. Implementation of a probabilistic test (different, uncertain or similar)
5. In silico validation on independent/multiple dataset.
6. Validation on cell lines genotyped on independent platform.

We have already seen some of the steps needed (1, 2, 3), we now pass to the following ones.

### 3.3.1 Implementation of a probabilistic test

Other important questions which we have to answer to when designing a genetic test are:

- What is the threshold on the genotype distance to call two samples 'identical' ('similar') or 'different'?
- How confident would the call be?
- What is the minimum number of loci needed for a robust test?

It could be useful to have a probabilistic test to determine if the measure of the test is correct at with which level of confidence. We can use a probabilistic approach to compare observations with expectations (gold standard).

Under the assumption that SNP calls at different loci are independent, we can think in terms of Binomial distribution. Each SNP can be considered as a trial,  $n$  = number of SNPs in the assay,  $k$  = number of matches,  $p$  is the probability of match and  $(1-p)$  of mismatch. Then the probability of having  $k$  matches (successes) out of  $N$  SNPs (trials) follows the binomial distribution.

With  $n$ ,  $np$  and  $np(1-p)$  large enough, we can use the Gaussian approximation of the Binomial distribution with  $K_{mean} = np$  and  $sd = \sqrt{np(1-p)}$ .

Using something that simple we can add a probabilistic test in our assay, defining an area of confidence given by  $K_{mean} \pm m \cdot sd$  where  $m$  is the number of standard deviations used to define the thresholds which will lead to have a smaller or wider confidence area.

### 3.3. BUILDING A SNP-BASED GENETIC TEST

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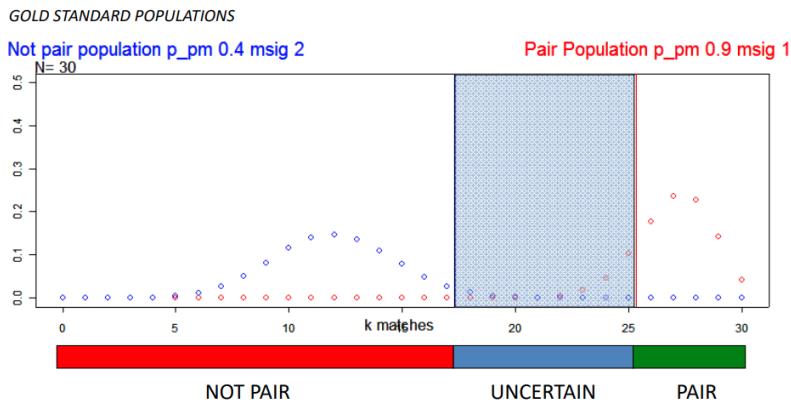


Figure 3.7

For example: given two unrelated samples, we reason in terms of 'what is the probability of having a certain number  $k$  of matches over a total number of  $n$  SNPs, therefore a certain value of  $D$ ?'.

The probability mass function for unrelated individuals is shown in figure 3.7 with a blue dotted line and indicates that there is a low probability of having both a very low and a very high number of matches.

We can also think in the opposite term: given two related samples, what is the probability of having matches? As represented by the red dotted line, in this case there will be a high probability of having many matches.

Using these probabilities we can set two thresholds which will define 3 regions:

- A '**not pair**' region for which the two samples will be considered as '**different**'
- a '**pair**' region for which the two samples will be considered as '**similar**'
- and an '**uncertain**' region, a grey zone, for which no certain result can be produced.

Then we can move the grey area based on what we want to be certain of and on how many SNPs we have.

By decreasing the number of SNPs, the grey zone will become more tiny, making the result more difficult to interpret. For example, a difference of only 2 matches could lead to opposite conclusions.

By contrast, with more SNPs the area will be wider and easier to interpret. Hence using a number of SNPs greater than the minimum number is better, otherwise there will be many uncertain calls.

## Further considerations and examples

In the past, before sequencing area and SNPs array area, short tandem repeats were commonly used for genetic fingerprint. They were used on gels to distinguish related and unrelated individuals, e.g., for the initial paternity test.

**Inherited copy number variants** can be used too for a fingerprinting test, but not all of them. The more amenable for this test are the loss type of CNV. In the population there will either a copy number of 2 or 1 or 0. If both parents have heterozygous pair of CNVs it will be possible that I have a homozygous deletion. If both parents have 2 copies at a site that is polymorphic in the population,

### 3.3. BUILDING A SNP-BASED GENETIC TEST

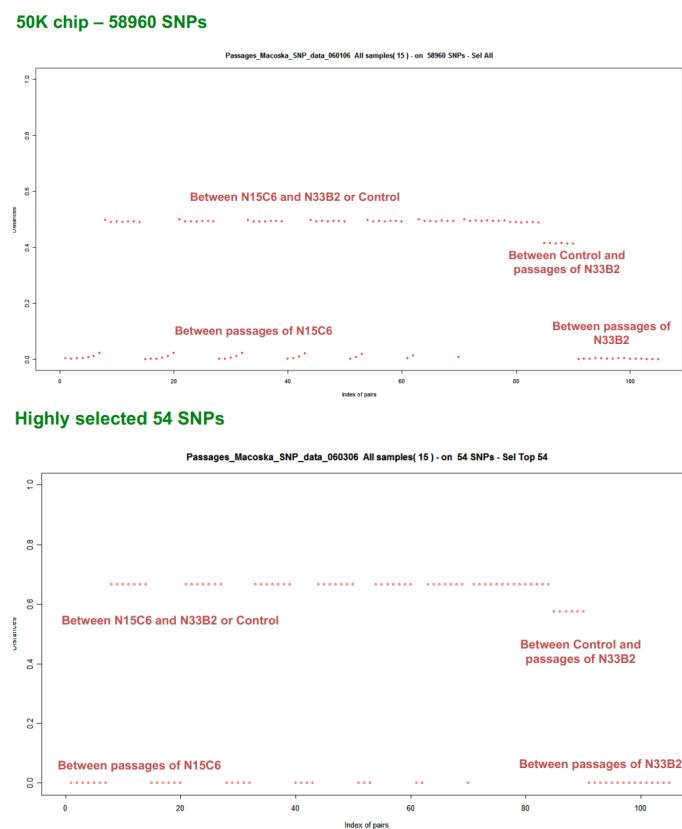
we will have a genotype equal to 2 copies.

If we think about gain of CNV then it becomes messy, because when combining multiple copies and have a add up we cannot distinguish what comes from what pair, so we cannot use them to identify an individual.

#### 3.3.2 Example 1: Cell line passages

A massive use of these genetic tests is done to assess genetic changes in in-vitro cultivation (also, in studies in tumor evolution, lineage plasticity, heterogeneity across metastasis across individuals or a single tumor).

Cell lines go through multiple passages in which they are used and stored. Genetic fingerprinting can be used to assess if among different passages the cells have remained the same, if they were mislabeled or if major genetic drifts happened.



**Figure 3.8**

In the example 3.8, two types of prostate cell lines which underwent multiple passages were used: N15C6 (passages from 48 to 63) and N33B2 (passages from 21 to 39). The cell lines were profiled with a SNPs array and the assay was run.

All passages of each cell lines were compared with all other passages. We expect all passages to have the same genetic fingerprinting in the same cell line.

### 3.3. BUILDING A SNP-BASED GENETIC TEST

However the results obtained using the full array of SNPs (50k), showed that some pairs which should be exactly identical (distance equal to zero) are actually a bit different (points at the bottom-left). By contrast, by using a set on only 54 SNPs, this diversity is not detectable, indicating that using the perfect number of SNPs could make us loose some information.

In order to understand this increase of distance, they looked at each chromosome to see if there were problems that justified increase the increased distance expected to be equal to zero in that cell line. All chromosome were tried. If we focus only on the SNPs spread across Chromosome 11, we observe that there is a major difference for certain passages with respect to the initial ones, only for one cell line (N15C6). This was due to the way the cells were immortalized (insertion in chromosome 11).

#### 3.3.3 Individual's Relatedness (genotype-distance)

The HapMap consortium sequenced hundreds of individuals for different ethnicities and also used trios. Trio sequencing is a technique which involves the sequencing of the genome of mother, father and son/daughter. Trios provide major information for haplotype blocks, for identifying regions related to inheritance, ecc.

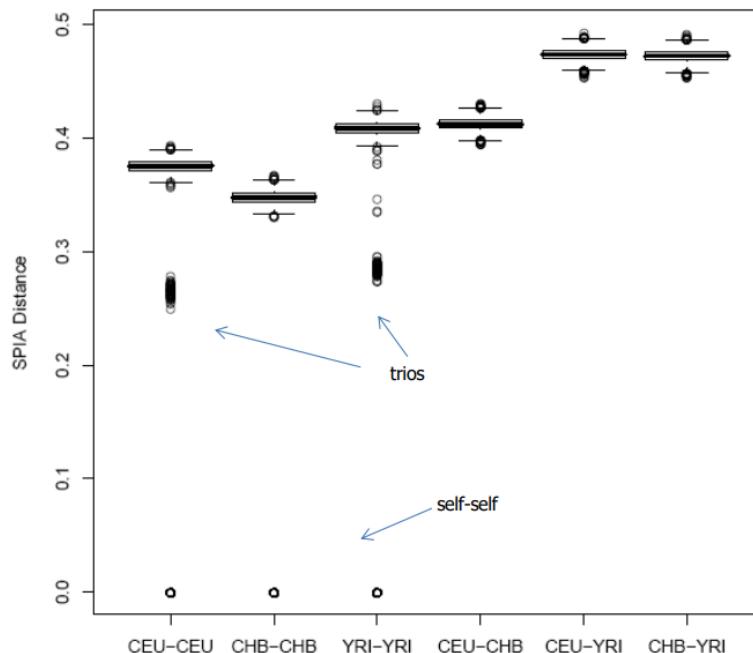


Figure 3.9

By looking at the data based on SPIA Assay (a genotype base assay which measures distance) at figure 3.9 we see that self-self pairs have distance zero, as expected; samples within each ethnicity have a certain average distance, which is lower than the distance observed among different ethnicities. Differences in distance among mixed samples are due to the fact that the SNPs used had on average higher MAF in some populations than in others. We also notice that in trios the distance is not

### **3.3. BUILDING A SNP-BASED GENETIC TEST**

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0 and is not equal to the median distance of unrelated individuals. This can be used for paternity tests or even in forensic science.

#### **3.3.4 Example 3: Cancer susceptibility test**

The data showed refers to a study where they were looking for polymorphisms that increase the likelihood of prostate cancer. In these studies, if relatives are present in the cohort, only one of them is taken to avoid skewing the results.

When looking for signs of cancer susceptibility by performing genetic fingerprinting, the division based on the degree of relatedness was determined 'for free' and could be used to remove unwanted samples from the cohort.

#### **3.3.5 Genetic structure of the human population**

One relevant aspect of the human genome is that it contains everything needed to learn about the genetic structure of the human population.

Understanding the genetic structure of human populations is of fundamental interest to medical, forensic and anthropological sciences.

Some of the reasons as to why knowing the genetic substructure of data is important:

- The goal of association studies is to identify DNA variants that affect disease risk or other traits of interest. However, association studies can be confounded by differences in ancestry.
- Misleading results could arise if individuals selected as disease cases have different ancestry, on average, than healthy controls. If in a study all controls are of the same ethnicity and the test is done on an individual of a different ethnicity than the test is biased.
- If we run a GWAS study using two ethnicities and we want to use the same markers of susceptibility worldwide, it won't work.

Especially in medicine and in the study of human evolution it is important to track the genetic background of individuals that are involved in studies in order to understand if the individuals are from a homogeneous population or from genetically distant ones.

More and more, clinical studies must have declarations of the checks and interpretation of the data of the genetic background of the individuals present in the study. It is very important to come to results for which we know exactly what is the applicability. To avoid spurious results, association studies often restrict their focus to a single continental group.

Advances in high-throughput genotyping technology have improved the understanding of global patterns of human genetic variation and suggest the potential to use large sample sets to uncover variation among closely spaced populations. One important piece of information to consider when developing methods to understand the genetic structure of a population, is to think in terms of variance, which is also relevant for human diseases.

Many SNPs have different MAFs in different populations. If we use those, and are able to have all of them in a simple computational way, we could be able to infer what is one individual's genetic background in terms of origins (e.g. Chinese origins).

The easiest mathematical approach to assess how well SNPs can distinguish ethnicity is by using **Principal Component Analysis (PCA)**. By running a very simple PCA on a set of SNPs including SNPs with different MAF in different populations we can, in a space, distinguish different ethnical groups. And we could also start thinking at individuals' origins.

How accurately can one predict an individual's geographic-ethnic background based upon his/her genetic barcode?

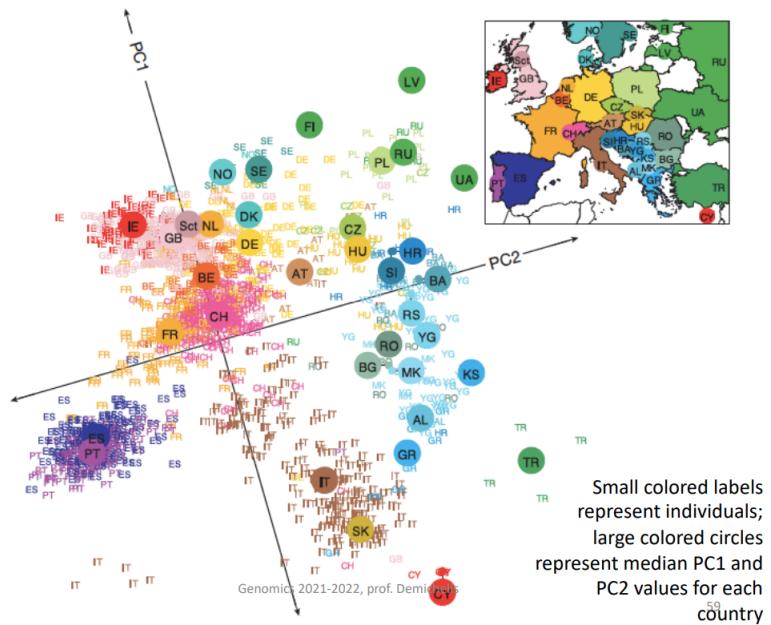
### 3.3. BUILDING A SNP-BASED GENETIC TEST

#### 3.3.5.1 Example paper: 'Genes mirror geography within Europe'

In the study seen during lectures they used a 500.000 (500k) single nucleotide polymorphism array. Information about the country of origin of grandparents, parents and other relatives was used to determine the geographical location that best represents each individual ancestry.

They run a combined study where they used a supervised search to find the best SNPs to make inference and then they tested it on another set of individuals.

By using high confidence data (individuals with high confidence origin data) and by using the genotypes of highly informative SNPs for specific region-related inheritance, they were able to rebuild the map of some of the countries in Europe 3.10.



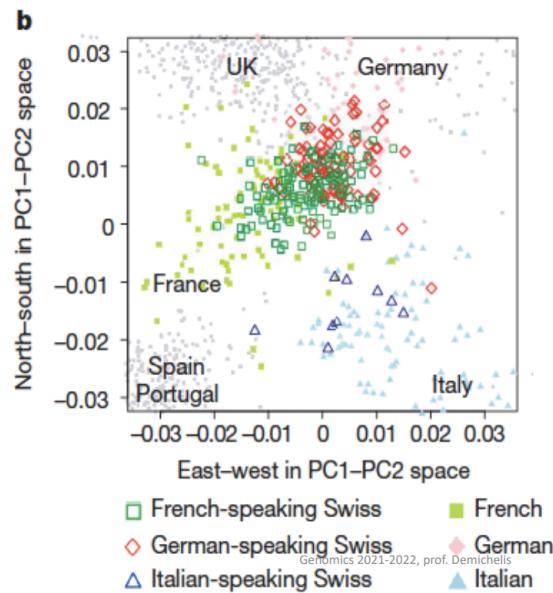
**Figure 3.10**

This result might be a little bit of a push, but it is true that by using properly selected variants it is possible to distinguish individuals coming from different countries. The way those SNPs are selected is very similar to the process saw for genetic fingerprint, but pushing for the selection of variants that are different in terms of MAF in different populations.

Clusters that are a bit more dense and distant from the others (like the Spain/Portugal cluster) could be due to the fact that many SNPs selected are typical of that area and are therefore able to maximize the difference with respect to that area (so it is a data-related 'issue').

### 3.3. BUILDING A SNP-BASED GENETIC TEST

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**Figure 3.11**

Focusing on Switzerland, they could even make inference on the linguistic canton 3.11. Again this is a bit of a push, but it is possibly true that in country where some regions have very different habits (e.g. marriage within the same area) might lead to have similar genetic fingerprint.

#### 3.3.5.2 Summary and notes

Low-frequency alleles tend to be the result of a recent mutation and are expected to geographically cluster around the location at which the mutation first arose. Hence, they can be highly informative about the fine-scale population structure.

Despite low average levels of genetic differentiation among Europeans, close correspondence between genetic and geographic distances was found. When mapping the genetic basis of a disease phenotype, spurious associations can arise if genetic structure is not properly accounted for.

# Chapter 4

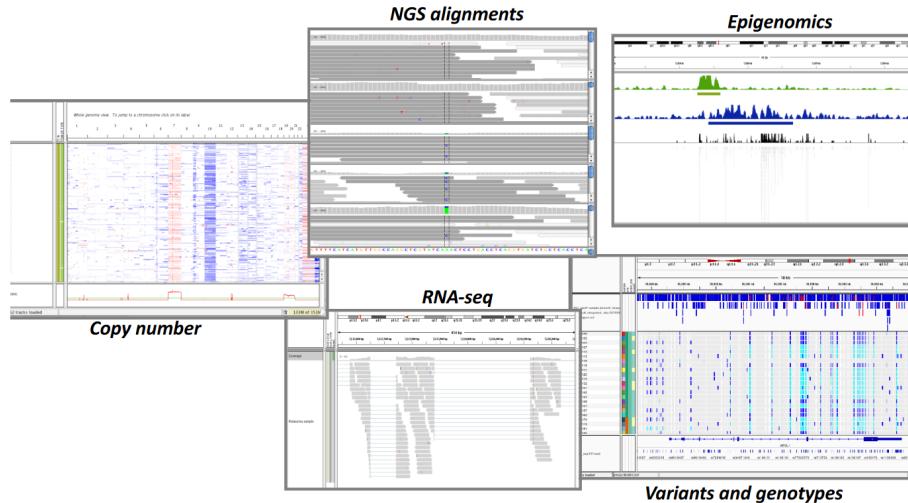
## IGV (Integrative Genomics Viewer)

The human genome nowadays is being explored extensively thanks to exons and whole-genome sequencing, epigenetic surveys, expression profiling of coding and noncoding RNAs, SNPs and copy number profiling, and functional assays.

Those findings are essential to pave the way for the future **precision medicine**, which is an approach for disease treatment and prevention that takes into account individual variability in genes, living environment, and lifestyle for each person. The scope is to administer the right drug, at the right time and at the right dose for each individual.

### 4.1 Main characteristics of IGV

Some utilization of IGV are: NGS alignment, epigenomics studies, copy number evaluations, RNA-sequencing and Identification of variants and genotypes. Below, some of the main utilization of IGV, also represented in figure 4.1.



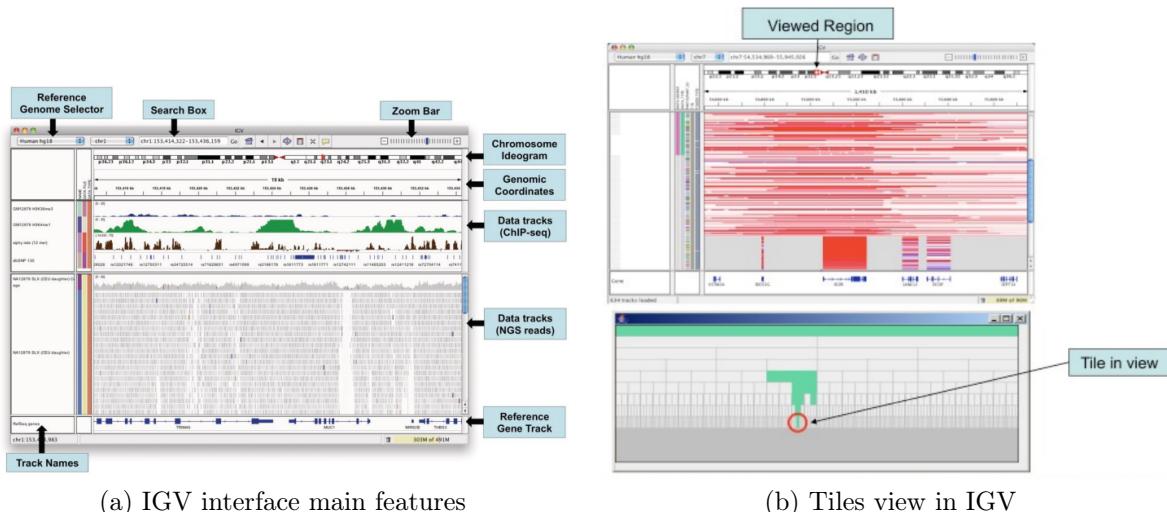
**Figure 4.1:** All the important usages of IGV

## 4.1. MAIN CHARACTERISTICS OF IGV

The IGV software is an **high-performance lightweight visualization tool** for interactive exploration of large, integrated genomic datasets. It supports a wide variety of data types, including next-generation sequence data, and genomic annotations. Data sets can be loaded from local or remote sources, including cloud-based resources.

It allows to move, zoom in and out quickly over different genomic scales (panel a) 4.2), and also to jump in precise positions of the sequence. It is possible to search for genomic coordinates or gene names. For each resolution scale (“zoom level”), the aggregated data is divided into tiles (panel b) 4.2) that correspond to a region viewable on a typical user display. Each tile is subdivided into bins, with the width of a bin chosen to correspond to the width represented by a pixel at that resolution scale. The corresponding data tiles for each zoom level are stored in the binary Tiled Data Format, or TDF, which has been optimized for fast tile retrieval.

A *tiled data file (TDF)* file (.tdf) is a binary file that contains data that has been preprocessed for faster display in IGV. TDF files are generated by using the *igtvtools* package (*toTDF* command).



**Figure 4.2**

Importantly, tile sizes for each zoom level are constant and small, and also, a single tile at the lowest resolution (spanning the entire genome) has the same memory footprint as a tile at the very high zoom levels (might span only a few kilobases).

Tiles no longer in view are discarded as needed to free memory. Navigation through a data set is similar to that of *Google Maps*, allowing the user to zoom and pan seamlessly across the genome at any level of detail from whole genome to base pair.

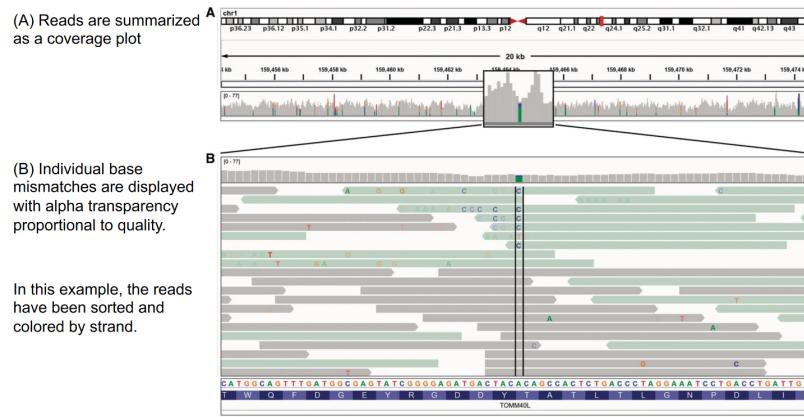
**Pixel resolution errors**, occurring when data density exceeds the constraint given by the number of pixels available for display, could be solved through data aggregation. As the user zooms below the 50 kb range, individual aligned reads become visible, like in figure ???. It is possible then to zoom further, and see the bases at each position.

Annotations for specific genomes could be found consulting the UCSC Table Browser (UCSC table).

## 4.1. MAIN CHARACTERISTICS OF IGV

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Other information regarding IGV are present in the Supplementary information - Integrative Genomics Viewer pdf file.



**Figure 4.3:** (A) Reads are summarized as a coverage plot .  
(B) Individual base mismatches are displayed with alpha transparency proportional to quality. In this example, the reads have been sorted and colored by strand. 20 kb genomic region, 1 individual. The coverage is [0-77]; at certain positions we have more than one base supported by the reads. E.g. green and blue column, some reads support C instead of A. The colored area is proportional to the allelic fraction for each base.

### 4.1.1 Igvtools

*Igvtools* comprise a set of utilities to prepare large files for efficient display. Some commands are reported in figure 4.4

<b>count</b>	- Computes alignment coverage from BAM files - Produces TDF or WIG files
<b>toTDF</b>	- Converts sorted data file to binary tiled data (TDF) - Supported file formats: WIG, bedGraph
<b>sort</b>	- Sorts file by genomic start position. - Supported file formats: BED, GFF, GTF, PSL, SAM, BAM, VCF
<b>index</b>	- Creates index for large genomic annotation files and alignments. - Supported file formats: BED, GFF, GTF, PSL, SAM, BAM, VCF

**Figure 4.4:** igvtools possible operations, the "count" function allows to generate coverage data, and it takes in input a BAM file. The obtained file could be then loaded with the "Load pre-computed coverage data" command

### 4.1.2 Session Files

Sessions allow users to share their data and views with other users simply and accurately. Session files describe the session in XML.

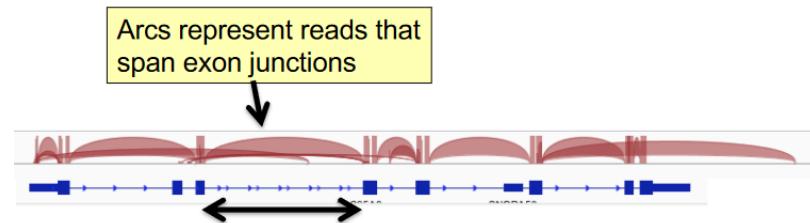
## 4.2. SOME OF THE MAIN UTILIZATIONS

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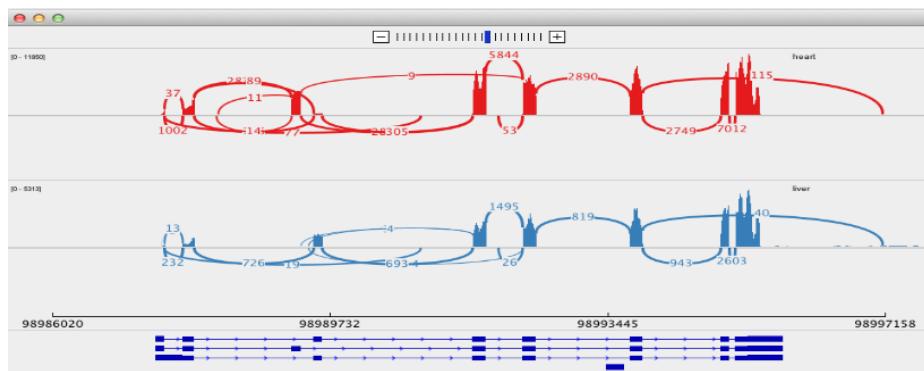
### 4.2 Some of the main utilizations

(Not all the passages needed to obtain the figures are described below, as they are included in the exercise file delivered by the professor)

#### 4.2.1 RNA-seq alignments



**Figure 4.5:** the height depends on the quantity of reads connecting the different exons.



**Figure 4.6: Sashimi plots:** The number of reads connecting exosomes are represented here on the curved lines. The peaks represent coverage within exons.

#### 4.2.2 Study of variants

It is possible to study variants from different samples.

### 4.3. INTERPRETING PAIR ORIENTATIONS



It is also possible to sort the samples in different ways and to group them considering different characteristics.

## 4.3 Interpreting Pair Orientations

We will now look at some aberrations' discoveries performed using IGV.

In IGV, each vertical bar corresponds to a read. If there is a colored sign, there is a polymorphism or difference with respect to a reference. The browser also gives info about the quality of the read and bases (and others from the BAM file).

While using a paired-end protocol, we can study inversions, duplications and translocations. A useful legend to navigate the subsequent example is reported in the list below.

- **LR** ( $\rightarrow \leftarrow$ ): Illumina (convention), the reads are left and right of the unsequenced part of the sequenced DNA fragment when aligned back to the reference genome.
- **LL, RR**: implies inversion in sequenced DNA with respect to reference.
- **RL**: implies duplication or translocation with respect to reference.

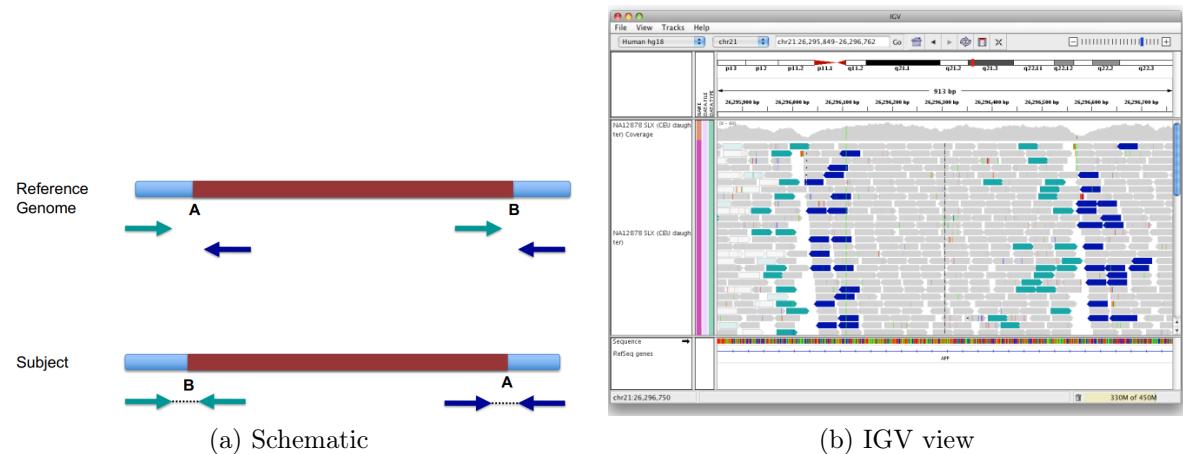
### 4.3.1 Inversion

To detect major aberration, like inversions, we need reads that span the breakpoint (either long reads, or, better, PE reads).

What's happening exactly at the breakpoint in figure 4.7? What's the coverage when looking at the data? When we map them on the reference, we see that the direction is LL and RR, meaning that there is an inversion. The sequence that we have in the molecule does not exist there and therefore that sequence does not exist in two copies in the molecule. We can also spot a drop in the local coverage. In the target molecule, either it does not exist or exist only in one allele and not the other one.

When interpreting structural variance, we need not to care only about end-orientation, but also coverage at the exact break point, which is due to the fact that the inverted breakpoint sequence does not exist in the reference genome.

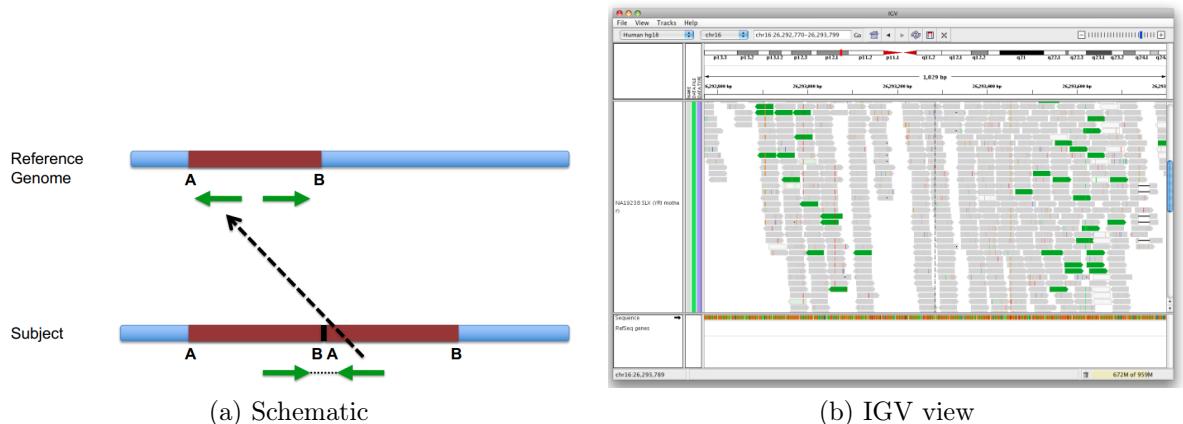
### 4.3. INTERPRETING PAIR ORIENTATIONS



**Figure 4.7:** Inversion discovering exploiting PE reads.

#### 4.3.2 Tandem duplication

Notice how in the tandem duplication (figure 4.8) all the reads that do not cover the junction point align perfectly to the reference. The coverage will be  $3/2$  of the expected value, proportional to the extra copy. B junction and A junction will not have modifications. If we had a read mapping BA, we would observe a partial alignment at B on the reference. We observe no drop of coverage because the sequences also exist in the reference.



**Figure 4.8:** Tandem duplication discovering exploiting PE reads.

#### 4.3.3 Inverted duplication

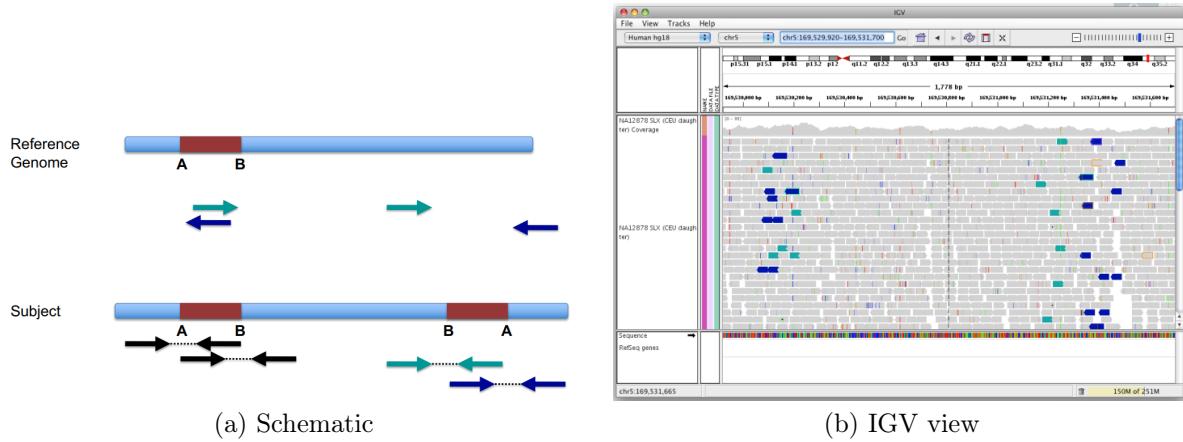
For the inverted duplication, in figure 4.9, we expect double coverage in the duplicated site in the reference genome.

Both A and B on the first segment on the subject are LR oriented, and the same occurs in the

#### 4.4. SUMMARY

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reference genome. When we add the second fragment the same holds, but direction will be LL and RR and the insert size will be significantly longer. In particular, we can notice that we have an overlapping of left and right reads on the reference. Furthermore, the coverage depth will highly increase due to the presence of multiple reads on the reference genome.



**Figure 4.9:** Inverted duplication discovering exploiting PE reads.

What if we have a deletion? How can you guess the size of it? We can look at the coverage or observed distance between the reads, which gives clean indication of the size of the deletion. For tiny deletions, smaller than the length of the read, we use the sequence within the reads and in this way discovering indels.

## 4.4 Summary

Summarizing, the elements to consider are:

- Pair ends relative orientation;
- Insert size length;
- Coverage within the aberrant region;
- Coverage outside of the aberrant region (flanking genomic segments);
- Coverage at the breakpoints.

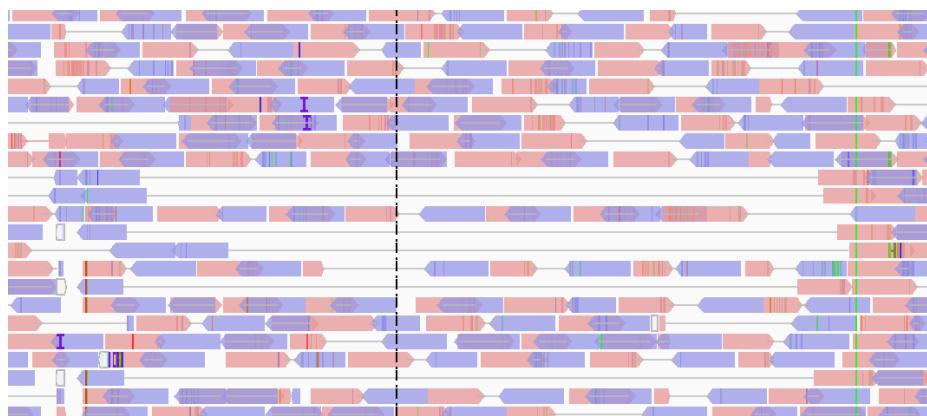
## 4.5 Exercise

The goal was to read pairs/end order/coverage/insert sizes at following coordinates (reference: hg19). Then to interpret, if possible, as inversion, inverted duplication, tandem duplication, or deletion. The description of the tasks can be found in the slides.

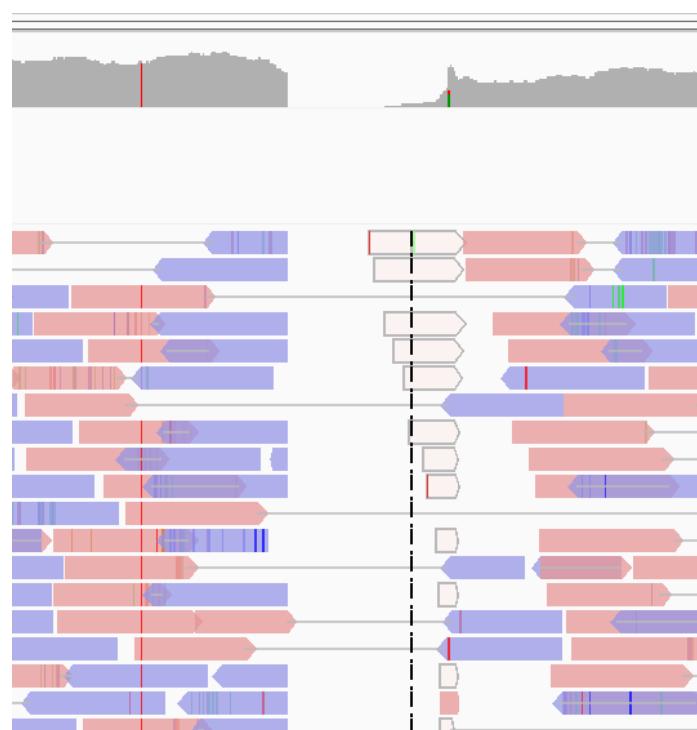
## 4.5. EXERCISE

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### 4.5.1 Task B



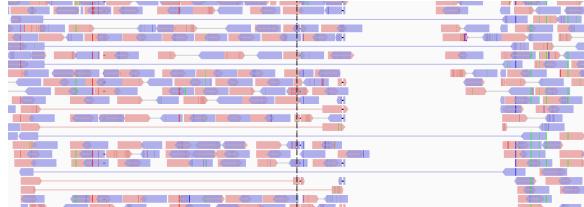
**Figure 4.10:** *chr1:11,050,009-11,055,137*: It could be a tandem duplication on one of the two alleles and a deletion on the other allele. The reason why I would suggest the presence of a deletion is due to the fact that the coverage remains quite constant, despite of the duplication.



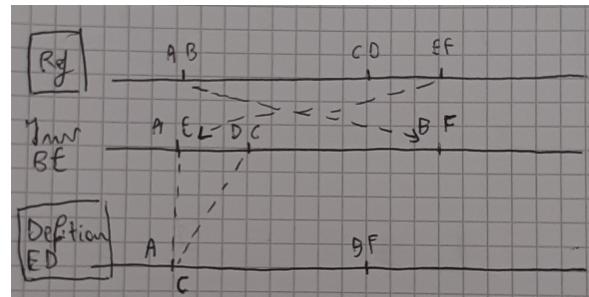
**Figure 4.11:** *chr5:9,410,315-9,413,699*: it is quite clear that both the alleles were deleted in that region, because of the decrease in coverage

#### 4.5. EXERCISE

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(a) IGV view



(b) written passages

Figure 4.12

# Chapter 5

## Tumor Evolution Studies via NGS data

Tumor board: organism research oriented (and not) hospitals, patient not strictly assigned to one doctor but many specialists. E.g. oncologists, pathologists, geneticists... These boards are also a training asset for complex case studies and teach young doctors how to manage difficult events.

### 5.1 Tumor evolution

It is important to understand what are the somatic events that occur during tumor genesis/evolution and when they arise.

Cancer cells accumulate mutations due to both cell division and toxic agents (radiations, UV light,...); these mutations are maintained by the cell and lead to clonal expansion. We can have a driver mutation kicking an oncogene or multiple mutations. Typical traits of cancer are:

- Cancer is a dynamic disease, that's why evolution of the disease is so important to track.
- During the course of disease, cancers generally become more heterogeneous, which is often related to treatment resistance.
- The bulk tumor includes a diverse collection of cells harboring distinct molecular signatures with differential levels of sensitivity to treatment.
- This heterogeneity might result in a non-uniform distribution of genetically distinct tumour-cell sub-populations across and within disease sites (spatial heterogeneity) or temporal variations in the molecular makeup of cancer cells (temporal heterogeneity).

#### 5.1.1 Tumor heterogeneity

Every site of the genome with somatic mutations (point, inversion, ...) is going to be differentially represented in tumor samples. Heterogeneity provides the fuel for resistance and is an obstacle to cancer treatment.

Therefore, an accurate assessment of tumor heterogeneity is essential for the development of effective therapies. Emerging techniques to study with considerable potential to dissect the complex clonal

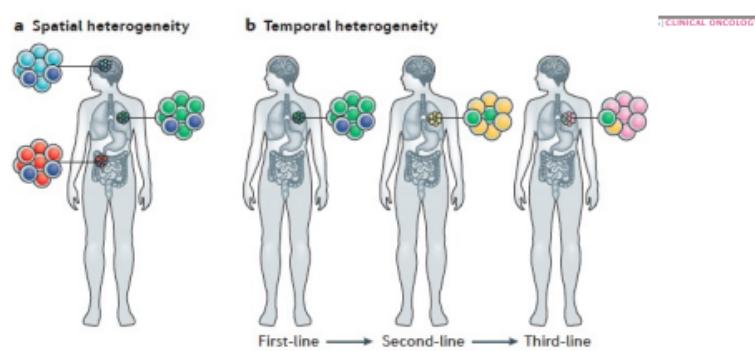
## 5.1. TUMOR EVOLUTION

architecture of cancers heterogeneity are: multi-region sequencing, single cell sequencing, analysis of autopsy samples, and longitudinal analysis of liquid biopsy samples.

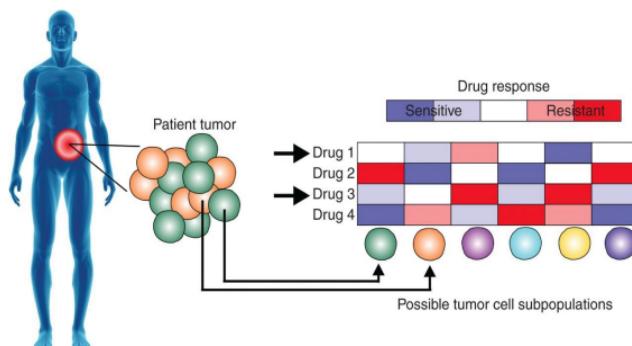
However, techniques to study tumor heterogeneity are hindered by intra-patient heterogeneity, which divide into **spatial** and **temporal** heterogeneity.

**Spatial heterogeneity** The patient has several independent tumor masses, which share certain cells, but have a unique genetic makeup;

**Temporal heterogeneity** One mass changes content over time, naturally or under particular pressures.



**Figure 5.1:** a) Spatial heterogeneity denotes an uneven distribution of cancer subclones across different regions of the primary tumor and/or metastatic sites. b) Temporal heterogeneity refers to variations in the molecular makeup of a single lesion over time, either as a result of natural progression of the tumor or as a result of exposure to selective pressures created by clinical interventions. Colors denote the presence of subclones with different genetic features.



Clare Fedele et al. *Cancer Discovery* 2014.

**Figure 5.2:** Certain cells of the tumor mass respond to treatment and some don't. Red = cell resistant to drug, blue = cell sensitive to drug.

## 5.1. TUMOR EVOLUTION

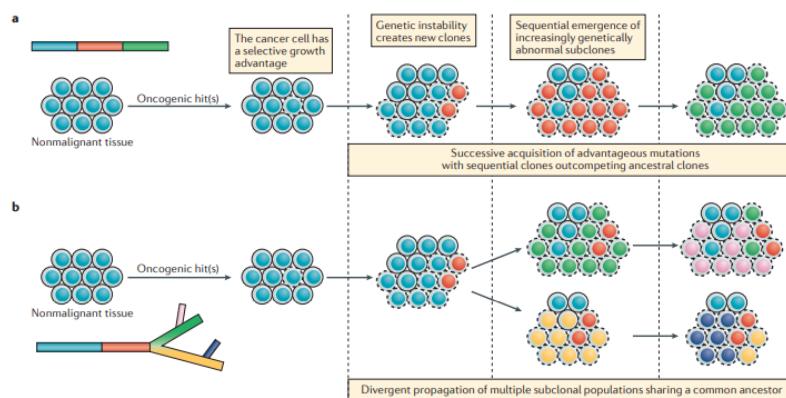
### 5.1.1.1 Linear an branching evolution

It could be possible that some cells positively respond to treatment and others not, creating a heterogeneous population in the mass.

The features of this set of cells changes over time. This evolution happens either because the new population **replaces** the older, or there's a **branching** and the tumor mass becomes heterogeneous.

**Linear evolution** Sequential genetic alterations confer a fitness advantage such that successive generations are able to outcompete the preceding clones, which lack this fitness advantage. Surviving dominant clones harbor the ancestral mutation.

**Branch evolution** Multiple genetically distinct populations can emerge from a common ancestral clone, with certain subclonal populations diverging from the common ancestor before others. The branching evolution is depicted in figure 5.3.



**Figure 5.3:** a) everything branches out from the monoclonal origin, but b) polyclonal origin, independent metastatic processes. Cells from independent lesions meet and form a highly diverse metastatic tumor.

How can we study tumor evolution? Example seen in class: prostate sample, multiple independent lesions. By browsing through the morphologies, we can find similarities for the expression of the same gene. Sanger sequencing can be used for very specific mutations. Through bulk DNA sequencing from each position, a representation of the tumor burden and features of all the different areas can be obtained.

### 5.1.2 Treatment resistance

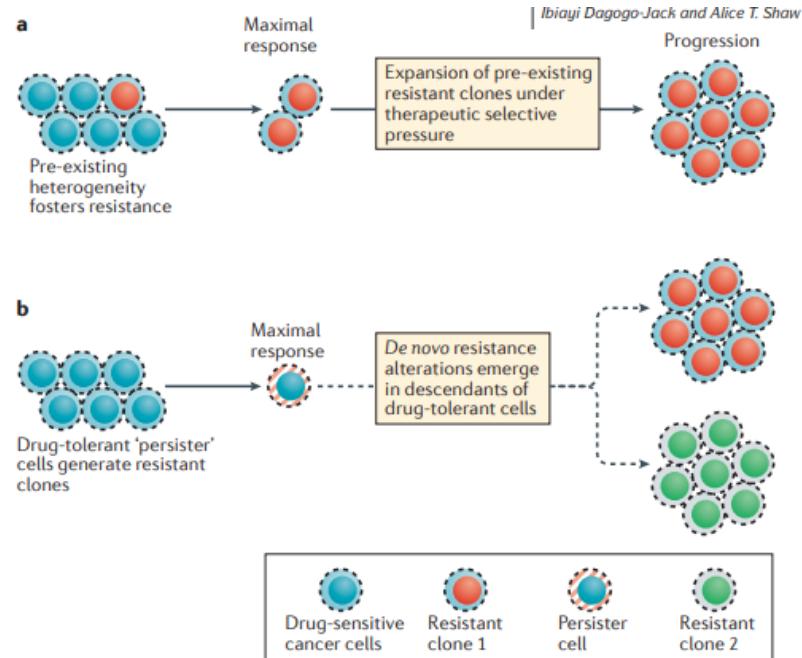
How does resistance to treatment arise in cancer cells? Is treatment resistance encoded in the original cells or is it driven by the treatment itself?

The two possibilities (also reported in figure 5.4) are selection of clones that provide resistance, or transformation of clones under treatment pressure.

**Primary resistance** Pre-existing heterogeneity fosters resistance. Only susceptible cells die and resistance cells continue dividing, allowing the tumor to regrow (high mass tumor).

## 5.2. FROM SKETCHES TO SEQUENCING DATA EVOLUTION INFORMATION

**Acquired resistance** Drug-tolerant, “persistent” cells generate resistant clones. At the time of diagnosis there are no markers, the de novo resistance alteration is developed afterward. Eventually, the resistant cells can form new tumors not responding to the drug.



**Figure 5.4:** Tumor cells evolution driven by treatment.

## 5.2 From sketches to sequencing data evolution information

In order to study tumor evolution, we can use common and private lesions across multiple samples from the same individual to reconstruct the path. Conversely, we can learn from multiple individuals at the same time points (select the most clonal lesion), with the goal of building a common clonal evolution map. E.g. tumor suppressor gene NKX3-1 and PTEN lesions are shared between many individuals. Overall, in prostate cancer it is likely to have a subsequent PTEN mutation if CHD1 mutation is present.

In general, we are interested into finding out which lesions occurred first and which is the model that fits better the data.

### 5.2.1 Tumor evolution and heterogeneity

To summarize, the difficult tasks one has to deal with when studying tumor data, and always need to study and take into account are:

- Intra tumor heterogeneity.
- Inter tumor/intra patient heterogeneity.
- Inter-patient heterogeneity.
- Clinical/treatment relevance.
- Time dependency.
- Admixture DNA (tumor purity).

### 5.3. USEFUL MEASURES FROM NGS PIPELINE

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If properly investigated, they provide for insightful hints in the analysis.

#### **5.2.1.1 Admixture**

Whenever we take a piece of tissue from a patient, we have multiple cell types. The same happens with a biopsy, which will never be composed 100% by cancer cells. This concept is referred to as **DNA admixture**.

**Tumor purity** is computed as 1-DNA admixture. Purity is related to lesion aggressiveness (low purity is linked to less severity) and lesion classification (clonal or subclonal). In fact, every interpretation of somatic data needs to be interpreted in the context of tumor purity. Lesion is clonal if all tumor cells have it, a less present lesion instead might be subclonal.

## **5.3 Useful measures from NGS pipeline**

Also in this case we will be exploiting the polymorphic information that is present in the genetics of every individual. SNPs are very helpful in all somatic analysis.

- **Minor allele frequency, (MAF)**, frequency at which the alternative allele at a polymorphic site is represented in the population.
- **Allelic fraction, (AF)**, how many times at a specific locus in a single individual we see the alternative base being represented. How many reads represent the alternative allele, how much support.

### **5.3.1 Informative SNPs in cancer studies**

Informative SNPs are SNPs at which one individual has a heterozygous genotype; we can count allelic fraction and assess the proportion of reads supporting the alternative base. If we have a deletion spanning a genomic area, at every informative SNP we will see that the allelic fraction, instead of being 50, will be either 0 or 100. Therefore, a diseased tissue will have an allelic distribution peaking at 0 or 1.

$N_{ref}$  is the percentage of reference base in the non-deleted allele.

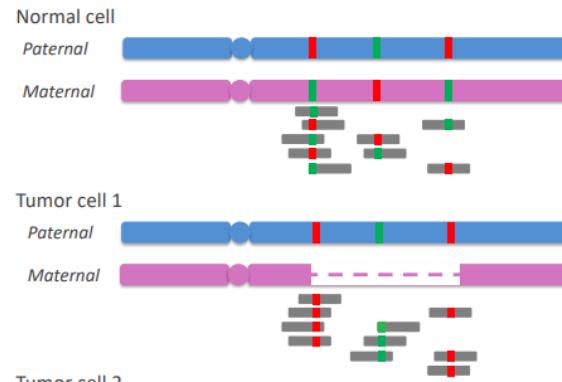
We can also track **beta ( $\beta$ )**, the percentage of neutral reads. If both alleles are equally represented, beta will be equal to 1. Conversely, when only one allele is represented, beta is equal to 0. How much beta is different from 1 is an indication of either admixture or subclonality of the lesion. When only some cells in the sample are tumor cells, the distribution of AF will have two peaks.

In each genomic segments that we are studying we many informative SNPs, at least two, to drive some conclusions. For example, figure 5.5.

How do we select informative SNPs? Use dbSNP database and select all heterozygous genotype sites, patient-centric analysis regardless of MAF. Obviously in the case of a target assay, it is possible to enrich for SNPs for high MAF, maximizing likelihood of finding informative SNPs (should be carefully designed).

### 5.3. USEFUL MEASURES FROM NGS PIPELINE

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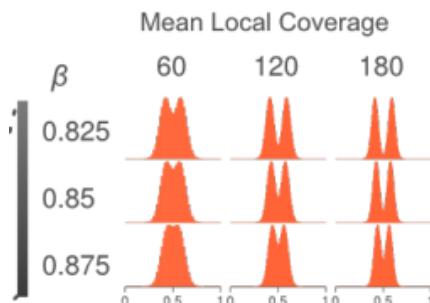


**Figure 5.5:** Thanks to the presence of informative SNPs is very easy to detected the loss of an allele in the tumor cell.

An example of the use of AF and beta measures for tumor data analysis is depicted in figure 5.6.

#### 5.3.2 Coverage and AF properties

Does the mean coverage of the experiment impact on the ability to use the AF properties? Intuitively, the deeper the sequencing the more likely it is to distinguish distribution that are not so close to each other. It is especially important when  $\beta$  is close to zero. An example is shown in figure 5.7.



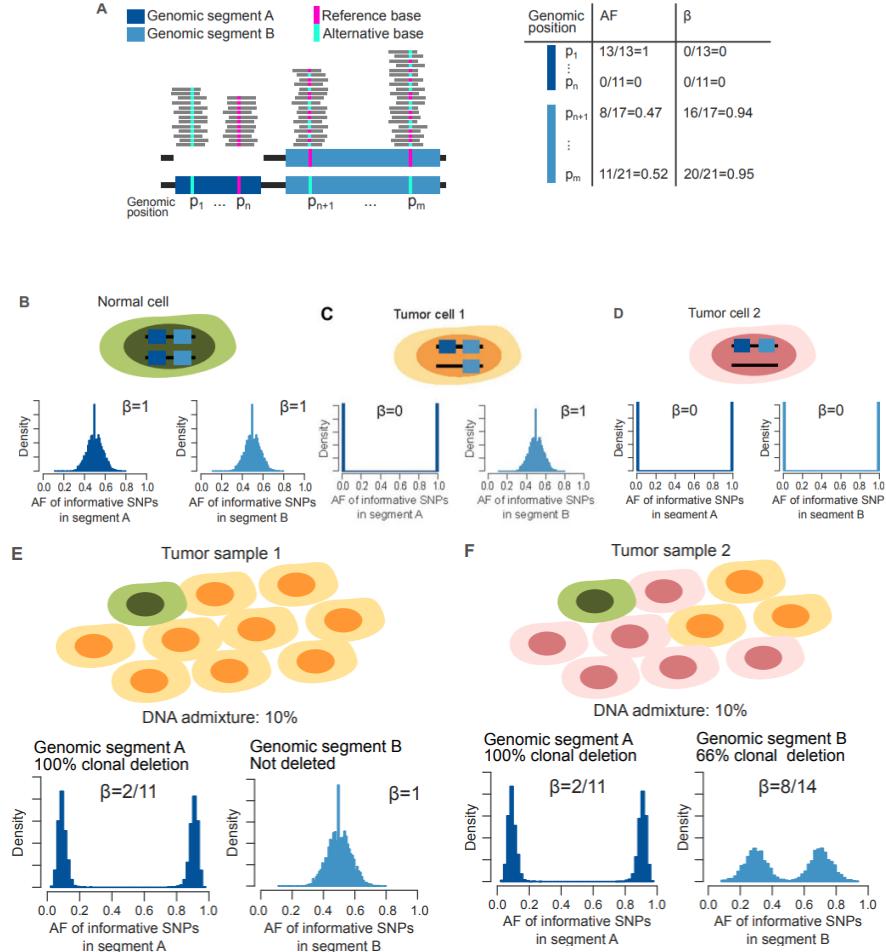
**Figure 5.7:** 2X experiment: on average we have 2 reads per gene, very easy to miscall.  
10X experiment: easy to do call, but in the case of tumor cells it becomes difficult. The higher the sequencing depth, the more accurate the calls will be (as it becomes easier to tell when a distribution has two peaks).

#### 5.3.3 Computing Beta

We can compute beta for each genomic segment S:

1. compute the observed distribution of the AF of informative SNPs in the genomic segment S;
2. find the values of Beta and Nref such that the expected distribution of the AF matches the observed AF;

### 5.3. USEFUL MEASURES FROM NGS PIPELINE



**Figure 5.6:** **A)** Example of the allelic fraction (AF) and beta ( $\beta$ ) computed in five genomic positions ( $p_1$  to  $p_m$ ). Positions  $p_1$  to  $p_n$  are within a hemizygous deleted genomic segment A, while genomic positions  $p_{n+1}$  to  $p_m$  lie within a wild type genomic segment B.

**(B-D)** Examples of a normal cell and two different tumor cells. Tumor cells 1 and 2 differ for the status of genomic segment B. Histograms below cell cartoons report the expected distribution of the allelic fraction of SNPs in genomic segments A and B together with the associated beta values.

**(E-F)** Examples of two different tumor samples. Tumor sample 1 includes one normal cell and nine tumor cells with deleted genomic segment A and wild type genomic segment B. Tumor sample 2 differs from tumor sample 1 in the presence of six tumor cells with a hemizygous deletion of genomic segment B. Expected distribution of the AF of informative SNPs together with estimated beta are depicted below each tumor sample cartoon.

## 5.4. GLOBAL VS LOCAL ESTIMATES OF ADMIXTURE

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3. compute uncertainty around Beta as a function of:
  - (a) the mean coverage of S;
  - (b) the number of informative SNPs in S;

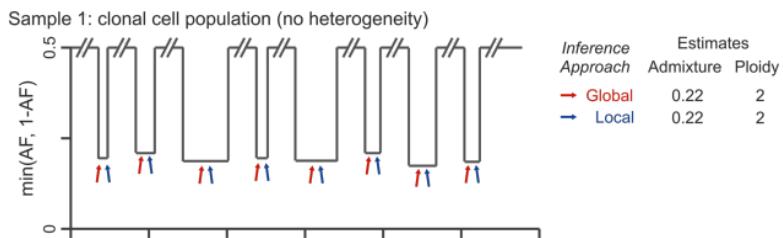
### 5.4 Global vs Local Estimates of admixture

We'll discuss two types of sample and how to determine the differences in cell population (admixture). In sample one depicted in figure 5.8 there's a clonal cell population, meaning no heterogeneity. On the X axis we have genomic coordinates indexed by informative SNPs for that individual, on the Y axis the half AF.

For all the informative SNPs present in this chunk of DNA, we see drops in AF that are smaller or wider, but more or less similar for each one of those lesions (drops). Meaning a drop or a gain in the amount of DNA that is almost identical in all of these chunks.

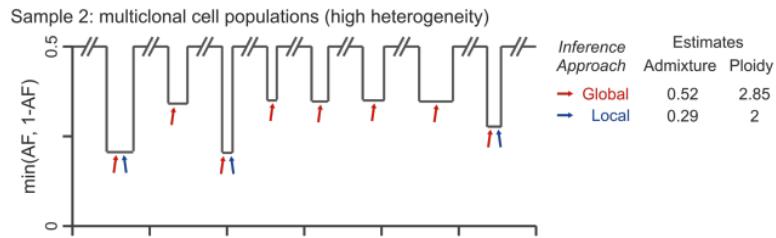
The representation of the lesions is supported by the same data along the stretch of DNA. Thinking in terms of how much the AF distribution from 0 and 1 and the center is basically the same across all of them. Meaning, the level of admixture, both globally and locally, is the same.

Or, again, the amount of cells that have the first, second, third lesion etc. is the same.



**Figure 5.8:** In the clonal cell population how much the distribution deviates from AF is the same globally and locally.

In picture 5.9 below we can see another sample. We can clearly discover multiclonal cell population. The depth of the lesion is proportional to the number of cells that carry the lesion. In this case the definitions of local and global admixture change: a **global** value is a global value of tumor purity, a local value is a value of the clonality of the lesion in the diseased cell population.



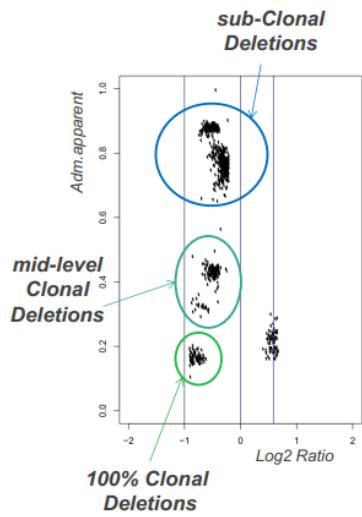
**Figure 5.9:** We observe heterogeneity, the global and local values are different. A global value is relative to tumor purity, local to clonality. Admixture = 1-tumor purity

## 5.5. A CHALLENGING CASE (PR-2741\*)

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### 5.4.0.1 Estimate of DNA admixture (1-Purity)

We will now translate the concepts expressed in the previous sub-sections 5.4 in a 2-dimensional space, as shown in figure 5.10.



**Figure 5.10:** Multiple clusters, where each dot is a genomic segment. Lower clusters are used for the admixture, the others are subclonal.

sions.

Moreover, the closest the points are, the most probable it is that the lesions happened close in time, and viceversa.

## 5.5 A challenging case (PR-2741\*)

Data of a real case of prostate cancer in which we can see a clear drop in coverage in region 2 of the 5th chromosome, while region 1 and 3 have equal coverage. The DNA present in region 2 could come either from admixing cells or from cells that do not have the deletion.

Looking at the AF of region 1, 2, 3, both from the tumor and the match normal normal sample we observe more or less the same two modes of distribution.

In the tumor sample in fact we do not see two peaks in 0 and 1 as expected. It could be signal of intervening normal cells, that bring the modes to the center, or subclonality event.

On the X axis we have the Log2 ratio measure and on the Y axis the admixture apparent, which is proportional to the beta value.

The Log2 Ratio is basically tumor over normal in the log2 space. It allows to interpret info about every segment in the genome when coupled with the beta measure.

The admixture apparent is calculated as

$$Adm. \text{ apparent} = \frac{\beta}{2 - \beta} \quad (5.1)$$

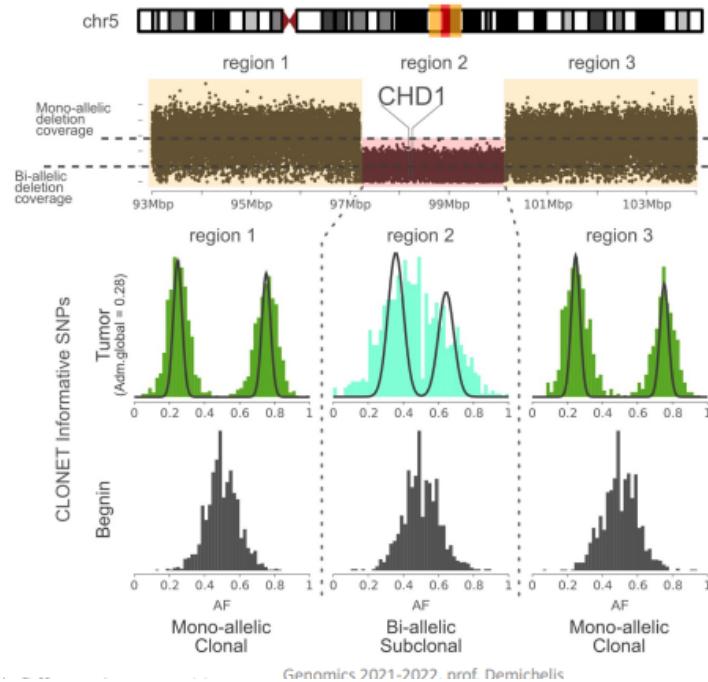
This measure associates an apparent DNA admixture to each monoallelic deletion. It is useful to calculate the clonality values, for which the formula is:

$$\text{Clonality: } \frac{1 - Adm. \text{ apparent}}{1 - Adm. \text{ global}} \quad (5.2)$$

The lowest cluster is the one used to assess admixture and the other ones are subclonal le-

## 5.5. A CHALLENGING CASE (PR-2741\*)

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**Figure 5.11:** The distribution of heterozygous SNPs in benign cells is peaked in 0.5, while we observe two modes in tumor cells. The distance between the two modes is equal in 1 and 3, it's proportional. In the middle we see that the two modes are moving towards the center, suggesting that the deletion is not likely 100% clonal or it is clonal and shift is due to lack of deletion in 1 and 3. Subclonality: lesion with admixture

# Chapter 6

## Tumor evolution studies (continued)

### 6.1 Analysis of clonality

#### 6.1.1 Recalls from previous chapter

At the basis of tumor evolution is the concept of how to use informative SNPs:SNPs for which a specific individual has heterozygous calls so that set of SNPs is unique for every individual. This property is connected to the fact that when we have the loss of an allele, the allelic fraction of the informative SNPs within that lesion will be informative of the lesion and its depth (clonality = what's the fraction of tumor cells that very likely harbor that lesion).

We can also have different population of cells, when a set of lesions is present in every population it is said to be clonal whereas when a specific set of lesion is harbored only by a subpopulation it is defined as subclonal.

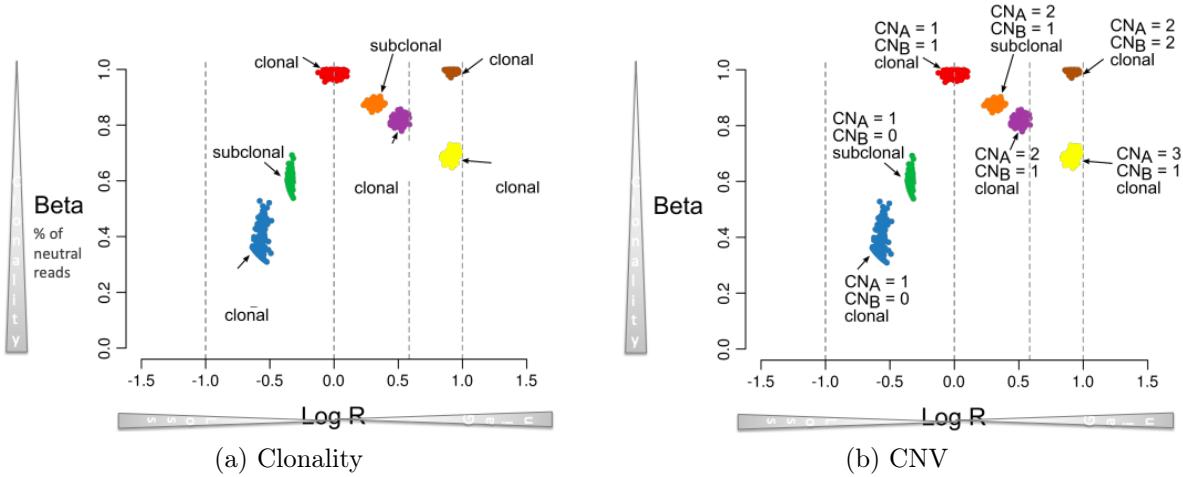
- The **Log2 ratio** is the log<sub>2</sub> of the ratio of the tumor over the normal that applies to array data signals (intensity of the signals) but also to the local coverage of a tumor BAM file over a normal BAM file.
- **beta** is a variable that goes from 0 to 1 and provides information of the number of reads that equally represent the two alleles; when beta is equal to 1 the concept of admixture (1-purity) is equal to 1 meaning that purity is equal to 0 if we are at the top of the y scale it means that there's no signal related to tumor content, while the lower we go, so the closer we get to 0, the higher the tumor content and the level of clonality is.

#### 6.1.2 Cluster analysis, an example

Referring to the image 6.1, we can derive some conclusions.

## 6.2. EVOLUTION MAPS

---



**Figure 6.1:** Lower clusters are more clonal, the more left the deeper they are in terms of loss of DNA.

Looking at panel A of figure 6.1

- The blue cluster with deletions is the most clonal one.
- Both blue and green clusters have deletions, since they have a negative log2 ratio, but the green one is less clonal than the blue one.
- In  $\log_2 R = 0$  and  $\beta = 1$ , where there's the red cluster, we have a status of no copy number changes (wild-type status in terms of copy numbers). This basically represents a total number of alleles which is the same in both the tumor and normal sample.
- All the other clusters with a positive log2 ratio had a gain of DNA.

In panel B of figure 6.1 the number of copies that correspond to all the clusters in the space is also reported.

- Blue: one copy of DNA, so there's a deletion
- Green one: also one copy of DNA but with subclonality

This is how we can map in the space the status of clonality and the number of copies for a specific segment in the genome.

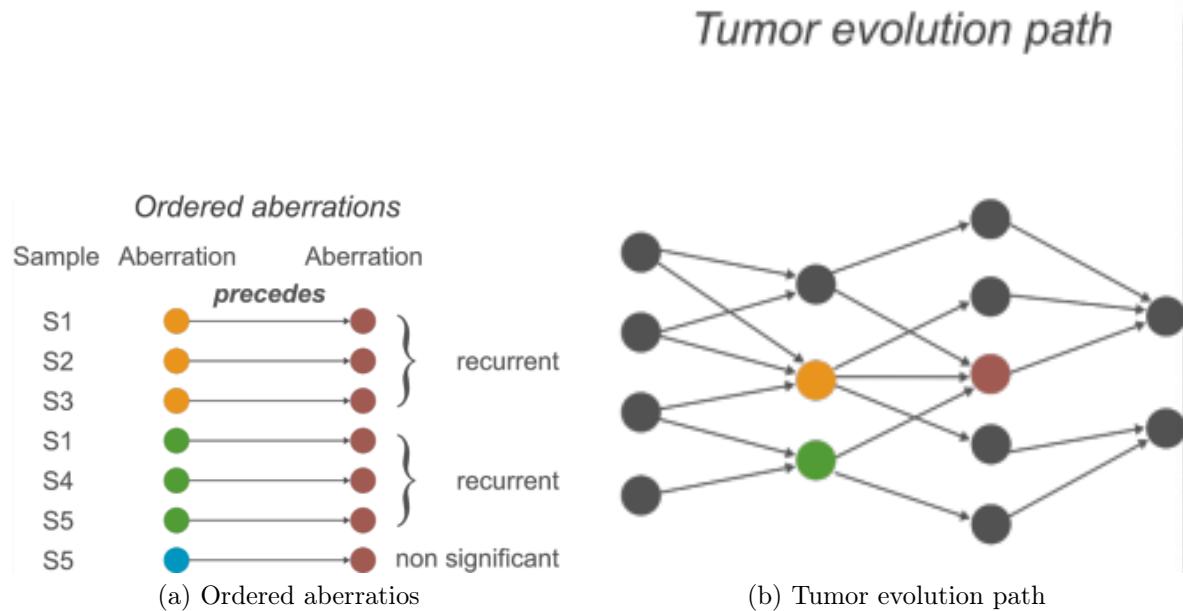
## 6.2 Evolution maps

We can use the information on clonality to build **evolution maps**. We wish to track the evolution of tumors over time with specific conditions e.g. treatment.

## 6.2. EVOLUTION MAPS

### 6.2.1 A toy example

The first thing to do is to look, within each individual, at concomitant deletion where one is subclonal to the other one.



**Figure 6.2:** Lower clusters are more clonal, the more left the deeper they are in terms of loss of DNA.

In panel A of figure 6.2:

- In sample 1 the brown lesion is subclonal to the orange one, and that same lesion is also subclonal to the green one.
- In sample 2 we have again the support of the relation between the brown and orange lesion with the same level of subclonality (brown subclonal to orange).
- In sample 3 is the same as in sample 1 and 2.
- Samples 4 and 5 have the same concomitant green and brown lesions again with the same level of subclonality.
- In sample 5 only we also have another concomitant lesion (blue subclonal to brown). The blue lesion is not included in the tumor evolution path, as it is not significant - we cannot build an arc.

The statistical support of each ‘arrow’ will depend on the number of observations and the total number of co-occurrences.

We then perform this analysis for all the concomitant lesions in our sample and we start drawing edges to keep track of what is subclonal to what. We compile this list across all individuals and look for how many times we see support for the same relationship in the same direction.

In our case we can say that the relationship going from orange to brown is supported by 3 out

## 6.2. EVOLUTION MAPS

of 5 individuals; the same can be said for the green going to brown. The blue one is instead not significant since it's supported by only one individual.

So having multiple observation supporting that aberration x precedes aberration y (i.e. aberration y is subclonal to aberration x) we can build an evolution chart.

In panel B of image 6.2 we have a graph representing the possible tumor evolution path. The orange and the green which have no relationship between them, are at the same level on the x axis in the path and they both go into brown. So one can assume that the more clonal a lesion is the more likely it is that it occurred earlier during the evolution (time is on the x axis of the path), and we can look for recurrent relationships among lesions.

In principle we can say that the grey ones at the beginning happened at the same time point and then at a second time point, the tumors in our set of samples, underwent loss of orange and green genes and then later they both underwent loss of the brown gene.

### 6.2.2 Working with real data

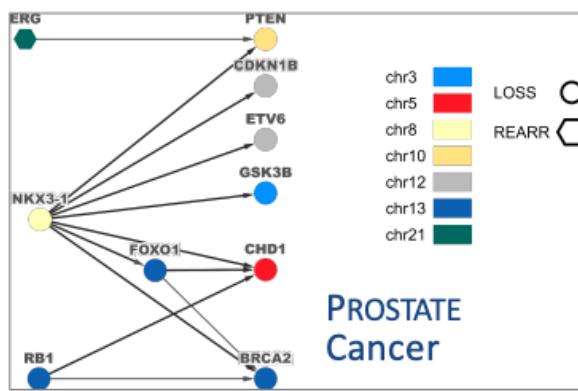


Figure 6.3

If we perform the same analysis in large datasets (lung cancer melanoma, prostate cancer ...) we can come up with all the dependencies that were observed and that were supported by more than one individual (e.g. in prostate cancer we can say that a loss in NKX3-1 precedes the deletion of PTEN).

Even if we have hundreds of BAM files on whole exon sequencing data from large collections all that we can build are evolution maps with at most three layers (pretty disappointing).

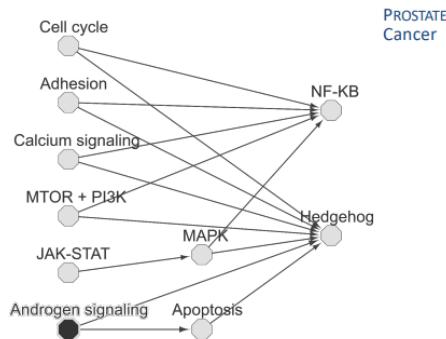
One reason for this is that to build a relationship which is statistically significant between two genes we need to have multiple instances of that relationship (in many samples). Meaning that we need to have co-occurrence of the two lesions and subclonality of the second lesion with respect to the first in a significant number of individuals compared to the total number of individuals that have co-occurrence. So if co-occurrence occurs in N individuals and subclonality of the second lesion to the first one occurs in a fraction of those, only if this fraction is significant with a proportion test out of the total number, then we can build the path. We are tremendously limited by co-occurrence of lesions.

## 6.2. EVOLUTION MAPS

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### 6.2.3 Pathway based evolution analysis

To boost the reconstruction of these paths gene families or **pathways** have been exploited, like for prostate cancer in figure 6.4



**Figure 6.4:** Pathway evolution study example for prostate cancer

E.g. if we are dealing with PTEN, which is a tumor-suppressive gene relevant in a specific pathway (PF3K), then it doesn't matter if we have deletion or inactivation of the same genes in the same pathway, what matters for the tumor evolution is that that specific pathway is altered and so what we can do is start aggregating signals from genes that belong to the same pathway.

So if individual 1 has a relationship between gene A and some gene in a specific pathway (PF3K) and individual 2 has a relationship between gene A and a second gene in that same pathway, then we can assume that maybe they have the same effect and so we can aggregate the information on the landing gene.

Basically, instead of going from gene 1 to gene 2 we go from pathway 1 to pathway 2, and in terms of numbers what we gain is that the co-occurrences are counted including all the gene lesions with the same function in pathway 1 and all the gene lesions with the same function in pathway 2 (if we consider the inactivation of the gene then we have to consider all the lesions that inactivate the gene and not others).

With this method we start having some more data to look for major changes during the evolution of the tumor pathway.

E.g. in prostate cancer we'd identify a set of pathways that are more or less at some level altered in earlier staged disease and that then trigger or are precedent to our pathways. Doing so we can learn more in terms of the biology of the disease evolution.

There are also more complicated ways to make inference of tumor evolution. Some try to avoid the hypothesis that the more clonal a lesion is the more likely it is to happen early, because we know it's not always the case; it might be in untreated samples but not in treated samples. In a treatment regimen, because of drug pressure selection, specific resistant clones harboring a specific lesion can take over due to their higher rate of proliferation. In this case, if we see a lesion that appears to be more clonal, it doesn't really mean that it happened earlier, it may be that it had a higher proliferation and so it's taking over (and we see it as apparently clonal but it's in fact a late event).

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

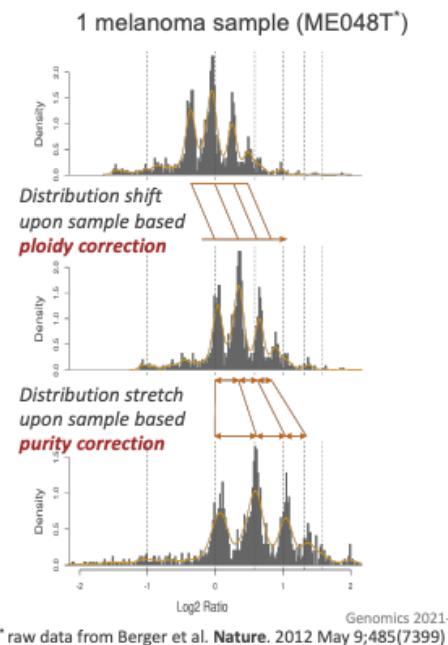
## 6.3 Ploidy and purity correction on $\log_2(\frac{T}{N})$ data

How can we use measure of the tumor purity and the effect of the tumor ploidy? How can we compare two different samples for which we quantify completely different levels of tumor content? For example, suppose we have a sample a 100% pure and with 50% of clonality (a lesion present in 50% of the cells) and a second sample with a tumor purity of 10% and a clonality of 100% (a lesion present in 100% of the cells). We need to compare numbers without having to convert every time, for every lesion, the depth of the lesion based on the tumor content.

The accuracy in calls for pure or admixture tissues (with same coverage) is different, we are likely to witness many more FP calls in admixture case. In order to compare, we need to **adjust the signal for tumor purity and ploidy**.

The coverage makes data coming from different samples comparable because we normalize everything to the total coverage, but when we deal with diseased cells we can have contamination from the admixture, so we need an extra step. The step, once we know how to assess the tumor purity and ploidy, is quite simple: we need to adjust the data for tumor purity and ploidy. An example is reported in figure 6.5. These corrections are part of standard preprocessing.

### 6.3.1 Melanoma example



**Figure 6.5:** Highly aberrant melanoma sample: genome duplication at some point + intervening lesions. The ploidy correction shifts the distribution around zero. For purity correction, the nominal value is around  $-1 \rightarrow$  stretch distribution, set peaks at the right position. Raw data from Berger et al. Nature. 2012 May 9;485(7399)

In the figure we are looking at one tumor sample: a whole genome sequencing of one melanoma sample. We see multiple peaks which correspond to different copy number states.

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

Most of the time, no matter NGS or array data, the best way to detect CNV is to compare tumor signals with normal signals. If we have tumor data with no somatic changes; if we match it to a normal genome, the two samples will be really similar (just some noise). The histogram of the log2ratio will look like a Normal distribution centered around zero.

Instead, when we have a tumor sample with many heterozygous or homozygous deletions; the distribution will still have a bulk around zero, but also two bums towards -1 (first heterozygous, then homozygous deletion). Extra copies would be represented by bums towards +1. In the case of admixture samples, the noise effect will prevent us to clearly identify bums, as they will be closer to the central peak.

Let's suppose we have a genome with a backbone of three copies but we sequence a bulk and we don't have 100% purity but 80% (so 20% is contamination).

#### **6.3.1.1 Ploidy correction**

Computationally we assess the ploidy through the copy number space and then correct the data. From the tumor and the match normal we obtain something like the first plot (top-left in picture 6.5, and we could wrongly assume that the main peak is always in 0 (wild-type state of the genome), but it shouldn't. In fact, if we assess the ploidy and overall we see a backbone state of three copies for our genome, then the main peak should be shifted toward three.

So, the *ploidy correction shifts the distribution towards the right* (second graph, 6.5).

#### **6.3.1.2 Purity correction**

We correct our data and the *purity correction causes a stretch between the peaks*, since tumor admixture dilutes the signal. So, the effect of purity correction is a wider spread between the peaks (third graph, 6.5).

#### **6.3.2 Melanoma example with 25 samples**

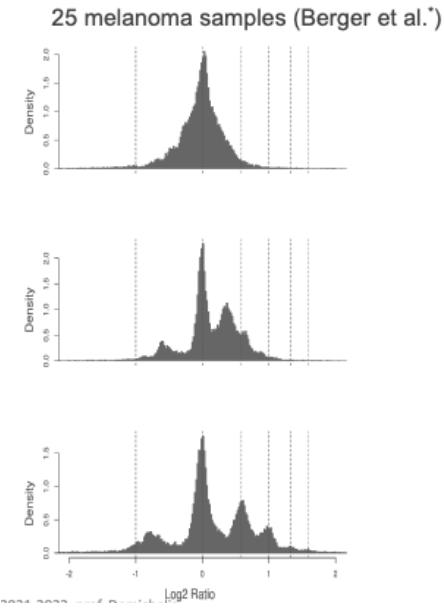
If we have one extra copy in our tumor, the log2 ratio will be around 0.58 and so we would expect that the signal will peak around that value; for two extra copies we'd expect a peak around 1 and so on.

We'll have the peak of the normal state around 0 and then if we have an underrepresented allele in our tumor we'd get another peak around -1 for the hemizygous deletion and then the homozygous deletion.

If our signal is not 100% pure tumor (so diluted by normal cells), the peak at -1 and 0.5 would be closer to the 0 peak for uncorrected data.

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

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**Figure 6.6:** 25 melanoma samples. Ploidy correction: start seeing interpretable signal. Purity correction: even though it is not perfect (hemizygous is different from -1), it is still quite clear. Raw data from Berger et al. Nature. 2012 May 9;485(7399)

- 1<sup>st</sup> graph (6.6): the distribution of the log2 data of uncorrected signal, every melanoma sample is highly aberrant with a ploidy that is different between different individuals and a purity that is also different between different individuals. But we do have the tumor ploidy and purity so we can correct the data and get rid of the noise.
- 2<sup>nd</sup> graph (6.6): we correct for ploidy.
- 3<sup>rd</sup> graph (6.6): we correct for purity too.

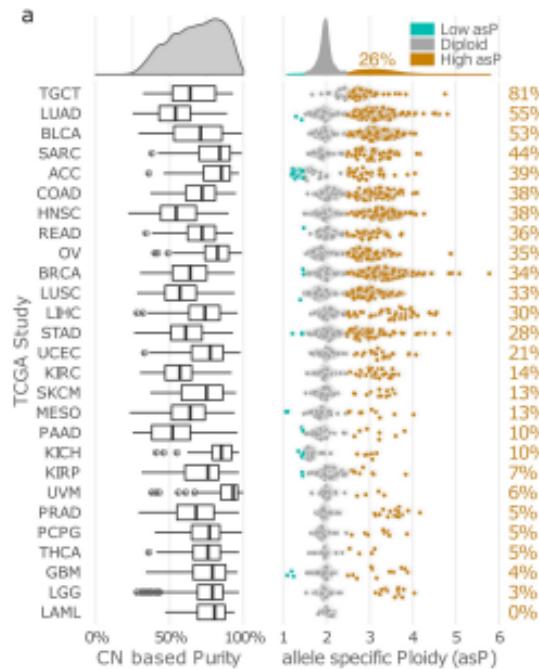
From the corrected data we learn that:

- A lot of tumors have a backbone ploidy of two;
- There are some hemizygous deletion not perfectly centered in one but closer to one in the 3<sup>rd</sup> graph if compared to the 1<sup>st</sup>;
- Some signal is compatible with homozygous deletion;
- We have a reasonable amount of signal for three copies which could come from a threeplloid status of some tumors.

#### 6.3.3 TCGA example

How commonly does suboptimal tumor purity affect proper copy number data analysis? How common it is that purity is not equal to 100% and ploidy is not equal to 2 in any primary disease?

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA



**Figure 6.7**

In figure 6.7 we can see a list of tumor types, where every draw is a tumor type (lung carcinoma, bladder cancer, colon cancer, ovarian ecc.). On the x axis we have tumor purity (1-admixture) going from 0 to 100% and for each type we can see the distribution of the tumor purity analysis of all the samples from the TCGA dataset. Every tumor type has a different number of sample profile. Looking at the GBM (glioblastoma multiforme), the middle vertical line is the median signal of the distribution, there are outliers shown and the black horizontal line represents the interquartile range.

Altogether, 8,183 primary cancer samples matched to 27 tumor types profiled with WES from TCGA. 4,950 cases with overall high tumor cellularity were identified. The majority had 69% tumor purity. There are some outliers e.g. ovarian cancer has high purity.

#### 6.3.3.1 Ploidy in TCGA example

Looking only at ploidy: what is the fraction within each tumor type with a ploidy significantly above two?

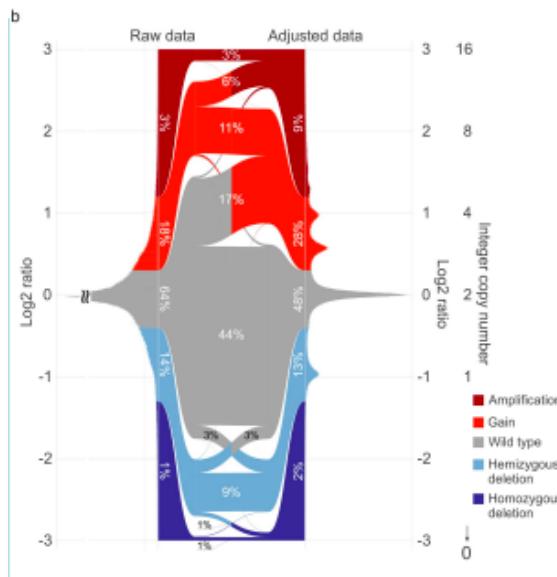
In the plot 6.7 they are sorted by decreasing percentage of tumors with a ploidy higher than two; for example, for the first and second tumor type, more than 50 % of the primary tumors have a ploidy status above two. Meaning, either they underwent whole genome duplication (4 or more copies) or triploidy (3) etc.

Then we have some tumors with very low ploidy (blue dots) where at least one copy of the entire genome is completely lost - low allele specific ploidy assessment.

Figure 6.8 shows what happens to data when we correct for ploidy and purity.

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

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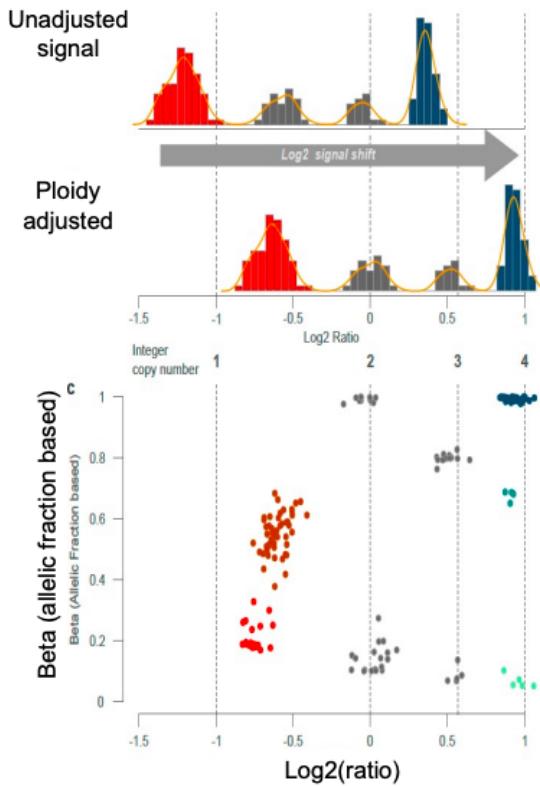
**Figure 6.8:** Y axis is the log2 ratio. On the left side we have the raw data, On the right side the adjusted data.

We can see where correction for ploidy and purity takes the signal. Focusing just on the first half we can see that we have the same noise we've seen for the melanoma uncorrected data.

The correction of the data results in the reclassification of 30% of the totality of the segments (if we don't correct we have a wrong copy number classification in 30% of the cases).

What's interesting is that the correction led to the doubling of the homozygous deletions that we were able to observe (these are very important because it means that the protein product won't be there at all).

### 6.3.4 Allele-specific analysis



**Figure 6.9:** Top panel: loss of an allele on A so we'll have 2-1-2 copies. Bottom panel: same situation on allele A but allele B is doubled so we'll have 3-2-3 copies. So, in this situation, the gene x will have two copies but both of them coming from the same allele (B).

By computing the log2 ratio in this situation we'll have the  $\log_2(2/2)$  which will lead to the collocation on the 0 axis but on the lower part (due to the clonality).

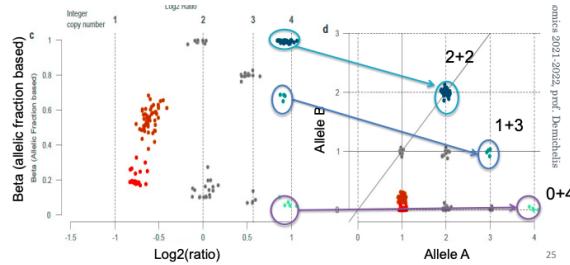
In figure 6.9 the data is adjusted. Then samples are represented in the beta-log2 ratio space where we can see that the data underneath the peaks are belonging to specific clusters.

This suggests that by only looking at the log2 ratio we are unable to distinguish the presence of clusters with different clonalities.

The most interesting information is the lower cluster (on the x=0 axis). Even when the  $T/N = 1$  (tumor/normal ratio) there is a status of one copy, and one copy -or something- that equally gives a log2 ratio equal to 0, which still represents copy neutral loss of heterozygosity (CN-LOH), so two copies on one allele and zero copies on the other.

The log2-beta statuses allows us to distinguish the copy-neutral LOH. There are equations that allows us to go from here to a space where our coordinates are the number of copies of allele A and number of copies of allele B, as depicted in figure 6.10.

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA



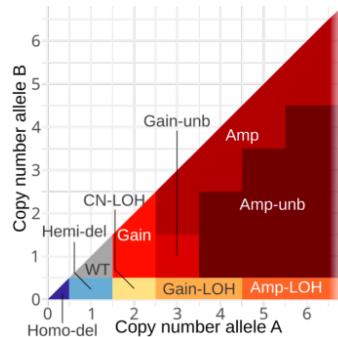
**Figure 6.10**

For four copies we can have different combinations:

- 2 copies of A + 2 copies of B,
- 3 copies of A + 1 copy of B
- 4 copies of A + 0 copies of B

The equations will not be discussed, what's important is that once we have corrected the data then we can shift our analysis up to the level of number of copies of each allele for each gene.

*Why is this important?* Let's imagine that for gene X we have one copy lost on allele A and a point mutation on the allele B which leads to unfunctional product so full loss of the protein. If we instead are in the second case and the point mutation happened after the duplication then we'll still have an allele functioning, whereas if it happened before the duplication, we'd have again full loss of functional protein. If we are able to distinguish the alleles we are able to also distinguish in which situation we are (which means we can distinguish between what's functional and what's not).



**Figure 6.11:** Extra graph with the same space allele a/ allele B where we can divide the space in terms of total number of copies and also what happens on both.

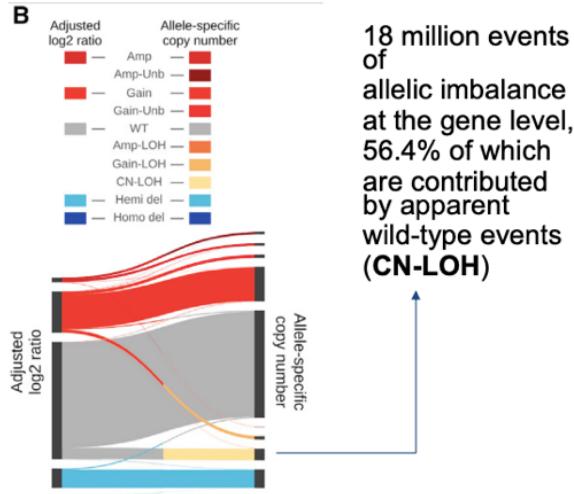
So, this whole computation allows us to reclassify copy number status in the space by shifting and stretching and also to assign a copy number A and B to every segment of the genome, which means to every gene.

If we do that we can see that many of the segments that have a total number of copies equal to two are in fact 2+0 and not 1+1. This means that there is a significant fraction of the genome which is apparently wild-type but which actually underwent loss from one an allele and a gain on the other. This event is called copy-neutral loss of heterozygosity (**CN-LOH**). Copy-neutral because

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

the number of copies doesn't change but there's been loss of heterozygosity.

From the TCGA data, they observed a relevant fraction of high copy number levels (4-5 copies) which all came from the same allele (one allele was lost and the other underwent multiple cycles of duplication).



**Figure 6.12:** Ciani et al, Cell Syst 2022

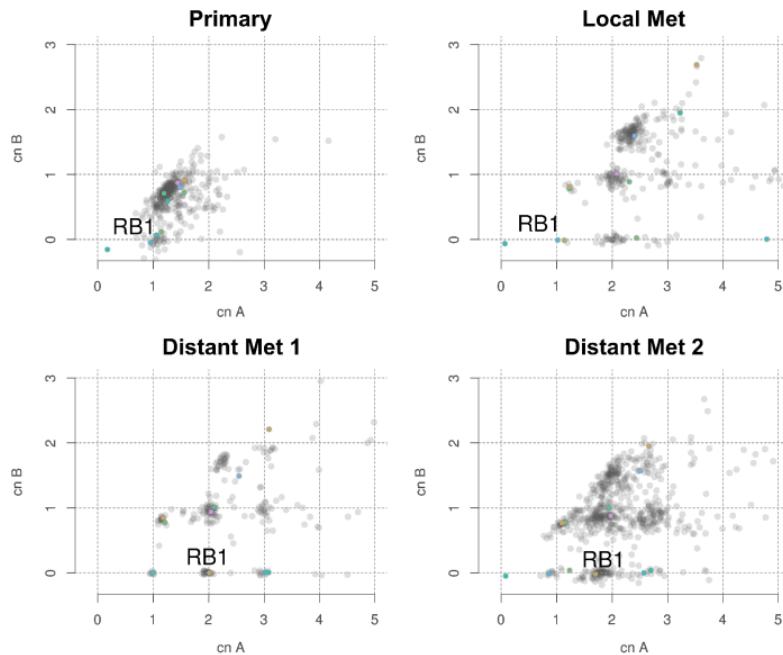
So, looking at the copy number only we'd say there's a gain (which is true) but we wouldn't have all the complete information (we also have to perform the allele analysis).

This information is relevant in precision medicine because there are ways to target genes exploiting loss of heterozygosity and up until now it was only used for deletions but now that's known, even if we have an apparent CN-LOH or we have a copy number gain LOH we can still consider to use the same approach.

#### 6.3.5 A Case study $CN_A$ , $CN_B$ real data example

We have one patient and we're looking at a primary sample, for which we plot the whole sequencing data in the copy number allele space and what we see (from the first plot in figure 6.13) is that:

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA



**Figure 6.13:** Case study -  $CN_A$ ,  $CN_B$  real data example with multi-sample data from the same patient. J Clin Invest. 2020 Apr 1;130(4):1653 -1668. doi: 10.1172/JCI131041

- There's a cloud of dots (every dot is a gene) which has a total number of copies around two;
- There's a cluster that underwent hemizygous deletion so we only have one copy of all the genes in there;
- There's one gene with a homozygous deletion (0,0).

Then we have three other metastatic sites for which they had biopsies so that they could run whole genome sequencing and perform the analysis of the data in the same space. We have a local metastasis and two distant mets.

What we see is:

- In distant met 1 there's no homozygous deletion<sup>1</sup> No DNA can be regained, it's impossible that the gene is reacquired, so probably the seeding of the distant mets happened before the loss of the gene
- In both the distant mets the gene RB1 gained an extra copy on allele A;
- In all the mets there are extra gains of copies of all the genes (maybe there's been a whole genome duplication of some sort);
- In distant met 1 the data are as clean as to allow us to state that the data point in yellow/grey over the 1 is subclonal (if we have genes with 1+1 copy is equivalent to say it's a subclonal hemizygous loss, it means that all the cells have at least one copy and then some cells also have a second copy);

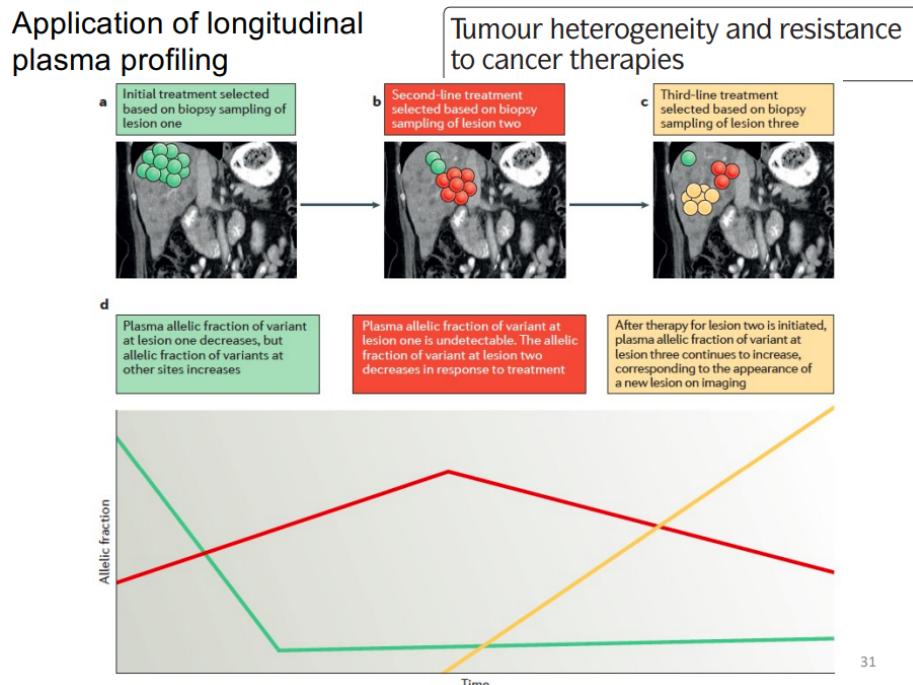
<sup>1</sup>How's possible that there's a homozygous deletion in the primary tumor which is then absent in the distant mets?

### 6.3. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

- In terms of evolution, very likely extra copies of the whole genome also in the local met after the loss of the second copy of the gene;
- CN-LOH of many genes, including RB1;
- Level of subclonality overall not high;

#### 6.3.5.1 Application of longitudinal plasma profiling

Another way to track evolution is to have **time points**.



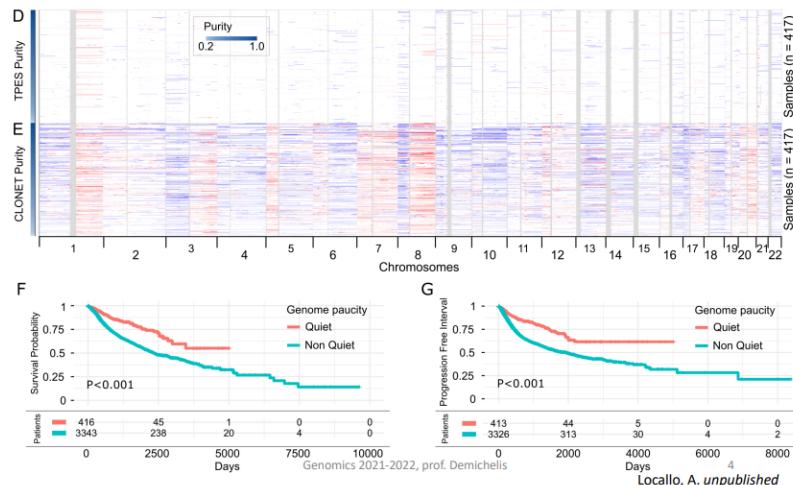
**Figure 6.14: Application of longitudinal plasma profiling.** Longitudinal monitoring of alterations in circulating tumour DNA has the potential to enable molecular relapses to be detected before the emergence of disease relapse on imaging. In a hypothetical example: **a)** A biopsy sample from lesion one (green) leads to the use of a targeted agent directed at the alterations in lesion one. **b)** A failure to also sample lesion two (red) might then lead to outgrowth of clones harbouring alternative molecular alterations, prompting the use of a combination of targeted agents or use of a single targeted therapy capable of overcoming both molecular alterations. **c)** The emergence of lesion three (yellow) might then be missed by biopsy sampling until this lesion becomes detectable on imaging. **d)** Longitudinal analysis of liquid biopsy samples would enable the detection and determination of the allelic fractions of the variants at all three lesions before their detection on imaging. This figure illustrates the ability of the molecular analysis of plasma to convey the full spectrum of resistance alterations and shows the dynamic nature of resistance.

If we deal with biopsies over time we can track the evolution using the allelic fraction of a lesion. Reasoning in terms of point mutations, let's say we have a point mutation at time point 0 in certain allelic fractions, which correspond to different subsets, we track the fractions over time. Remember: allelic fraction at any time point needs to be corrected for tumor content, otherwise we would not be able to compare multiple time points from the same patient.

# Chapter 7

## Tumor evolution studies via NGS data: SNVs-based methods

There is a large number of tumors where copy-number aberrations are minimal. Consequently, it is difficult to use copy number based approaches for these kind of tumors. It is estimated that about 3% of primary tumors present flat genomes, like the one in figure 7.1, meaning that they display very few copy number changes. These types of tumors are correlated to a better prognosis both in overall survival and progression-free interval, but relapses are still present so the assessment of these tumors is important.



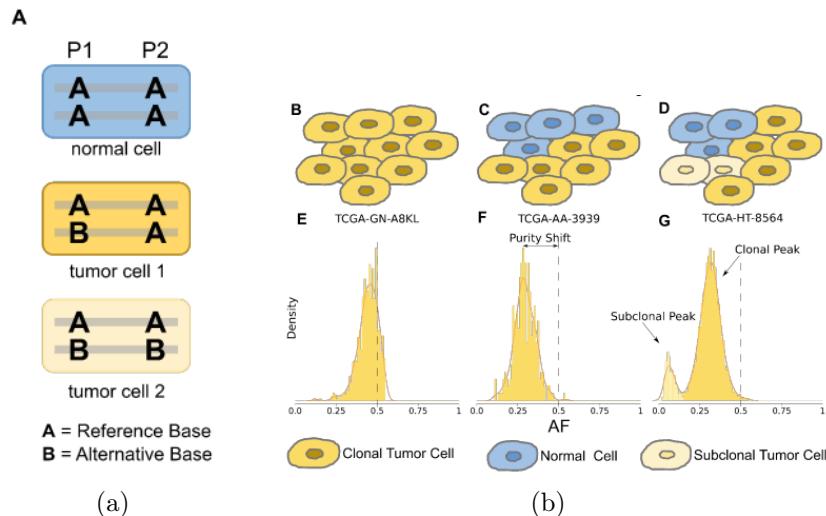
**Figure 7.1:** Data on 'quiet genomes', that make about 1-3% of all primary tumors.

Multiple Tumor Purity Assessment assays have been proposed, based on: RNA, DNA methylation and SNVs. In this chapter we will be focusing on the methods exploiting SNVs.

## 7.1. RATIONALE OF SOMATIC POINT MUTATION BASED ASSAYS

### 7.1 Rationale of somatic point mutation based assays

Consider figure 7.2. Peaks shift for a clonal tumor cell population and some mixed populations. Considering two genomic locations, healthy cells have genotype AA-AA, clonal tumor cells AB-AA and subclonal tumor cells AB-AB, where B is the alternative allele associated with a somatic point mutation



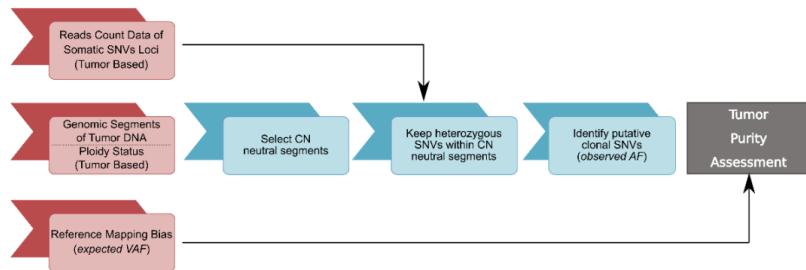
**Figure 7.2:** a) genotype of normal and tumor cells, P1 and P2 are single nucleotide positions.  
b) B,C,D) Three possible tumor tissue compositions: B) 100% pure clonal tumor population, C) clonal tumor population (60%) admixed with normal cells (40%), D) two subclonal tumor populations (50% + 20%) admixed with normal cells (30%). E,F,G) Histograms of the VAF values of SNVs in tumor cell populations B), C) and D), respectively.

The distribution of allelic fractions of the clonal population only is symmetric, with the main peak around 0.5. A mixed population of clonal tumor and normal healthy cells shows a shifted peak. The distance from 0.5 to the peak is proportional to the fraction of normal cells, because normal cells contribution moves the peak towards the side from the center (purity shift displayed in 7.2). A subclonal point mutation is identified with a second peak towards 0, because its allelic fraction is probably far distant from 0.5.

### 7.2 TPES (Tumor Purity Estimation)

It is important to consider the **Reference Mapping Bias**: a polymorphic locus carrying a non-reference base is less likely to be mapped during the alignment process. With a perfect SNV (clonal, monoallelic, in highly pure tumor), allelic fraction will not be 0.5 because the aligner considers the variation as an error and sometimes discards the read containing it: some signal is lost.

### 7.3. HOW MANY SNVs ARE NEEDED TO ASSESS TUMOR PURITY?



**Figure 7.3:** Workflow of the TPES algorithm

The tool TPES is extensively described in chapter 16. However, the main steps are:

- **Selection of CN-neutral segments:** point mutations that are flat in terms of copy-number are perfect for flat genomes and easier to deal with. This is the first filter implemented by this tool: a threshold is set on the log<sub>2</sub> of the tumor over the normal.
- When considering the **allelic fractions** of all the somatic mutations of whole genome, a major peak is expected around 0.5 (expected VAF). Other peaks can be originated from things that escaped the previous filter or from monoallelic mutations with copy-neutral LOH (loss of heterozygosity): in this case the allelic fraction results doubled. So another threshold on allelic fraction is needed (maxAF=0.55).
- Identification of **putative clonal SNVs**: the peak closer to 0.5 is the most useful to determine tumor purity. The others are related to subclonal events.

With enough point mutations and after peaks identification, purity is assessed with the following equation:

$$1 - \text{purity} = \text{admixture} = 1 - \frac{\text{observedVAF}}{\text{expectedVAF}}$$

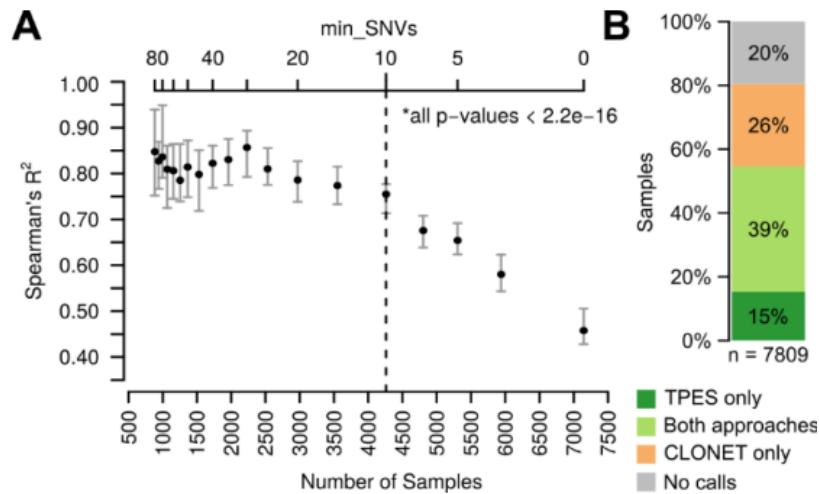
### 7.3 How many SNVs are needed to assess tumor purity?

The number of SNVs changes for each tumor type, so not all tumor types guarantee enough SNVs. The minimum number of SNVs needed to obtain reliable results can be assessed with a **comparative analysis**. The Spearman's correlations between the results of two different purity calling algorithms using decreasing number of SNVs are computed. The subsampling approach (which SNVs to consider) is to subsample the SNVs as many time as possible to have higher confidence on the results. At each iteration, as many samples as possible are used, but the number decreases when the number of SNVs increases.

The computations determined 10 as the minimum number of SNVs needed to infer tumor purity, as reported in figure 10.3. With this number, tumor was detected in 80% of samples by combining TPES and CLONET (CN-based). The 20% could be tumor-free or not detected samples. Since both SNVs and CN based methods failed, this 20% could be possibly detected with methylation.

## 7.4. COMPARISON BETWEEN PURITY CALLERS

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**Figure 7.4:** A) Correlation between two purity estimating algorithms (TPES and CLONET) with decreasing number of SNVs considered. B) Percentages of samples where tumor purity was assessed by the two tools (TPES considering with 10 SNVs)

## 7.4 Comparison between purity callers

TPES was compared to other tools that do the same thing but with a range of different methodologies: good correlation between the results was found, in particular with the CN-based algorithms. This shows that genomics is more reproducible in general to assess purity, while methods relying for example on image analysis give different results.

The best solution to assess tumor purity is to couple a CN-based and a SNV-based approach: some samples are only detected by one of the two so a combination gives the best results globally.

## 7.5 Pros and Cons of SNVs-based tumor purity assessment

Pros:

- Best-suited for CN neutral tumor genomes
- Applicable to a range of NGS techniques
- Fast and low demanding in terms of computational resources
- TPES is available as R package on CRAN

Limitations:

- Needs a reasonable number of putative clonal somatic heterozygous SNVs per sample
- Sensible to subclonal cell populations which could influence clonal peak detection

# Chapter 8

## Liquid biopsies in oncology

It is more feasible to track the tumor progression stage for a patient from liquid biopsies (panoramic overview at a time point), rather than from tissue biopsy (highly accurate snapshot in a specific site at a timepoint). How accurate can a liquid biopsy is depends on many factors: if there is homogeneity the quality is really similar to tissue biopsy. We might have different representations according to sites. The major advantage of liquid biopsy is being minimally invasive. Furthermore, it is easier for early diagnostics (screening), quantify the presence of minimal residual of disease.

### 8.1 General considerations

The main difference between tissue and liquid biopsies are listed in table 8.1.

## 8.1. GENERAL CONSIDERATIONS

---

Tissue Biopsy	Liquid Biopsy
Accurate and detailed view of one tissue only	Landscape overview, with resolution depending on tumor burden, releasing rates, metastases and tumor heterogeneity. It is possible to get an aggregated signal of different tumor cell populations
Single tumor	Possibility of getting signal from multiple tumor masses
Signal relative to a specific point in time	At a certain point in time, but multiple serial samples can be collected
Invasive and painful for the patient, not feasible for all the tissues or in presence of metastatic sites	Minimally invasive (so it is possible to collect samples multiple times) and can be coupled with a routine blood draw
	It is possible to design specific assays to detect minimal quantities of tumor cells, for example the ones left behind after surgery. This is useful to detect minimal residual disease (MRD) and avoid tumor recurrence
	The collection of serial samples allows for example to track clonal evolution of the tumor over time, to catch treatment resistances early on and to monitor the patient's response to the treatment
	It can be used for early detection of cancer, many studies are trying to reach this objective

**Table 8.1:** Main differences between tissue and liquid biopsy I.

Other considerations are reported in table 8.2.

## 8.2. ISSUES IN THE INTERPRETATION OF CFDNA DATA

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Tissue biopsy	Liquid Biopsy
	Material availability
From needle biopsies, biopsies, surgical resections (if some material is left after the clinical protocol and the patient agrees to a research protocol)	From circulating tumor cells, extracellular vesicles, cell-free DNA (the most interesting). In healthy donors there is 4ng/ml cell-free DNA (below 10 anyway), in tumor patients 100s ng/ml (but the range is really wide). The numbers are higher if the tumor is metastatic and the treatment is also very influential on the quantity of cfDNA. Tumor patients under treatment have cfDNA quantities comparable to healthy people. Anyway, cfDNA quantity is influenced by a number of factors in addition to cancers so it is not a good diagnostic feature by itself
	Tumor content
Tumor content can be assessed with a microscope: the proportion of tumor cells compared to healthy cells is measured based on morphology with a simple staining of the tissue slide. So tumor content is assessed by counting cells and considering the magnification of the image. If subtyping is needed, a staining for markers is performed. Computational methods are also available	The fraction of circulating tumor DNA (ctDNA) is inferred with methods based on genomics (or possibly also methylation)
	Tumor ploidy/aneuploidy
Tumor ploidy: assesed with cytogenetics, FISH, or from NGS data	Average ploidy: inferred computationally based on genomics but it is quite tricky

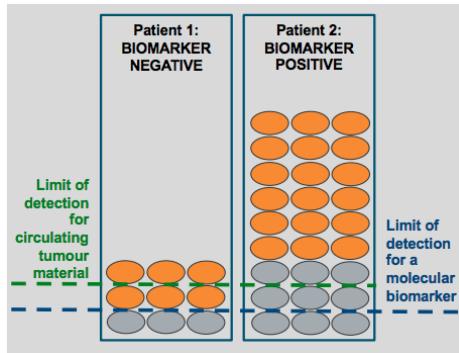
**Table 8.2:** Main differences between tissue and liquid biopsy I.

## 8.2 Issues in the interpretation of cfDNA data

### 8.2.1 Normalization on tumor content

When interpreting data from liquid biopsies, it is fundamental to contextualize a mutation after observing it. In order to associate a particular mutation to a particular diagnosis, the signal has to be normalized based on tumor content. Without normalization, tumor content is the most influential variable on the patient's prognosis and this can be misleading. For example, one mutation could look like it is linked to a specific type of tumor when it is actually present in other types too, but it is not detected due to the low tumor content of some samples (example in figure 8.7). For this reason not all the literature available about liquid biopsies is reliable: lack of normalization leads to completely wrong conclusions. This applies to all kinds of assays: from microarrays to the sequencing of extracellular vesicles.

## 8.2. ISSUES IN THE INTERPRETATION OF CFDNA DATA

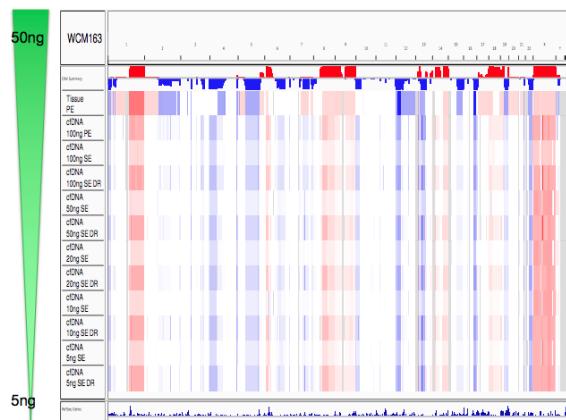


**Figure 8.1:** The two samples have the same percentage of tumor cells, however the first one results negative for the marker because of the low tumor content

### 8.2.2 Quantity of input material

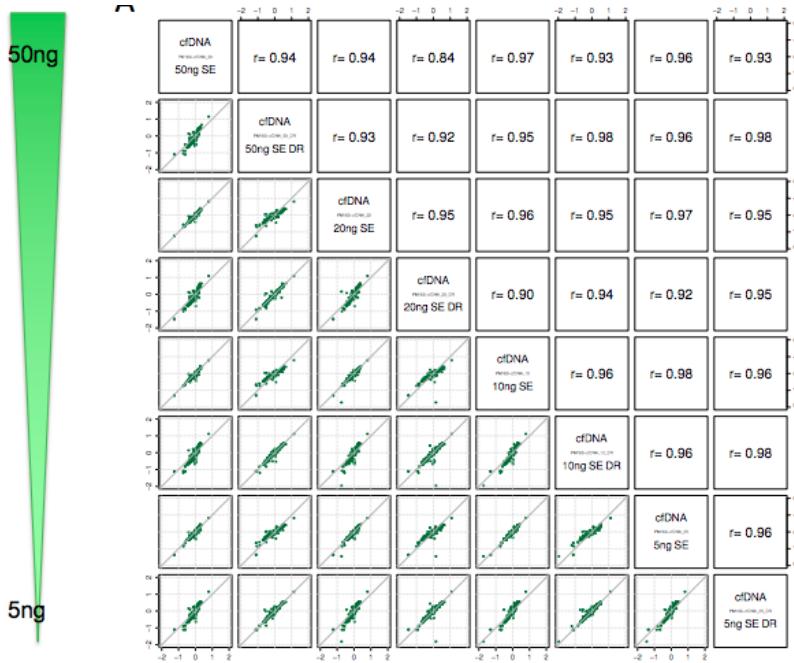
Another source of errors in the interpretation of cfDNA data is the amount of input material: if the patient's tumor content is high, the results could be obtained with a limited amount of extracted nucleic acid, but if the tumor content is low, too little material can lower the chances of detecting tumor cells (figure 8.2). The problem is that in most cases the tumor content is unknown before the analysis and this must be considered when designing an experiment. Usually the standard procedure is to begin with 2ml of plasma, the DNA content should be from 50 ng to 5ng. If no tumor is detected, one should repeat the assay with more material (or sequence another vial and combine the results) to be sure that the tumor is not present and not just undetected. In some cases some information about the state of the patient is available: for example if a patient is in remission more material is required.

Keep in mind that if the sample is pure, 10 ng of DNA should correspond to around 1500 diploid tumor genomes.



**Figure 8.2:** The same analyses are repeated with different quantities of starting material: for a patient with high tumor content the results do not change even with as little as 5ng of cfDNA

### 8.3. SNV DETECTION IN LIQUID BIOPSIES



**Figure 8.3:** Copy number signal correlates very well between different initial amounts of cfDNA for a sample with high tumor content

## 8.3 SNV detection in liquid biopsies

Technical problems we have to face if we want to detect SNVs through liquid biopsies are:

- PCR artifacts;
- Sequencing errors: one mutation should be validated by multiple reads to be confirmed;
- Problems related to the depth of coverage: the required coverage should be estimated considering the expected tumor content of the sample and deeper sequencing may be required;

But we also biological problems:

- Low tumor content: ctDNA/cfDNA ratio
- Clonal hematopoiesis (when a hematopoietic stem cell starts making cells with the same genetic mutation): to distinguish the signal coming from clonal hematopoiesis, compare it with what has been sequenced before from solid tumors. It is rare to observe something in liquid biopsy that has never been noticed in solid ones.
- Copy number variations and ploidy: with a whole genome duplication and a SNV only present on one allele, the signal corresponding to the mutation is only 25% and has to be correctly interpreted.
- **Intra-patient tumor heterogeneity:** very low allelic fractions for SNVs that are not clonal can be difficult to observe

## 8.4. REQUIREMENTS DEPEND ON THE APPLICATION

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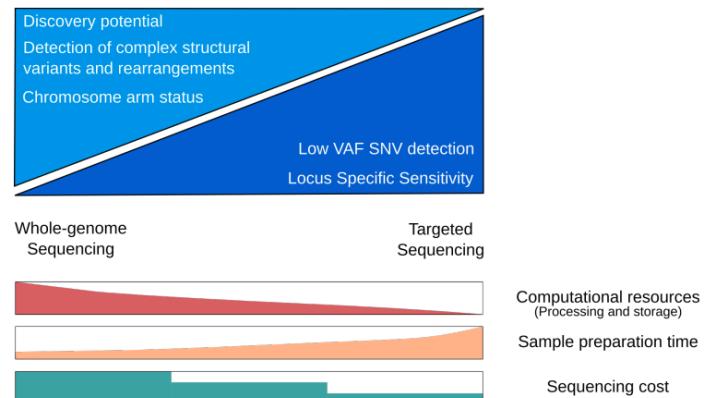
Multiple **tools** are available to detect SNVs. Each tool will probably give different results (or partially concordant ones). Each tool can be tuned to favour some types of calls, so the tuning parameters should be carefully selected.

## 8.4 Requirements depend on the application

Application	Requirements
Early tumor detection, MRD detection, Recurrence detection	Tumor quantity is low so a low signal is expected: higher quantity of starting material is required but there needs to be a balance between the number of false positives (with too much material) and false negatives (with too little) that can be produced
Tumor dynamics, Treatment response, Mechanisms of resistance	The assay should be designed in order to be able to distinguish between different clones (sub clonality analysis)
Single biomarker assessment	The only important thing is to detect whether one point mutation is present or not, so in this case tumor content is not important. A targeted assay is used and specific locations associated with the SNV are sequenced as deep as possible to detect the mutation

## 8.5 Whole genome vs targeted sequencing

Whole genome sequencing has higher computational cost, while targeted assays have higher sample preparation time. The sequencing cost is higher for whole genomes but it does not decrease evenly



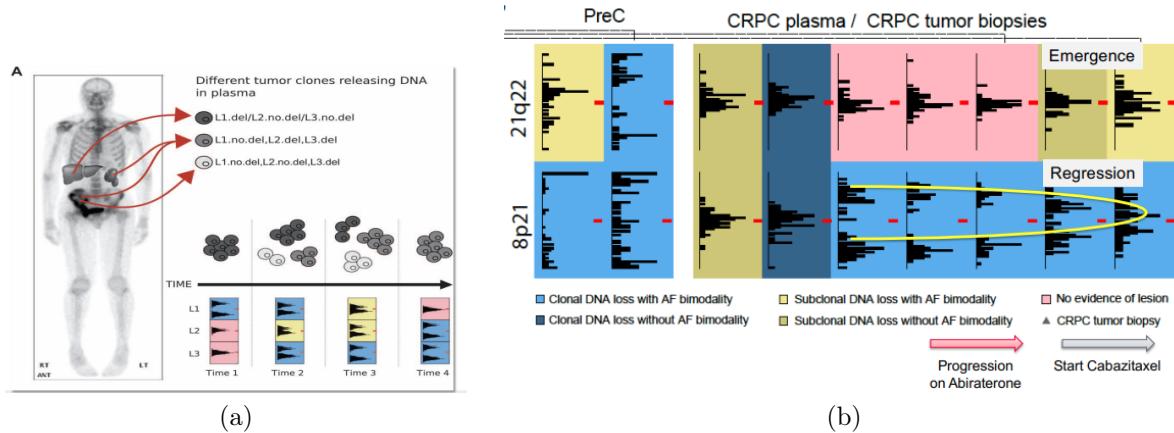
**Figure 8.4:** Whole genome vs targeted sequencing

## 8.6 Case studies

### 8.6.1 Case I

Assay to study specific signal distribution for the disease of interest (prostate cancer). By analyzing this as a dynamic process, we might see the overall distribution of clonal DNA. One of the lesion

## 8.6. CASE STUDIES



**Figure 8.5**

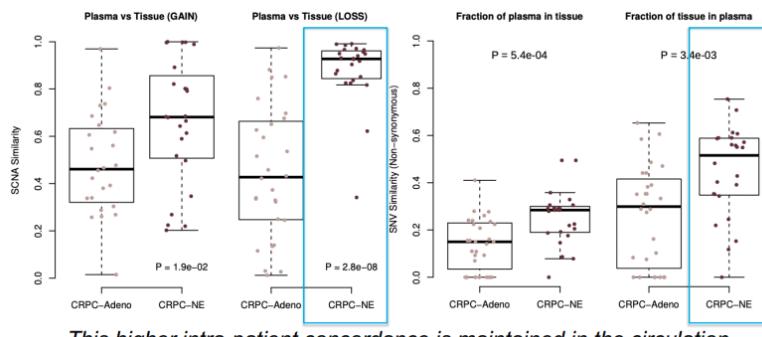
tracked was 21q22: the distribution on the top and the bottom are different at each time point with different dynamics. When the patient regressed 8p21, 21q22 emerged. Figure 8.5.

### 8.6.2 Case II

Non-AR driven castration resistant prostate cancer, really rare as a de novo, but rising incidence especially after potent AR-pathway. Hard to treat and to diagnose. Both tissue and liquid biopsy analysis to find:

- Potential biomarkers for liquid biopsy
- Distinguish the transition to severe stage

First tissue heterogeneity study, see how similar metastases were. Resulting in homogeneity higher with the most aggressive phenotype (NE). The same was observed in liquid biopsy, confirming the potential NE biomarker usage. A liquid biopsy is identical to lymph node metastasis, very clear signal. In another patients, certain genes switch from one cluster to another from tissue to plasma, suggesting that the metastatic representation was partially heterogenous. We have a landscape overview.



**Figure 8.6:** Beltran, Romanel et al, JCI 2020

## 8.7. TAKE-HOME MESSAGE

Man initially diagnosed with adenocarcinoma, switched to NE (clinical assessment). Plasma sample before NE diagnosis and multiple tissue biopsies. It was noted that the liver metastasis (NE) had lesion represented in the plasma sample, suggesting that a clone that was transforming was already present at past time. In certain cases, a liquid biopsy can be informative of something that would only emerge later clinically.

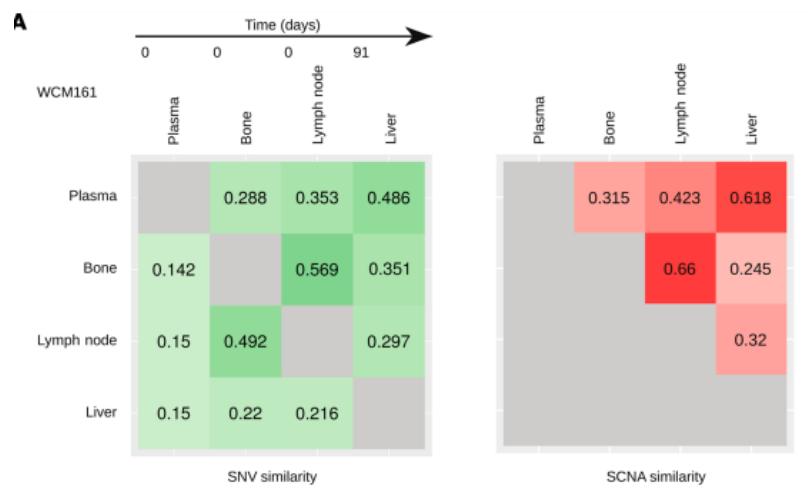
By comparing the genomic content of each metastasis with liquid biopsy, we can come up with a measure of the modification contributing more to the disease.

## 8.7 Take-home message

**Possible exam question:** what type of assay should be run and which are the requirements for a specific situation.

### 8.7.1 Challenges in the tracking of tumor evolution

- ctDNA content (fraction of tumor content in circulation)
- polyploidy (allelic imbalance events)
- ability to detect signal is gene region dependent and individual dependent
- different DNA release rates from different metastases



**Figure 8.7:** xMetastatic biopsy time points during clinical progression from CRPC-Adeno to CRPC-NE. Plasma sample at time of CRPCAdeno with lymph node and bone metastases displayed a genomic ctDNA profile most similar to the CRPC-NE liver metastasis observed on imaging and biopsied 3 months later at time of progression on abiraterone.

# Chapter 9

## Extracellular vesicles

*Introduction to Extracellular vesicles as disease biomarker carriers in the circulation of patients*

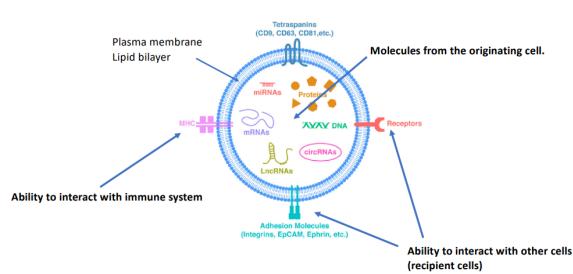
We will be trying to answer three main questions: what are EVs? Why are we interested in them? How can we use them to study cancer? (or any other disease)

### 9.1 Definition

"Extracellular vesicles are membrane-enclosed nanoscale particles released from essentially all prokaryotic and eukaryotic cells that carry proteins, lipids, RNA and DNA."

The outside layer is made of the lipidic membrane of the cells from which the vesicles were originated. The content can vary: RNAs of different length, DNA, proteins. They also come from the cell that originated the vesicles.

On the membrane there are proteins that are able to interact with the immune system, receptors, adhesion molecules and tetraspanins (they span the membrane four times, and are markers, or recognition proteins of the vesicle). Figure 9.1 represents all the main components of a vesicle.

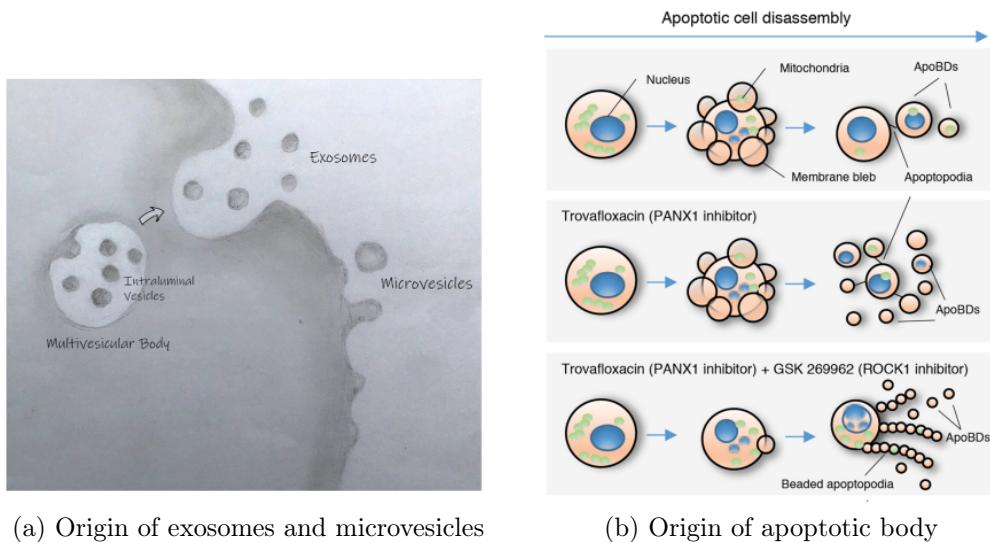


**Figure 9.1:** Sketch representing the main components of an EV.

### 9.2 Characterization

Extracellular vesicles are very different from each other. Especially in older studies, each research group used to study EVs from a certain site and gave the EVs a specific name (for examples, EVs from the prostate were called *prostatosomes*, from a tumor sample were called *oncosomes* and so

## 9.2. CHARACTERIZATION



**Figure 9.2:** caption

on).

A consortium was created to make some order in the nomenclature and which are the parameters to characterize and study them.

### 9.2.1 Size

The smallest vesicles are the microvesicles (100 -1000 nm), then we find the exosomes (50 - 150nm), finally the apoptotic EVs and apoptotic bodies (100-5000 nm). Note that the categories overlap, there's no clear cut. Exosomes could represent a new class of EV with no lipid bilayer and are 30-50nm long.

### 9.2.2 Origin

Exosomes come from the endocytic pathway, microvesicles and apoptotic bodies from the plasma membrane.

Microvesicles come directly from the membrane of the cell, while the exosomes come from the multivescicular body, which contains the intraluminal vesicles, as shown in panel a) of figure 9.2.

Apoptotic bodies instead are the result of cell death. In the experiment performed in panel b) of figure 9.2, the researchers induced apoptosis using different methods and the vesicles had different sized.

The main steps in the origin of exosomes, the EVs of main interest, are:

- Endocytosis: the cell either capture everything in the ECM, or the substrate is selected by receptors.
- Formation of early and late endosomes: lysosomes are organelles which go through a process of maturation, in the late endosomes enzymes complete the packaging of the substrate.

### **9.3. THE IMPORTANCE OF EVS**

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- Formation of multi Vesicular bodies: Multivesicular bodies contain intraluminal vesicles, the precursors of the exosomes.

#### **9.2.3 Content**

Exosomes and microvesicles mainly carry proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs). But which type of nucleic acids? The vesicles are really smalls and cannot contain big fragments of DNA. Further studies however proved the presence of longer, protein coding transcripts. A lot of RNA transcripts have important regulatory functions (like miRNA, circRNA etc).

Apoptotic bodies instead are the entire representation of the cell's cytoplasm. They have an equal representation of the cell content, whereas for exosomes there's an active process of selection of the cargo.

How does the cell select the cargo?

- Abundant RNA
- Fragmentation
- Particular sequence motifs
- Unique secondary structures
- RNA modification (eg.mRNA uridylation)

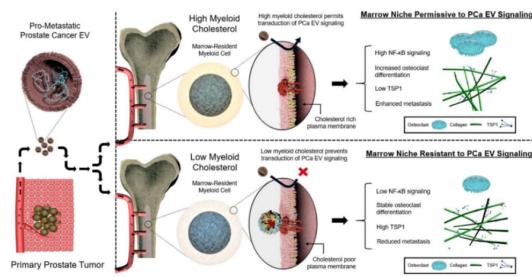
## **9.3 The importance of EVs**

Main reason: each EV carries information about its cell of origin and its putative function! What can be the function of the EVs, how does it act in presence of other recipient cells, what's the effect on other cells?

### **9.3.1 Role in cancer**

In cancer, EVs have important functions. In prostate cancer it has been shown that exosomes are able to modulate the immune system, by changing the preferential maturation of the cells of the immune systems. They aid in the proliferation of endothelial cells, in stromal fibroblast differentiation, creating population that are pro/anti tumorigenic. They act on the remodeling of ECM, which is extremely important for metastasis, as EVs provide for a way for cells to harbor to a different substrate and create metastatic sites (especially for bone cancer). An example of how exosomes can boost metastasis is reported in figure 9.3.

#### 9.4. HOW CAN WE USE THEM TO STUDY CANCER? (OR ANY OTHER DISEASE)



**Figure 9.3:** Prostate cancer extracellular vesicles mediate intercellular communication with bone marrow cells and promote metastasis in a cholesterol-dependent manner. *Stephen E. Henrich, JExtracell Vesicles. 2020.*

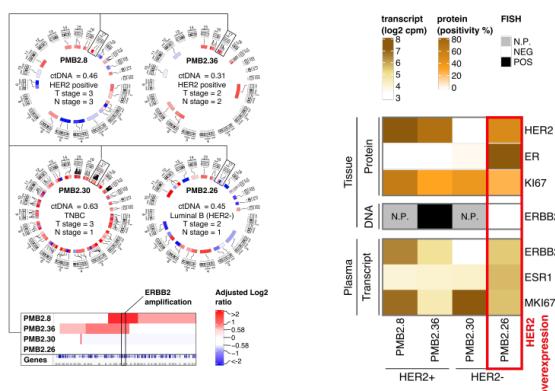
Exosomes from prostate cancer travel in the body, arrive to the bone and boost metastasis.

#### 9.4 How can we use them to study cancer? (or any other disease)

Use liquid biopsy as a novel tool for cancer detection and monitoring. By just drawing some blood, in a serial matter, we can retrieve a lot of EVs, coming from all of the tissues of the body, including the cancer cells.

In the same tumor there could be different populations, each harboring different mutations and having different proliferation rates. They respond in different ways to therapies.

In the bloodstream we have cells coming from the tumor, free cDNA, EVs and ribolipoproteins. We can perform the analysis of the liquid biopsy with a Multi-analyte approach: using different molecules/analysis (es. DNA, RNA) to detect tumor related signal.



**Figure 9.4:** 4 Breast cancer patients. On the left: Whole Exome Sequencing of cfDNA from plasma.

Breast cancer usually stratifies in different subtypes and one of the main molecular features is the presence of hormone receptors, in particular the one expressed by HER2. Two patients that were HER2 positive and two patients HER2 negative. The two positive patients were confirmed by high protein level, but one of the negative patients has a signal for the protein, but no amplification

## 9.4. HOW CAN WE USE THEM TO STUDY CANCER? (OR ANY OTHER DISEASE)

(performed with FISH). At the end, the clinicians classified this ambiguous patient as... ambiguous. By integrating the RNA form the EVs, for the HER2 positive we have high expression of ERBB2 (concordant with the previous results), but also for a HER2- patient. This is concordant with the immunoistochemistry but not by FISH. Why? The regulation of HER2 is not only regulated by the amplification, but also by over-expression.

If we only perform the geneic analysis we would have missed important info, because with EVs we were able to identify over-expression even in absence of amplification.

### **9.4.1 Tracking tumor signal in serial samples**

Serial approach: tracking the signal of different biomarkers in time. Es: tracking response to a drug treatment. In this

Sequence/perform digital PCR on the samples from the blood and follow the biomarkers in time, to discover how well the patient is responding to the treatment.

### **9.4.2 Different EVs isolation methods**

# Chapter 10

## Epigenetic profiling of cell-free DNA

Gian Marco Franceschini - *gian.franceschini@unitn.it*

### 10.1 Introduction

All the cells of the human organism present the same genetic information but they give rise to different types of tissues and cells. This happens mostly thanks to epigenetics. The main epigenetic modifications are:

- **DNA methylation:** in humans they are mainly found on CpG islands (genomic regions with high CG content)
- **Histone post translational modifications (PTMs)**
- The **chromatine architecture**
- ...and many others

All levels of epigenetic controls are often dysregulated in cancer: these variations usually go in favor of cancer cells survival. For this reason, epigenetic reprogramming has recently been added to the hallmarks of cancer.

The epigenetic landscape is very different from the genetic one. DNA mutations are directional: they cannot be reverted so they accumulate with subsequent cells generations. The epigenome is plastic, so it can be reverted (possibly through therapy but this can happen physiologically). Moreover, the human epigenome is tissue/cell specific while the genome is unique.

### 10.2 DNA methylation

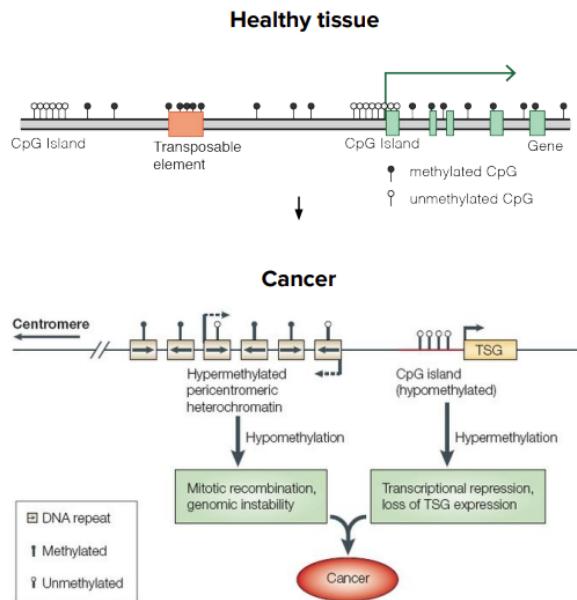
DNA methylation is the addition of methyl-groups to cytosines in CpG islands. It is regulated by enzymes that are responsible for regulating the cell-specific transcriptional state. These enzymes can be:

- Cis-factors: local control
- Trans-factors: genome-wise control

### 10.3. HOW IS DNA METHYLATION MEASURED?

CpG islands are spread through the genome and when they are in a promoter they regulate gene expression through transcriptional silencing of the corresponding gene if they are methylated. The mechanisms are multiple and still not completely clear: DNA methylation could for example impair the binding of transcription factors or recruit repressing proteins. This methylation landscape is highly regulated and tissue-specific.

In cancer tissue, hypomethylated and hypermethylated regions are often observed, leading to an abnormal regulation of gene expression. In addition to that, hypermethylation of pericentrometric heterochromatin in cancer can lead to mitotic recombination and thus genomic instability 10.1.



**Figure 10.1:** Methylation patterns altered in cancer

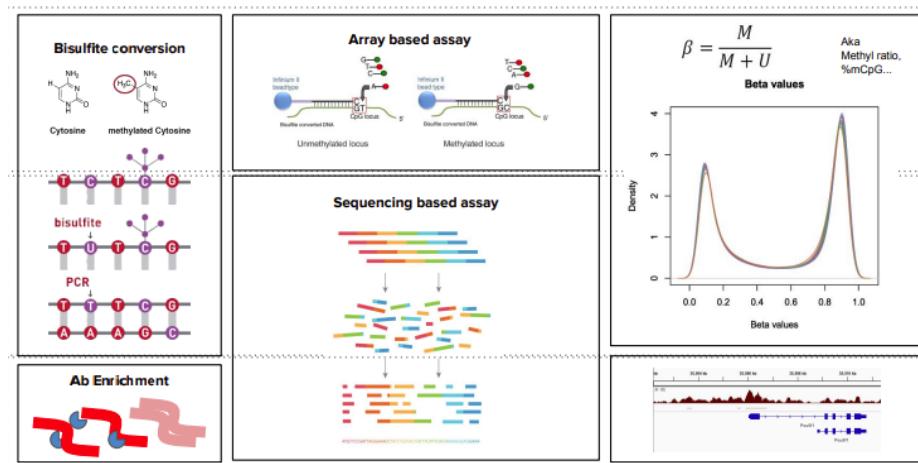
This landscape of regulation is very complex: DNA methylation can regulate gene expression but it is not the only regulating factor, some histone modifications also contribute for example.

DNA methylations are not inherited across generations, so there is no accumulation of methylation variants, as it happens with regular DNA mutations. Each individual is born with a brand new methylation landscape that is then disrupted during life (not only due to cancer or disease). Interestingly, it could be possible to exploit variations in the DNA methylome to measure age by computing how many cell divisions led to that specific methylation state.

## 10.3 How is DNA methylation measured?

The first step is the **bisulfite conversion**: thanks to bisulfate ions, unmethylated Cs are converted into Us. With some particular alignment algorithms that are aware of these modifications one can detect the errors and thus methylations. Both array-based and shotgun-sequencing-based assays are used to this aim. The result of such an assay is a series of **beta values**: the fraction of reads corresponding to one genome site that is methylated.

#### 10.4. TISSUE-SPECIFIC VS DISEASE-SPECIFIC DNA MARKERS



**Figure 10.2:** Main methods for DNA methylation measurement

Immunoprecipitation-based methods are also available, but the most frequently used methods are based on whole genome profiling.

It is useful to analyze the sequencing result at the **single-read level**: different methylation configurations can lead to the same global methylation level but have different biological interpretations. For example one methylation level of 0.5 can be the result of one completely methylated allele with the other one unmethylated or two half-methylated alleles. This kind of information is important in order to determine, for example, if the sample contains different types of cells or if it there is some disrupting pathological situation.

#### 10.4 Tissue-specific vs disease-specific DNA markers

- **Aspecific** → most of the genome
- **Tissue-specific** → methylations that regulate gene expression to activate the tissue-specific functions of cells
- **Disease-specific** → CpG hypermethylation, genome-wide hypomethylation and other modifications usually correlated with cancer
- **Tissue+cancer-specific** → methylation patterns specific of cancer in a certain tissue. These markers allow to discriminate between different tumor types.

#### 10.5 DNA methylation based liquid biopsy

When a cancer cell dies, its DNA is released in circulation and it is potentially possible to get it with a liquid biopsy. The goal is to analyze methylations of cfDNA to retrieve information about the state of the patient, and possibly detect early-stage tumors.

For this purpose, when compared to genomic DNA, the analysis of the methylation landscape has some positive and some negative aspects. For genomic DNA, the percentage of actually informative

## 10.5. DNA METHYLATION BASED LIQUID BIOPSY

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signal on the whole information that is obtained can be small and difficult to observe, on the other hand, for DNA methylations it is difficult to discriminate between what is aberrant and what is not because the modifications are tissue-specific and it is difficult to obtain clear background references to make a comparison.

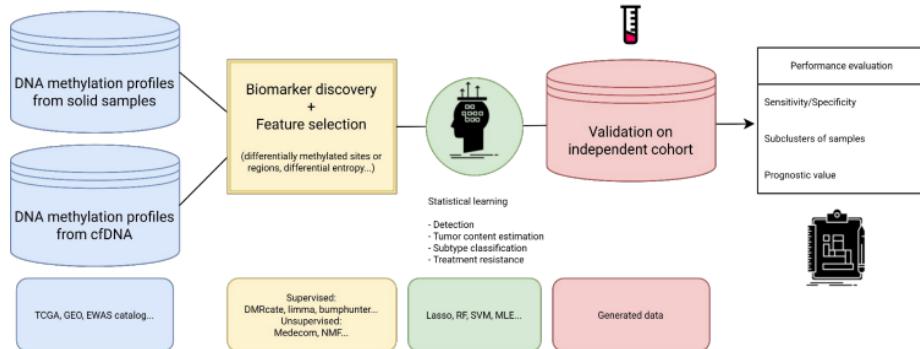
	Genomic DNA	DNA methylation
<b>Molecular signal</b>	Signal is limited to genomic alterations, and thus might be low for SNVs or quiet tumors	Extended and multi-facet signal, amenable to genome wide detection
<b>Background/reference</b>	A single well known background: the normal human genome, as profiled by the control germline sample	Multiple cell populations with distinct profiles, each contributing to the DNA methylation signal
<b>Variability</b>	Low rate of biological variability, discrete signal and overall acceptable technical errors	Discrete degree of biological variability, continuous signal with variable confidence ("coverage, platform, experimental approach...")
<b>Information content</b>	Limited to genomic information (SNV, SCNA...) but possible fragmentomics applications	Could potentially capture transcriptional state of cancer cells, offering a snapshot of processes such as lineage switching
<b>State of the art</b>	Highly characterized and interpretable, extended literature and high quality samples are available to aid interpretation	Fewer datasets available, but promising results in the past few years. Currently a mostly uncharted territory

**Figure 10.3:** Comparison of genomics vs methylation for cancer detection

### 10.5.1 Workflow

First, the data is sequenced from solid and liquid samples: the methylation profiles from solid samples are needed as reference. The reference profiles for liquid biopsies analysis are derived from white blood cells and from the cancer type of interest. White blood cells are the background reference for cfDNA, since the most frequent genomic material in circulation originates from this type of cells. If a methylation pattern different from the one of blood cells is found in cfDNA it means that cells of some other tissue are dying and their material is going into circulation and it is not a positive signal. With these patterns as reference, the goal is to discover biomarkers and perform feature selection. Subsequently, a model is fitted and optimized to perform predictions on new data. The last step is performance evaluation.

## 10.5. DNA METHYLATION BASED LIQUID BIOPSY



**Figure 10.4:** Common analysis workflow

### 10.5.2 CCGA study

The Circulating Cell-free Genome Atlas (CCGA) is a study conducted by Grail designed to characterize the landscape of genomic cancer signals in the blood of people with and without cancer. The study enrolled approximately 15,000 participants. Their goal is early and simple detection of cancer from analysis of methylations on cell-free DNA.

They performed whole genome methylation profiles after choosing between three different independent methods (the other two were targeted sequencing and whole genome sequencing for CNVs but they decided to further develop the methylation path). In the second phase they developed an assay for a targeted methylation study: the best features to discriminate between the two classes (cancer vs non-cancer) were selected in order to sequence the areas with these modifications without whole genome analysis. A model for this classification was developed, trained and validated. The last step is a large-scale clinical validation with a 5 years follow-up that is still in progress.

The results are great but not for all cancer types: sensitivity is better for cancers of highly-vascularized tissues and metastatic tumors, while some types of cancer produce a lot of false negative results. Moreover, detection is obviously better when cancer progresses but the goal is early detection.

### 10.5.3 Deconvolution approaches

Deconvolution of cell-free DNA is another task to be performed on DNA methylation other than classification. The goal is to explain the observed signal with a combination of pure signals: discover the main contribution that led to a specific methylation landscape, one example is tumor profiling.

From liquid biopsies, it is possible to detect which are the main contributors to the cfDNA. These results can be compared with the ones obtained from cancer patients to determine which are the contributors to the difference in cfDNA that is observed and to infer data for tumor diagnosis or treatment resistance detection.

In order to perform deconvolution, **high-quality reference atlases** are needed: one was built with the contribution of Grail. They sorted healthy donors cells with FACS and profiled them. Cell type specific methylation profiles were built, so it is possible to use this atlas to select biomarkers, like a reference genome. They generated specific methylation patterns for 39 human cell types from 207 methylomes.

## 10.6 Targeted panel approaches for tumor content estimation

*Demichelis'group study*

Their interest is detection of treatment resistance in prostate cancer. The goal is to know when the tumor becomes resistant, in order to be able to change or calibrate the therapy. A sequencing panel was developed to detect the amount of cancer-derived DNA in circulation, and interestingly only 50 regions are sufficient to get a satisfying estimation. A model is built to know how much ctDNA is expected after treatment and it is possible to get a score that estimates the level of resistance.

## Part II

# Papers

# Chapter 11

## Role of non-coding sequence variants in cancer

### 11.1 Abstract

Patients with cancer carry somatic sequence variants in their tumour in addition to germline variants in their inherited genome. Numerous studies have noted the importance of non-coding variants in cancer. The overwhelming majority of variants occur in non-coding portion of the genome.

#### 11.1.1 Introduction

One of the most important benefit of whole genome sequencing is the identification of variants in non-coding regions of the genome, with most of them lying in such regions. One of the biggest challenges is to identify driver mutation and distinguish them from passenger mutations.

### 11.2 Genomic sequence variants

The general properties of sequence variants are applicable to non-coding variants. They range from single nucleotide variants to small insertion and deletion of less than 50bp or indels, to larger structural variants. The latter can be copy number variants CNV or copy-number neutral. An average human genome contains 4 million germline sequence variants, whereas a tumour genome contains thousands of variants relative to the same individual germline DNA. Somatic variants are rarer in healthy tissues. Somatic mutation frequency varies across different cancer types. Some germline variants may be responsible for tumorigenesis (high penetrance) or modulate the effect of somatic variants (low penetrance). The germline variants associated with increased cancer susceptibility do not have a fitness effect at reproductive age, which can be the reason for the continued prevalence of such variants in the population. Germline variants show LD that increase the difficulty in disentangling the causal disease variants. A much higher fraction of somatic variants consist of structural variants and unlike germline variants they happen on a specific tissue. However germline variants can have a functional effect in specific tissue if they occur in regions of closed chromatin or if they disrupt a binding site of a tissue-specific transcription factor. Kataegis is characteristic only of somatic variants. Moreover somatic variants are not inherited and so they are not subject to meiosis and do not show LD.

## 11.3 Non-coding element annotation

Non coding elements can have diverse roles in the regulation of protein-coding genes. They consist of cis-regulatory regions and ncRNAs. They are identified by functional genomics approaches or sequence conservation and display cell and tissue specificity.

### 11.3.1 Cis regulatory regions

Cis-regulatory regions include promoters and distal elements which regulate gene expression following binding by TFs. TFs bind to specific DNA sequences within their larger regions of occupancy which can be identified using chromatin immunoprecipitation followed by sequencing assays. They bind DNA in regions of open chromatin identified using DNase I hypersensitivity assays and DNase I footprinting. DNA methylation and other histone modification can modulate TF accessibility. Several histone marks are associated with specific putative functions. Most of sequence-specific TF and chromatin marks lead to highly localized ChIP-seq signals, other marks are associated with large genomic domains. Epigenetic changes can alter TF accessibility in different cellular states and can change the activity or regulatory elements.

### 11.3.2 Distal regulatory elements

Distal regulatory elements may regulate gene expression by interacting with promoters in the 3D structure of the genome. Linking them to their target region is crucial to understand the effects of sequence variants in them. Multiple approaches have been used to link chromosome conformation capture: regulatory sequences can control transcription by looping to and physically contacting target coding genes that are located tens or hundreds of kilobases away. It probes one-versus-one contacts in the 3D space of the genome. Other variations control one-versus-all, many-versus-many and all-versus-all contacts. Other approaches include correlation of histone marks at enhancer regions and target gene expression across multiple cell lines. Links between repression quantitative trait loci and associated genes. The resulting linkages can be studied as a comprehensive network.

### 11.3.3 RNA-seq

RNA-seq reveals non-coding transcripts, which can be confirmed to not have protein-coding ability by the absence of open reading frames or proteomic analysis. Certain histone modification can also indicate ncRNA activity. ncRNA can be divided into categories and they act through different mechanisms to modulate gene expressions. In particular miRNA and lncRNA are important in cancer biology. miRNA inhibit target gene expression by binding to the 3'-UTR and causing mRNA degradation or repression of translations. The mechanisms of action of lncRNA remain unclear, but a number of lncRNA have been shown to act as molecular scaffolds that bind proteins, DNA or other RNA molecules and are able to modulate gene expression.

### 11.3.4 Transcribed pseudogenes

Transcribed pseudogenes are a type of ncRNA that bear a clear resemblance to functioning protein coding genes. They are copies of coding genes that have lost their ability to code for proteins owing to disabling mutations. They can be divided into duplicated and processed based on their formation from duplication or retrotransposition of the parent gene. Processed pseudogenes lack the promoter sequence and intronic structure and contain a 3'-poly(A) tail. These pseudogenes can be transcribed

## 11.4. ROLES FOR SOMATIC VARIANTS IN CANCER

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and regulate the expression of their parent genes, generating endo-siRNA and participating in the RNA interference pathway or by acting as molecular sponges.

### 11.3.5 Evolutionary conservation

Evolutionary conservation of genomic sequence across multiple species is used to annotate non-coding regions. Comparative analysis allowed the discovery of these ultra-conserved elements, the majority of which do not overlap protein-coding exons. Analysis of these sequence is important because they have been shown to have a role in cancer biology. Non-coding elements exhibit conservation among humans. Negative selection within the population can be estimated using enrichment of rare alleles and reduced density of single nucleotide polymorphisms. These can be important to identify elements that show human-specific conservation in functional non-coding categories. The ultra sensitive elements and have strong depletion of common polymorphisms and enrichment of known disease-causing mutations. Negative selection can be used to identify candidate cancer driving mutations.

## 11.4 Roles for somatic variants in cancer

Because cancer genomes contain a higher fraction of structural variants than germline genomes, variant detection becomes challenging. The depth of coverage needs to be more than typically used to account for the decreased purity and increased ploidy.

### 11.4.1 Gain of TF-binding sites

TERT encodes the catalytic subunit of the enzyme telomerase. This allows to lengthen telomeres, allowing cells to escape apoptosis and become cancerous. TERT expression is typically repressed, but it can be overexpressed in cancer. Recurrent mutation in the promoter of TERT in many different cancer types have been found. These mutations create binding motifs for the ETS family like TCF leading to their binding to TERT and upregulation of its expression. Tumours in tissues with low rates of self-renewal tend to exhibit higher frequencies of TERT promoter mutations. Gain of TF-binding site has been observed for enhancers, an important distal cis-regulatory elements that play a major part in gene transcription.

### 11.4.2 Fusion events due to genomic rearrangements

Genomic rearrangements can lead to fusion of active regulatory elements with oncogenes. Moreover somatic structural variants juxtapose coding sequences proximal to active enhancers during enhancer hijacking. So these genomic rearrangements bring oncogenes adjacent to active promoters or enhancers.

### 11.4.3 ncRNAs and their binding sites

Disregulation of ncRNAs is a cancer signature and it can be due to the presence of somatic variants in them. MALAT1 or metastasis-associated lung adenocarcinoma transcript 1 is an example of this. Mutation of MALAT1 might be under positive selection in the tumour. In another example copy number amplification of a lncRNA is thought to contribute to neuroblastoma progression. Mutation in the binding sites of ncRNA are linked to cancer.

#### **11.4.4 Role of pseudogenes in modulating the expression of a parental gene**

because of their resemblance to their parental protein-coding genes, transcribed pseudogenes are thought to have a natural way of affecting and regulating their parental counterparts. Pseudogene deletion or amplification can affect competition of miRNA binding.

### **11.5 Roles for germline variants in cancer**

Most of the non-coding germline variants associated with cancer susceptibility can be analyzed through WGS data from healthy and ill individual. Germline-non coding variants can affect gene expression in many different ways: point mutation can disrupt binding motifs. GWAS SNPs and the one in LD with them might help to identify the causal variants and shed light on their mechanism of actions.

#### **11.5.1 Promoter mutations**

Germline mutation can create binding motifs with functional effects in the tissues where the TF is expressed. Moreover they can upregulate the binding.

#### **11.5.2 SNPs in enhancers**

Multiple SNPs in a gene desert can increase the risk of cancer: this can be due to the fact that they happens in regions that act as enhancers . Tissue specificity might be the reason why they are associated with specific cancers. Hormone-regulated cancers have mutation in TF-binding sites that vary with age owing to a differential TF activity during a person lifetime.

#### **11.5.3 Variants in introns**

Variants in introns can affect splice sites and cause loss of regulatory repressor elements. Germline CNV spanning intronic inhibitor regulatory elements can lead to the overexpression of target transcripts, modulating cell proliferation or migration.

#### **11.5.4 SNPs in ncRNA and their binding sites**

Most cancer-associated polymorphisms are related to increased risk, some of them can be beneficial.

#### **11.5.5 Others**

Other methods to identify variants with functional consequences such as ECTS and allele-specific expression analysis have been used to interpret cancer-associated loci identified through GWAS. These reveal germline determinants of gene expression in tumours and help to establish a link between non-coding risk loci and their target coding genes.

### **11.6 Interplay between germline and somatic variants**

Cancer results from a complex interplay of inherited germline and acquired somatic variants. Loss of heterozygosity events affecting non-coding element have been observed. Somatic variants disrupt

## **11.7. COMPUTATIONAL METHODS FOR IDENTIFYING VARIANTS**

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the only functioning copy of the non-coding element. One example is the loss of miRNA or lncRNA. However some mutation can weaken the effect of a somatic variant.

## **11.7 Computational methods for identifying variants**

Computational prediction of drivers is a challenging task. Driver identification uses detection of signals of positive selection or prediction of mutations with high functional impact. Analysis of the recurrence of somatic variants from tumour samples in functional elements to identify regions under positive selection is similar to the burden test strategy. Such analysis can be done in a specific cancer type or across multiple cancers. In addition tools that try to do this need to account for genomic mutation rate covariants that lead to mutational heterogeneity across the genome. Computational identification of non-coding drivers is more challenging than the coding one because of their complex and varied modes of action. Non-coding mutation are also more abundant and the key mutations have to be distinguished from a larger set of passenger events. Some methods analyse the recurrence of somatic variants from tumour samples in functional elements. Tools exits to annotate and prioritize potentially functional non-coding variants with high impact. These tools can interpret SNV and indels or some structural variants. Some of them try to interpret the effect of cis-regulatory mutations at a nucleotide level of resolution by computing whether they create new TF-binding motifs. Biological networks can provide information about the connectivities of the target genes of non-coding variants. High inter and intra-species conservation tend to be an indicator of function.

## **11.8 Experimental approaches for functional validation**

Experimental approaches to understand the effects of cis-regulatory mutations in promoters and enhancers on cellular functions have main strategies. First they require introducing the sequence variants, determining the resulting molecular level effects on transcription using high and low throughput functional assays and demonstrating direct biological significance. One way to introduce sequence variants involves the use of CRISPR-Cas9 systems. Then the effect evaluated through sequencing screening or luciferase reporter assays. Analysis of the mutation in a high-throughput manner can be achieved using a modification of cis-regulatory element analysis by sequencing. Synthetic promoter libraries drive the expression of a common reporter gene and a downstream unique barcode sequence that identifies the upstream promoter. RNA-seq reveals the effects of promoter variants on the expression levels of their paired barcode sequence. The activity of enhancers is independent of their location, so they can be incorporated into high-throughput reporter assays using different reporter construct arrangements. In CRE-seq approaches the enhancer is placed upstream of the reporter gene and the barcode. The cloned libraries can be transfected into eukaryotic cells in pooled format and RNA-seq is used to assess the resulting expression level of the reporter driven by each variant element. Visible reporter assays using synthetic transcription reporter construct that contain the regulatory sequence of the reporter gene enable direct validation. Other approaches are needed to validate variants in ncRNA, UTR and introns. Monogene assays can be used to test the effects of intronic variants: the variant sequence is cloned into transcription-competent minigene vectors and transfected into mammalian cells. This is followed by examination of the splicing patterns of the transcripts. Functional screening help identify the best candidates but still needs tumour type specific validation. Functional validation requires demonstrating oncogenic properties that are increased owing to the variant in question. Wild type and mutants are compared in vitro and in vivo. Overall functional validation of non-coding variants is important to understand their biological consequence.

## **11.8. EXPERIMENTAL APPROACHES FOR FUNCTIONAL VALIDATION**

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High-throughput prioritization of putative functional mutations is crucial before testing of the most promising candidates in in vivo systems.

# Chapter 12

## Advances in understanding cancer genomics through second-generation sequencing

### 12.1 Abstract

The application of second generation DNA sequencing technologies is allowing substantial advances in cancer genomics. These methods are increasing the efficiency and resolution of detection of each of the principal types of somatic cancer genome alteration.

#### 12.1.1 Introduction

A near term medical impact is the elucidation of mechanisms of cancer pathogenesis, leading to improvements in the diagnosis of cancer and the selection of cancer treatment. It has become feasible to sequence expressed genes, known exons and complete genomes of cancer samples. Most of the genomic alteration that cause cancer are somatic. Studying these alteration can improve therapies targeted against the production of these alterations. Comprehensive genome based diagnosis of cancer is increasingly crucial for therapeutic decisions. Some genomic alterations in cancer are prevalent at a low frequency in clinical samples, owing to substantial admixture with non-malignant cells. These methods makes it feasible to discover novel chromosomal rearrangements and microbial infections and to resolve copy number alterations at very high resolution. The data generated from second-generation sequencing provides a statistical and computational challenge. This will be partly solved by systematic analysis of large cancer genome data sets.

### 12.2 Cancer-specific consideration

Cancer samples and genomes have general distinct characteristics from other tissue samples that require particular consideration.

### **12.3. EXPERIMENTAL APPROACHES**

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#### **12.2.1 Characteristics of cancer samples for genomic analysis**

Cancer samples differ in their quantity, quality and purity from the peripheral blood samples. Diagnostic biopsies from patients with disseminated disease tend to contain few cells, therefore the quantity of nucleic acid available may be limiting. An alternative approach to deal with small sample is whole-genome amplification, but it does not preserve genome structure and can create artefactual alteration. Nucleic acids from cancer are of lower quality due to formalin fixation and paraffin embedding necessary for microscopi histology. They will have undergone cross-linking and be degraded. Special experimental and computational methods are required. Moreover cancer specimens can include substantial fraction of necrotic and apoptotic cells. Moreover a cancer specimen will have a mixture of cancer and normal genomes and the cancer themselves can be highly heterogeneous and composed of different clones.

#### **12.2.2 Structural variability of cancer genomes**

Cancer genomes vary in their sequence and structure compared to normal genomes and among themselves. Cancer genomes vary in their mutation frequency, in global copy number or ploidy and in genome structure. The presence of a somatic mutation is not enough to establish statistical significance: it must be evaluated in terms of the sample-specific background mutation rate. The analysis of mutations must be adjusted for the ploidy and purity of each sample and copy number at each region. To identify somatic alteration, comparison with matched normal DNA from the same individual is essential.

### **12.3 Experimental approaches**

The application of second-generation sequencing has allowed cancer genomics to move from focused approaches to comprehensive genome-wide approaches.

#### **12.3.1 Whole genome sequencing**

Complete sequencing of the genome of cancer tissue to high redundancy, using germline DNA sequence from the same individual as a comparison has the power to discover the full range of genomic alterations using a single approach. So it is the most comprehensive characterization of the cancer genome and the most costly. The major potential is the discovery of chromosomal rearrangements. It also may be able to detect other types of genomic alterations like somatic mutations of non-coding regions as well as non annotated regions. The two main parameters to consider when performing WGS are depth of coverage and physical coverage. Sequence depth is measured by the amount of over-sampling, typically at least a 30 fold average coverage is needed. Physical coverage is important for detecting rearrangements. This is helped by paired-end sequencing. The expected distance between paired reads is used to place the reads on the reference genome. The distance between the paired reads can be increased creating jumping libraries by circularization. This has two limitation: the coverage is lower and point mutation resolution is lower. Second it requires large high-quality DNA, which may not be possible with all clinical cancer samples.

#### **12.3.2 Exome sequencing**

Target sequencing approaches have an increased sequence coverage of regions of interests at lower costs and higher throughput. Any subset of the genome can be targeted. Capillary-based sequencing

## **12.4. DETECTING CLASSES OF GENOME ALTERATIONS**

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has been proven powerful to focus sequencing efforts on the coding genes of interest. Uneven capture efficiency across exons can mean that not all exons are sequenced and some off-tagged hybridization can occur. The higher coverage make WES suitable for mutation discovery in cancer samples of mixed purity.

### **12.3.3 Transcriptome sequencing**

RNA-seq is a powerful approach for understanding cancer. Transcriptome sequencing is sensitive and efficient in detecting intragenic fusions like in-frame fusion events that lead to oncogene activation. Transcriptome sequencing can be used to detect somatic mutations by finding a matched normal sample. Mutation detection is hampering due to a lack of statistical power. RNA-seq allows analysis of gene expression profiles and is powerful for identifying transcripts with low-level expression. It can also detect novel transcripts, alternative splice forms and non-human transcripts.

## **12.4 Detecting classes of genome alterations**

Second-generation sequencing can provide a comprehensive picture of the cancer genome detecting each of the major alterations in the cancer genome.

### **12.4.1 Somatic nucleotide substitutions and small insertion and deletion mutations**

Nucleotide substitutions are the most common somatic genomic alteration occurring at a frequency of one in a million. Insertion and deletions are tenfold less common. The rate of mutations varies greatly between cancer specimens. Detection of somatic mutations in cancer requires mutation calling on the tumour DNA and the matched normal DNA, coupled with comparison to a reference genome. False positive are inaccurate detection of an event in the tumour and detection of a germline event in the tumour but failure to detect it in the normal. Noise can be due to machine-sequencing errors, incorrect local alignment and discordant alignment of pairs. Moreover it can be caused by failures to detect the germline alleles that differ from the reference sequence in the normal sample. False negative si often due to insufficient coverage. Statistical significance of an alteration can be assessed by comparison to the sample-specific background mutation rates in the specific nucleotide context and correcting for multiple hypothesis testing. Computational tools predict the effect of an amino acid change on the protein structure and function, and some tools aim to distinguish driver from passing alterations. Experimental validation is the most powerful method.

### **12.4.2 Copy number**

Second generation sequencing methods offer substantial benefits for copy number analysis, including higher resolution and precise delineation of the breakpoints of copy number changes. The digital nature allow to estimate the tumour to normal copy number ration at a genomic locus counting the number of reads in both tumour and normal samples in the locus.

### **12.4.3 Chromosomal rearrangements**

Second-generation sequencing has been shown to allow systematic description of the rearrangements in a given cancer sample. Extension of these approaches to large numbers of samples should lead to the discovery of the major recurrent translocations in cancer. Intrachromosomal rearrangements,

## 12.5. COMPUTATIONAL ISSUES

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inversions, tandem duplications and deletions, insertions of non-endogenous sequences like viral ones, reciprocal and non-reciprocal interchromosomal rearrangements and complex rearrangements like combinations of these various events can be detected through second-generation sequencing.

### 12.4.4 Microbe-discovery methods

In addition to somatic alterations many cancers are caused by microbial infections. Neither array methods nor directed sequencing approaches can identify new examples of microbial genomes that have inserted themselves into the human genome. Computational subtraction of the sequence from a sample from the human reference genome can detect non-human sequences and identify novel microbial infections associated with human disease. Challenges include low concentration of the microbial agent ,hit and run mechanisms, quality issue that cause artefacts and incompleteness of human genome reference samples.

## 12.5 Computational issues

The three main challenges in developing computational solutions are the need to simultaneously analyse data from tumour and patient to identify rare somatic events, ability to analyse very different and highly rearranged genomes and to handle samples with unknown levels of non-tumour contaminations and heterogeneity within the tumour.

### 12.5.1 Alignment and assembly

Reads must be aligned to the specific chromosome, position and DNA strand from which they are most likely to have originated. These are performed against reference human genomes using methods developed for normal samples. The uniqueness of every cancer genome and the difficulty of correctly assigning rearranged sequences from homologous regions mean that de novo assembly of cancer genomes is likely to become the most powerful approach.

### 12.5.2 mutations detection

As somatic genome alteration are rare, any method that detects mutations in cancer must do so with low false positive rates. The first report of a method specific for somatic mutation calling or SNVMix. Systematic analysis of false-positive and false-negative rates of the methods based on real cancer data is yet to be performed. A naive somatic mutation caller can be built by applying a germline single-sample mutation caller to the tumour and normal data sets: somatic events are those detected only in the tumour. Somatic mutation calling is more complex because cancer samples vary in purity and ploidy. A key parameter for each mutation is its allelic fraction: the expected fraction of reads in the tumour that harbour the mutation among all reads that map to the same genomic location. The allelic fraction captures the local complexity of the tumour genome, the non-tumour contamination levels and any mutation-dependent experimental or alignment bias.

### 12.5.3 Validation of mutation and rearrangement calls

Accurate estimation of false positive and false-negative rates is a challenge. The second can be estimated by validation of the event using an orthogonal technology: a genotyping assay such as mass spectrometric analysis. This is not sufficiently sensitive to validate mutations with low allelic fractions. Current efforts are focused on applying deep targeted second generation sequencing to

## **12.5. COMPUTATIONAL ISSUES**

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validate the events. For validating rearrangements the current methods require PCR amplification of the region surrounding the event followed by sequencing of this region. They are not high-throughput. A developing concept is to capture the rearranged sites using a similar protocol to the exon capture approach and apply deep sequencing.

# Chapter 13

## Integrative genomics viewer

### 13.1 Introduction

Experienced human review is essential in analysis of the datasets generated during genomic studies. The integrative genomics viewer or IGV is a visualization tool that enables intuitive real-time exploration of diverse, large scale genomic data sets. It supports integration of aligned sequence reads, mutations, copy number, RNA interference screens, gene expression, methylation and genomic annotations. IGV makes use of efficient, multi-resolution file formats to enable real-time exploration of arbitrarily large data sets over all resolution scales. The user can zoom and pan across the genome at any level of detail, from whole genome to base pair. Sample annotations can be defined and data divided into tracks. Annotations are displayed as a heatmap. Its scalable architecture makes it well suited for genome-wide exploration of NGS datasets, both basic aligned read and its derived results. As the user zooms below the 50kb range individual aligned reads become visible and putative SNPs are highlighted as allele counts in the coverage plot. Zooming in further individual base mismatches become visible, highlighted by color and intensity according to base call and quality. Reads can be sorted by quality, strand, sample and other attributes. IGV use paired ends reads to color-code paired ends if their insert sizes are larger than expected, fall on different chromosomes or have unexpected pair orientations. Intra and inter chromosomal events are readily distinguished by color-coding.

# Chapter 14

## Tumour heterogeneity and resistance to cancer therapies

### 14.1 Abstract

As a result of cancer heterogeneity, the bulk tumour might include a diverse collection of cells harbouring distinct molecular signatures with differential levels of sensitivity to treatment. This might result in a non-uniform distribution of distinct subpopulations across and within disease sites or temporal variations. This provides the fuel for resistance.

#### 14.1.1 Introduction

The stochastic nature of cancer initiation reinforces the notion that the development and progression of cancer does not follow a fixed course. The ongoing evolution of cancer might generate a molecularly heterogeneous bulk tumour consisting of cancer cells harbouring distinct molecular signatures with differential levels of sensitivity. Intertumoural heterogeneity is the heterogeneity between patients harbouring tumours of the same histological type. Intratumoral heterogeneity is spatial or temporal heterogeneity: dynamic variations in the genetic diversity of an individual tumour over time. Oncogenic drivers can be exploited to treat cancer, but almost all of them develop resistance to targeted therapies. Intratumoural heterogeneity drives the evolution of cancers and fosters drug resistance. A comprehensive understanding of tumour dynamics is essential for the development of effective and durable therapeutic strategies.

### 14.2 Causes of intratumoral heterogeneity

#### 14.2.1 Genomic instability

Instability might result from exposure to exogenous mutagens and aberrations in endogenous processes. Characteristic genetic signatures associated with some of these mutagenic processes have been identified by large-scale genomic sequencing. Exposure to chemotherapy might increase the mutational spectrum of a tumour and create genomic instability. Genomic instability can also result from chromosome-level changes that lead to gains or losses of whole-genome segments rather than point mutations.

### 14.3. THE SPECTRUM OF TUMOUR HETEROGENEITY

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#### 14.2.2 The clonal evolution and selection hypothesis

Genomic instability fosters genetic diversity by providing the raw material needed for the generation of tumour heterogeneity. Dynamic chromosomal instability can lead to copy-number imbalances and non-uniform loss of chromosomal segments harbouring specific alterations that can contribute to mutational heterogeneity across different regions. Increased levels of genomic instability promote the emergence of more competitive subclones. Genomic instability cooperates with other factors to promote the development of tumour heterogeneity. The clonal evolution method and or the selection framework are used to explain how clonal diversity is generated and maintained. This model is based on the hypothesis that tumour initiation occurs in a stochastic manner, beginning with an induced change that confers a selective growth advantage and leads to neoplastic proliferation. The genomic instability creates additional genetic diversity subjected to evolutionary selection pressures, resulting in the sequential emergence of increasingly genetically abnormal and heterogeneous subpopulations. Linear evolution describes evolution owing to the successive acquisition of mutations that confer a growth and survival advantage. Sequential clones have advantageous mutations and out-compete ancestral clones. Alternatively branching evolution denotes the emergence and divergent propagation of multiple sub clonal tumour cell populations that share a common ancestor. Branched evolution has a greater opportunity to create a more heterogeneous tumour. Moreover different sub clones might cooperate for tumour propagation in cancer.

### 14.3 The spectrum of tumour heterogeneity

#### 14.3.1 Spatial heterogeneity

Cancer can ignore growth suppression signals, invade local tissues and metastasize to distant organs. The molecular make-up of cancer cells in different sites can be different, owing to the variable influences of micro-environment related factor. Heterogeneity might exist among the cell present within the parent tumour. The uneven distribution of diverse tumour subpopulations across different sites and within a tumours is termed spatial heterogeneity.

##### 14.3.1.1 Heterogeneity at a single disease site

Primary tumours contain multiple geographically separated and molecularly distinct cellular subpopulations. This can result in an uneven distribution of key molecular alterations across different regions. It might manifest as the ubiquitous presence of key molecular driver alterations, with an unequal distribution of additional molecular alterations. The pattern of spatial heterogeneity observed is reflective of the specific evolutionary context. Multiregion sampling is an informative investigational strategy that improves the ability to determine the extent of spatial heterogeneity within an individual tumour. Many of the unevenly distributed passenger mutations are not expressed. Markers of different impact can be present in geographically distinct regions within the same tumour. Genomic instability is a better biomarker than the alterations detected. A substantial level of genetic diversity exists between individual cancer cells. Multifocal tumours (multiple histologically similar cancers within a single organ) pose a unique challenge because genetic homogeneity cannot be assumed. Moreover the potential exists for divergence.

##### 14.3.1.2 Comparison of spatially distinct disease sites

The genetic makeup of cancer cells at a specific metastatic site might differ from that of the parent tumour. The degree of genetic discordance might reflect whether the metastases occurred as late

### 14.3. THE SPECTRUM OF TUMOUR HETEROGENEITY

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events or arose through dissemination early in the course of tumour development. Comparison of the genetic make-up of different metastases reveal substantial levels of heterogeneity. In the simplest scenario, seeding of multiple metastatic sites by identical clones, all metastatic sites would have the same genetic signature. This uni-directional flow might not be a universal scenario: tumour self-seeding and exchange of tumour material between different metastatic sites can occur. Moreover polyclonal seeding can happen. In some cases distant metastases can arise from independent seeding by genetically distinct subclones originating from the primary tumour. Moreover site specific factors could promote genetic divergence after initial colonization.

#### 14.3.2 Temporal heterogeneity

Temporal heterogeneity refers to the dynamic variation in the genetic diversity of a tumour over time. Chemotherapy can alter the molecular make-up of tumours over time by creating shifts in the mutational spectrum. Mutations in genes that are fundamental to replication and cell-cycle regulation can contribute to genomic instability. Targeted therapies can exert selective pressures on oncogene-driven cancer cells.

##### 14.3.2.1 Genomic complexity might increase with exposure to targeted therapies

The efficacy of targeted therapies reflects therapeutic vulnerabilities resulting from a dependence on specific growth signals and the original location of the driver alteration. Resistance can arise through mutations, activation of bypass signalling pathways and cell-lineage changes. De novo resistance alterations can be present at low variant allele frequencies in pretreatment tumour specimens. Resistant clones merge from the selective expansion of pre-existing populations during treatment with targeted agents. The genomic complexity increases with exposure to sequencing systemic therapies: the single genetic snapshot depicted in a diagnostic biopsy sample might become outdated during the clinical course. Serial characterization of tumours at multiple time points is necessary in order to accurately capture the various temporal shifts that take place during clonal evolution.

##### 14.3.2.2 Longitudinal sampling provides insight into temporal heterogeneity

Longitudinal profiling has the potential to decipher the role of clonal evolution. Repeat biopsy sampling enables the tailored use of sequential therapies. Clonal evolution that arises from the selective pressures created by targeted agents is dynamic. Clonal dynamics are not always easily manipulated by treatment interruption. Longitudinal sampling might be most clinically relevant when used as a tool to enable the selection of subsequent treatment strategies.

##### 14.3.2.3 Residual drug-tolerant cells can foster temporal heterogeneity

A reliance on biopsy samples might fail to detect cancers at the early or intermediate stages of resistance. The residual disease left to therapies could harbour a small population of quiescent drug-tolerant cells that have survived owing to adaptive activation. Acquired resistance is attributed to selective expansion of pre-existing subclonal population. Data from some studies suggest that the ongoing evolution of drug-tolerant cells leads to de novo generation of resistance alterations. These can emerge from single-cell clones derived from drug-tolerant cells. This emphasizes the necessity of developing sensitive technologies that enable the early detection of resistance. The emergence of resistance highlights the need to develop therapeutic strategies that target the minimal residual disease state.

## 14.4. NONINVASIVE MONITORING OF HETEROGENEITY

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### 14.4 Noninvasive monitoring of heterogeneity

Analysis involving single-site biopsy sampling might result in underestimation of the degree of spatial heterogeneity, and sampling intervals tolerable by the patient might not enable the true extent of temporal heterogeneity to be captured. Liquid biopsies that facilitate longitudinal analysis of tumour-derived genetic material are a promising strategy for addressing the shortcomings of tissue sampling. Genotyping of circulating tumour cells, circulating exosomes and circulating cell-free tumour DNA or ctDNA had promising results.

#### 14.4.1 Analysis of ctDNA

Analysis of ctDNA is a sensitive and highly informative method of identifying clinically relevant genomic alterations with a high degree of concordance with tissue biopsy. ctDNA might enable the identification of alterations not detected by tissue genotyping. Optimizing ctDNA platforms to increase sensitivity for very-low-frequency mutations might enable the early detection of resistance and relapse. This is because the detection of variants associated with treatment resistance in plasma can precede the emergence of evidence of radiographic progression by 10 months in some patients. Longitudinal plasma analysis is an effective tool for gauging the influence of treatment on the molecular and genetic makeup of a patient's cancer over time. Plasma clearance might be predictive of a clinical response. Serial plasma analysis can enable the kinetics of dominant alterations present before treatment to be monitored and to capture clonal shifts occurring during therapy. Several studies found that genotyping of plasma samples enables the kinetics of intratumoural heterogeneity to be captured in a timeframe that is potentially conducive to guiding clinical decision making. Plasma samples contain ctDNA from multiple metastatic sites so it can enable the detection of clinically relevant alterations that are not identified through analysis of tissue biopsy samples. Reliance on tissue sampling alone often underestimates the degree of overlap between distinct driver alterations. Heterogeneity of alterations associated with resistance in plasma samples correlates with shorter PGS durations. Analysis of pretreatment plasma samples can provide some insight into the probable disease outcomes of patients receiving treatment. Sampling of multiple lesions during autopsy can improve upon the ability of tissue sampling to capture the extent of molecular heterogeneity present in cancers. Plasma genotyping might provide a more-comprehensive readout of tumour heterogeneity.

## 14.5 Overcoming heterogeneity

Higher level of intratumoural heterogeneity predispose patients to inferior responses to anticancer therapies, including to targeted agents. The degree to which subpopulations coexist will affect clinical outcomes. Cancers become more heterogeneous and complex with successive exposure to systemic agents: responses to subsequent lines of therapy are often not as robust as responses to initial treatments. The current paradigm of sequential treatment is suboptimal: it fails to address the heterogeneity that might underlie incomplete responses to treatment. A bulk solid tumour is a heterogeneous entity that predominantly consists of drug-sensitive cells: mathematical modelling might enable the design of dosing schedules that account for this inherent heterogeneity. Withdrawal of targeted therapy can negate the selective advantage conferred upon drug-resistant cells and enable repopulation of the tumour with drug-sensitive cells. Intermittent dose scheduling can temporarily suppress clonal outgrowth, although the effect is to subdue heterogeneity rather than to eliminate it. Combination approaches that target heterogeneous tumour populations have proven successful in preclinical studies. Plasticity between different signalling pathways is a potential manifestation

#### 14.5. OVERCOMING HETEROGENEITY

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of temporal heterogeneity under therapeutic selective pressure. Drug combinations targeting multiple signalling pathways could provide another means of addressing intratumoural heterogeneity. The characteristics of the tumour before treatment could be used to design therapeutic approaches. Drug tolerant cells can develop a wide range of resistance mechanisms and targeting this population offers another opportunity to curtail intratumour heterogeneity. Combinations are likely to be more effective than monotherapy. Many tumours lack actionable genetic alterations. In these cases strategies that target more ubiquitous sources of heterogeneity are likely to be most applicable. Genomic instability is a pervasive and ideal target. Countering the development of genomic instability is a more daunting task than silencing a dominant signalling pathway. This is likely to be most effective in patients with cancers that are prone to mutagenic stress.

# Chapter 15

## Unravelling the clonal hierarchy of somatic genomic aberrations

### 15.1 Introduction

#### 15.1.1 Abstract

Defining the chronology of molecular alterations may identify milestones in carcinogenesis. The analyses highlight the diversity of clonal evolution within and across tumour types that might be informative for risk stratification and patient selection for targeted therapies.

#### 15.1.2 Background

Cancer arises from clones that undergo intense evolutionary selection during disease progression. This process may lead to subclonal divergence resulting in genetic and molecular heterogeneity. Several methods have been developed to quantify DNA admixture and ploidy from SNP array data that use the relative abundance of specific allele signal ( $B$  allele frequency) and the tumour over normal signal ratio or Log R to measure the complexity of the cellular population. Using germline heterozygous SNP loci or informative SNPs tumour purity and ploidy are estimated analyzing allelic fraction values. Subclonal alterations will appear as outliers from the computed admixture and ploidy. Global methods are well-suited for tumour samples with homogenous genomic aberrations. These approaches are suboptimal with tumour samples with high heterogeneity. Local optimization creates estimates of purity and ploidy from few clonal events. The AF values of informative SNPs in a somatic deletion result from the composition of signal from non-tumour cells, tumour cells without the deletion and tumour cells harbouring the deletion. Modelling the probability distribution of the observed AF, a local estimate of the DNA admixture is computed, accounting for both normal cell admixture and subclonal tumour cell population. After the estimation for all deletions across the genome only selected regions contribute to the computation of the tumour sample global admixture.

## 15.2 Results

### 15.2.1 Clonality assessment of aberrations from sequencing reads

The reads mapped into a genomic window can be partitioned into a set containing reads that equally represent parental chromosomes and a set containing reads from only one parent chromosome. There are four steps that from neutral read counts, allow inference of clonality of any genomic window. First the percentage of neutral reads within a genomic segment are estimated independently of its Log R value. Then the Log R value is used to relate the neutral reads with a local estimate of DNA admixture. Local estimates are aggregated to estimate global admixture and clonality of somatic copy number aberrations. Aneuploidy genomes are identified and the analysis corrected accordingly. The analysis is then extended to point mutations and structural rearrangements. For each genomic segment  $Seg$  the expected  $AF$  of the informative SNP has a bimodal distribution that relates to the composition of the DNA sample. The distance between the two modes is proportional to the percentage of neutral reads  $\beta$ . The expected distribution of the  $AF$  varies accordingly with  $\beta$  and  $N_{ref}$ , the proportion of reference base reads in the allele represented by active reads. For each input segment  $Seg$ , optimization based on swarm intelligence finds a  $\beta$  that minimizes the difference between the expected and the observed  $AF$  distribution. Then the Log R of  $Seg$  allows computing a local estimate of the admixture. If  $Seg$  defines a mono-allelic deletion,  $\beta$  corresponds to the percentage of reads deriving from cells that do not harbor the deletion and relates to a local estimate of the percentage of admixed cells:

$$Adm.local = \frac{\beta}{2 - \beta}$$

Local admixture values are clustered and the lowest median determines the global admixture of the sample. The more the local admixture value differs from the global the more  $Seg$  is subclonal. The clonality of  $Seg$  or  $Cl_{Seg}$  is computed as the percentage of tumour cells in a sample harbouring  $Seg$ . If  $Seg$  is a gain  $Adm.local$  extends by rescaling the percentage of neutral reads  $\beta$  to recover the percentage of reads sequenced from cell that does not harbour the gain of  $Seg$ . Bi-allelic deletions are treated separately. If the deletion is clonal its  $AF$  has binomial distribution  $\beta = 1$  and represents DNA admixture. In case of subclonality  $\beta$  is proportional to the percentage of tumour cells that do not harbour the deletion. Aneuploidy causes a shift in the Log R vs  $\beta$  space. In any segment with an empty active reads set each allele has the same number of copies and  $\beta = 1$ . The ploidy of a sample is the shift in the Log R values of the neutral segment that best accounts for the observed Log R values. Log R data are corrected for ploidy and  $Adm.global$  to achieve better estimates of the segment copy number. Clonality estimates build on the assumption that reads supporting the alternative allele are representative of the amount of tumour DNA harbouring the mutation. The proportion of reads supporting the alternative allele of a pure and clonal hemizygous PM (point mutations) has symmetric binomial distribution.  $Adm.global$  represents the percentage of reads from admixed cells that have to be ignored to compute the correct value of AP (proportion of reads supporting the alternative allele). A PM is subclonal when its corrected AP has a low probability to be clonal. The same principle applies to REARRs (structural rearrangements). The total number of reads that span both sides of a breakpoint defining a REARR is a proxy of the number of cells harbouring the rearrangement. The difference between the expected and observed proportion of reads supporting the alternative allele is proportional to the subclonality of the considered REARR.

## 15.2. RESULTS

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### 15.2.2 Inferring the order of mutations in a tumour sample

The assessment of the clonality of each somatic aberration enables the deconvolution of the sequence of oncogenic events that occur during tumour initiation and progression. Assuming that clonal alterations pre-dates subclonal alterations within the same tumour, pairs of genes aberrant in the sample and across multiple tumours are considered to determine the directionality of the clonal-subclonal hierarchy. To minimize the number of false positives (clonal called subclonal) the estimation uncertainty around  $\beta$  is computed and propagated to clonality values. This enables robust comparison of aberration clonality across different tumour sample data. If a clonal aberration  $A_1$  and a subclonal  $A_2$  occur within the same sample  $S$ ,  $A_1$  has been acquired before  $A_2$  in  $S$  and  $A_1$  precedes  $A_2$  in  $S$ . The same dependency has to be found consistently across samples to derive the rule that links  $A_1$  and  $A_2$ . This can produce an evolution path draft and in the presence of adequate sample size and frequencies of co-occurring aberrations, the statistical significance of the relation between  $A_1$  and  $A_2$  can be assessed by testing the null hypothesis that the two aberrations are independent and consider a binomial distribution with number of trials  $n$  equals the number of samples where  $A_1$  is clonal and  $A_2$  is subclonal or vice versa.

### 15.2.3 In silico and in situ experimental validation

To assess if the coverage depth typical for large scale sequencing experiments has an effect on clonality estimates miSeq ultra-deep sequencing data was queried. Excellent agreement in downstream clonality calls for deletion was observed. CLONET did not assign clonality values to aberrations in which MiSeq does not confirm AP values. Next studying PMs and assessing high correlation of AP values between WGS and MiSeq data, suggesting that the study coverage dose not significantly impact the ability to assess aberration clonality. In order to validate the clonality status of complex structural genomic aberrations, in situ tests was used. The ability to assess rearrangement clonality was demonstrated focusing on well-characterized REARRs. Perfect agreement was evident. Also subclonal bi-allelic deletion was validated by fluorescence. The prediction highlights a small subclonal bi-allelic deletion within a larger clonal mono-allelic deletion, suggesting that selective evolutionary pressure is acting on the genomic region.

### 15.2.4 Comparative analysis reveals different mechanisms of tumour deregulation

The mean number of events classified as clonal or subclonal by means of the proportion test with FDR correction. Deletions are more heterogeneous than gains in prostate and lung cancer, while melanoma had the opposite behaviour. Comparing the proportion of clonal/subclonal losses and gains the prostate and lung samples are statistically indistinguishable. This suggests that temporally distinct mechanisms lead to loss and gain across the three tumour types. Prostate cancer in terms of PMs exhibits more subclonal events than melanoma, suggesting a more central role of PMs in melanoma oncogenesis compared with prostate cancer. Aggregated values reflect only part of the story: great variability in the percentage of clonal events within a single combination of tumour and aberration is observed. Then the distribution along the genome of the variability in the clonality status of aberrations was assessed. Commonality between the three tumour types in some regions can be observed. Then the capability of clonality analysis to highlight tumour specific mechanism of deregulation was investigated. Considering PTEN deletion, which is involved in many cancer types, it was seen how the timing of the alteration is different and may point to differential roles for pathway inactivation. The focal and subclonal deletion in prostate samples suggests that evolutionary

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pressure is acting later and may promote cancer progression at a later stage. PTEN is homogenously lost in metastatic melanoma. In lung cancer this loss is more rare. CLONET can identify tumour lineage specific subclonality.

#### 15.2.5 Clonal hierarchy of genomic aberrations

The temporal evolution of driver aberrations was analysed to build evolution maps capitalizing on the information from multiple individuals' samples in the absence of multiregion samples. Given the sample size and the mutation frequencies, drafts of evolution maps were built by implementing the following rule. In particular, an arrow from  $A_1$  to  $A_2$  is drawn if:

- $A_1$  and  $A_2$  co-occur in at least two samples.
- $A_1$  preceded  $A_2$  in at least one sample.
- $A_2$  does not precede  $A_1$  in the considered dataset.

The sensitivity of CLONET allowed the identification of additional genes whose loss precedes the homozygous deletion. No contradictory relations were detected in independent datasets. In order to investigate common patterns of progression across tumour types, a large set of putative cancer genes was interrogated and applied pairwise intersections of identified paths. The evolution of known cancer signalling pathways was explored: both common themes across tumour types and tissue-specific patterns emerged. Recurrently deregulated pathways where detected as early drivers. The timing of dysregulation along the evolutionary paths can be independent across tumour types.

## 15.3 Materials and methods

### 15.3.1 CLONET pipeline

SNPs have been extracted from BAM files using an in-house procedure, SCNAs were detected using SegSeq from tumour and normal sequencing-based data, PM coordinates were as in original corresponding manuscripts and REARRs were identified by means of dRanger and Breakpointer. To avoid germline background effects, genes that intersect significant with known germline copy number variants were filtered out.

### 15.3.2 CLONET on exome and targeted sequencing data

The analysis of samples with few SCNAs provided that informative SNPs read counts and Log R values are available is enabled. Individual specific informative SNPs can be identified from matched normal DNA samples. Appropriate Log R values can be obtained for exome genome segments with platform specific strategies and provided to CLONET as input. Array-based segmented data or SCNA segments directly inferred from exome data with recent well-performing tools. CLONET combines segment input with exome-derived read counts to estimate purity and ploidy. Then subclonal aberrations are called based on sequencing data. Copy number calls derived using custom control regions and very high-coverage allowed for CLONET based clonality estimation even in the case of low tumour content.

### 15.3.3 Expected distribution of the allelic fraction of a genomic segment

Consider a genomic segment that spans a set of informative SNPs for the individual of interest. For any of them with coverage  $cov$  the total number of reads  $r$  supporting the reference base is the

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sum of the neutral reads  $r_n$  and the active reads  $r_a$  supporting the reference base.  $\beta$  is the ratio between neutral reads and the total number of reads spanning the SNP of interest. The probability of having  $k$  reference reads is the convolution of the probability of observing  $\beta \cdot k$  neutral reads and  $(1 - \beta) \cdot k$  active reads:

$$P(r = k, 0 \leq k \leq cov) = Conv(P(r_n = \beta \cdot k), P(r_a = (1 - \beta) \cdot k))$$

$P(r_n = \beta \cdot k)$  is assumed to follow a binomial distribution with trials  $\beta \cdot cov$  and probability of success  $ps$ . All active reads support the reference or the alternative base.  $N_{ref}$  is the proportion of informative SNPs within the aberration that carry the SNP reference base in the allele represented by the active reads.  $P(r_a = (1 - \beta) \cdot k)$  follows a categorical distribution with values equal to  $N_{ref}$ .

$$P(r = k | cov, \beta, N_{ref}, ps) = (1 - N_{ref}) \cdot B(k | \beta \cdot cov, ps) + N_{ref} \cdot B(k - (1 - \beta) \cdot cov | \beta \cdot cov, ps)$$

Where  $B(m | n, p)$  is the probability mass function of a binomial distribution, the probability of  $m$  successes in  $n$  trials with success probability  $P$ .

#### 15.3.4 Estimated proportion of neutral reads for a genomic segment

$\beta$  and  $N_{ref}$  can be inferred from the sequencing coverage at informative SNPs within the segment. Given a segment  $Seg$  and a set  $I$  of informative SNPs in  $Seg$ , each SNP in  $I$  is a sample from the distribution described earlier. Optimization can allow for the identification of values  $\beta$  and  $N_{ref}$  for each segment using the Kolmogorov-Smirnov for the likelihood that  $I$  are a sample of the distribution and a particle swarm optimization finds a candidate  $\hat{\beta}$  and  $\hat{N}_{ref}$  that best represents the distribution of the allelic fraction of the SNPs in  $I$ .

#### 15.3.5 From neutral to non-aberrant reads

Consider a  $Seg$  if the  $\text{Log } R$  value of  $Seg$  support a SCNA  $C$ , reads that cover  $Seg$  from cells harbouring  $C$  are considered aberrant. If  $Seg$  is a candidate mono-allelic deletion  $\beta$  corresponds to the percentage of reads that cover  $Seg$  and are sequenced from cells harbouring both alleles. If the Log R value supports a gain with  $cn > 2$ ,  $\beta$  has to be rescaled to obtain the percentage of sequenced cells that have copy number  $cn$ . If  $cn$  is odd, the number of neutral reads is the sum of the neutral from admixed plus the neutral of the gain.  $\beta_{cn}$  of reads from cells with  $cn$  is computed from  $\beta$  by removing neutral reads due to the gain:

$$\beta_{cn} = 1 - cn_G \cdot (1 - \beta)$$

If  $cn$  is even and one copy difference between allele is allowed  $\beta$  is closed to one.

#### 15.3.6 From aberrant reads to aberrant cells

Given a somatic mono-allelic deletion  $M$  the local admixture  $Adm.local$  is the proportion of cells not harbouring  $M$  over the total number of cells. Let  $a$  define the total number of reads supporting the alternative allele, as the sum of neutral  $a_n$  and active  $a_a$  reads. For any informative SNP within  $M$ , the local admixture is:

$$Adm.local_M = \frac{\frac{r_n + a_n}{2}}{\frac{r_n + a_n}{2} + (r_a + a_a)}$$

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The proportion of non-aberrant reads covering  $M$  is:

$$\beta_M = \frac{r_n + a_n}{r_n + a_n + r_a + a_a}$$

#### 15.3.7 Uncertainty assessment and its propagation to clonality estimates

To optimize sensitivity and specificity the estimation uncertainty  $\epsilon$  around  $\beta$  is computed. The value of  $\epsilon$  varies upon the mean coverage and the number of informative SNPs. The mean coverage controls the ability to discern the two modes of the AF distribution. Higher  $\beta$  requires higher coverage. The procedure to infer the value of  $\beta$  is independent from its Log R value. Segments aggregate into cluster corresponding to copy number and define a clonality status. Restricting to putative somatic mono-allelic deletions,  $B_{min}$  with the lowest median of  $\beta$  would represent 100% clonal deletions.  $B$  is the set of  $\beta$  values of all the putative somatic mono-allelic deletions.  $B_{min}$  is the smallest subset of  $B$  such that  $\min(B)$  in  $B_{min}$  and for all  $\beta'$  in  $B$  and not in  $B_{min}$ ,  $\max(B_{min}) + \text{err}(\max(B_{min})) < \beta' - \text{err}(\beta')$ . The median value is selected as candidate  $Adm.global$ . Given a somatic copy number  $C$  in a sample, the local and global admixtures are computed. The clonality  $Cl_C$  of  $C$  is the percentage of tumor cells in a sample harbouring  $C$ :

$$Cl_C = \frac{1 - Adm.local_C}{1 - Adm.global}$$

The more the value local differ from the global the more  $C$  is subclonal.

#### 15.3.8 Clonality of bi-allelic deletion

For subclonal bi-allelic deletion the allelic fraction signal comes from cells with two or one allele. Consider a subclonal bi-allelic deletion where  $n$ ,  $m$  and  $b$  denote the proportion of cells with two, one and zero alleles. The local estimate of the admixture can be computed. This is the proportion of cells with two alleles in the subpopulation of cells with one or two alleles,  $n = Adm.local(n+m)$ . The proportion of normal cells in the sum is equal to the global DNA admixture. The clonality of a bi-allelic deletion  $Cl_B$  is the percentage of cells harbouring the bi-allelic deletion over the number of cells with a mono- or a bi-allelic deletion  $\frac{b}{m+b}$ .

$$Cl_B = \frac{Adm.global - Adm.local \cdot Adm.global}{Adm.local \cdot (1 - Adm.global)}$$

# Chapter 16

## TPES: tumor purity estimation from SNVs

### 16.1 Abstract

Tumour purity is the proportion of cancer cells in a tumour sample. It impacts on the accurate assessment of molecular and genomics features.

#### 16.1.1 Introduction

Genomic and molecular analysis of tumour samples require the quantification of tumour and admixed normal cells proportion. In order to assess the somatic lesion detection boundaries and to perform comparative analyses several tools were built to quantify TP from NGS data. The approaches based on SCNA fall short for samples with quiet genomes. To solve this purity can be estimated through the distribution of variant allelic fractions within copy number neutral tumour segments.

### 16.2 Materials and methods

The VAF distribution of a set of clonal monoallelic SNVs from a pure tumour sample should be centred in 0.5. Technical and cancer specific factors may influence the VAF value as reference mapping bias. Moreover in the case of subclonal events the VAF is altered. Clonal monoallelic SNVs in a diploid segment are suited for TP estimation and are named p-SNV. Given a set of p-SNVs, TP could be computed as:

$$\frac{\text{observed VAF}(pSNP)}{\text{expected VAF}}$$

Where *observed VAF* is computed from the tumour data while *expected VAF* is the value expected from a pure tumour sample accounting for reference mapping bias. p-SNVs are selected with a conservative procedure. To minimize the number of false positive p-SNVs for each sample, TPES introduces two main filtering steps. In the first SNVs are selected from copy-number neutral segments applying a conservative filter on the Log R value of each genomic segment. Moreover the log R is adjusted for ploidy and SNV are retained only with a number of reads mapping the alternative base and AG above and below threshold. Chromosome X and Y are excluded to avoid

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gender stratification. This nominates a set of heterozygous copy-number neutral SNPv. The second filter TPES removes putative subclonal mutations. Observed VAF distribution is smoothed by kernel density estimation. Local maxima of the underlying distribution can be observed. The peak with the highest VAF value is the candidate observed VAF.

# Chapter 17

## SNP panel identification assay (SPIA): a genetic-based assay for the identification of cell lines

### 17.1 Abstract

Experiments reported in the scientific literature may contain pre-analytic errors due to inaccurate identities of the cell lines employed. To address this a simple approach to enable accurate determination of cell line identify has been developed by genotyping SNP.

#### 17.1.1 Introduction

Cell lines are important in the identification of therapeutic targets and in understanding molecular pathways related to drug-tumour interactions. One recognized risk in cell line maintenance is human error, either by mislabelling or cross-contamination. In an effort to identify latent cross-contamination or other errors SNPs are considered. SNPs as DNA markers have been shown to be well suited for different purposes such as animal identification, identification of population ancestry and for forensic purposes. The ability of high-density oligonucleotide arrays to genotype hundreds of thousands of SNP loci in parallel provides a molecular fingerprint of each sample. To this end an assay that employs 30 to 50 single loci and capable of distinguishing any two DNA sample has been developed. This assay can identify a given sample comparing its genotype with a reference dataset.

### 17.2 Material and methods

#### 17.2.1 Genotype distance

To evaluate the similarity of two DNA sample the similarity measure  $D$  is introduced.  $D$  is proportional to the number of genotype mismatches between the samples and is normalized to the number of genotype calls available for both samples. Given a set of  $N_{SNPs}$  of individual SNPs,  $CL1$  and  $CL2$  are ordered sets of genotype calls of two samples and  $vN_{SNPs} - Card(T)$ , where  $T = \{i : cl1_i \neq NoCall \cap cl2_i \neq NoCall\}$ . For  $vN_{SNPs} > 0$ ,  $D$  is defined as:

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$$D(CL1, CL2) = \frac{1}{vN_{SNPs}} \sum_{i=1, \dots, N_{SNPs}} d(cl1_i, cl2_i)$$

Where:

$$d(cl1_i, cl2_i) = \begin{cases} 1 & \text{if } cl1_i \neq cl2_i \\ 0 & \text{if } cl1_i = cl2_i \vee cl_i = NoCall \end{cases}$$

The distance is normalized over the number of available calls. Moreover the algorithm evaluates:

- The count of mismatches where the two samples are homozygous for different alleles.
- The count of mismatches where one is homozygous and the other is heterozygous.
- The count of homozygous matches and the count of heterozygous matches.

For each mismatch the algorithms reports the identifier of the sample with largest number of heterozygous calls. The implementation of the distance can be modified to weight different types of mismatch.

### 17.2.2 SNP panel selection procedure

Using a small number of SNPs samples can still be accurately distinguished. Initial filters on the selection of SNPs where:

- SNPs with the rs identifier.
- SNPs represented on the 10K Affymetrix oligonucleotide array.
- SNPs not in intronic regions.

On the training set the minor allele frequency, the heterozygosity rate and the call rate for each SNP across all sample have been computed. Then SNP satisfying the Hardy-Weinberg equilibrium applying elastic boundaries and having SNP call rates more than 80% have been filtered. Then on the test set, the heterozygosity rate of the identified SNP has been computed. At each iteration a variable number of SNPs has been computed. SNPs have been ranked according to the selection rate.

### 17.2.3 SPIA probabilistic test on cell line genotype distance

A double probabilistic test to apply on the genotype distance is applied to discern when two cell lines are close enough to be called similar. The test score depends on the number of matches and on the total number of SNPs evaluated. The test relies on the probability of the evaluated distance belonging to the population of real matched pairs or to the population of real non-pairs. If the output is not clear a second panel of SNPs would need to be investigated. If the SNPs are independent and the genotype call probability being the same at each SNP, the probability of having  $k$  matches out of  $N$  SNPs follows the binomial distribution:

$$P_k = \binom{N}{k} P^k Q^{N-k} = \frac{N!}{k!(N-k)!} P^k Q^{N-k}$$

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Where  $P$  and  $Q$  are the probability of match and mismatch and  $N$  is  $vN_{SNPs}$ . By knowing the probability of match at a single SNP for a real matched pair  $P_M$  and for a non-matched pair  $P_{non-M}$  the distribution of real matched pair and non-pair can be drawn. For a given  $vN_{SNPs}$  then areas corresponding to “different”, “uncertain” and “similar” can be defined. The area limits depend on the level of confidence needed. The mean number of successes  $k_{mean}$  is equal to  $NP_M$  and the standard deviation  $sd_{kmean} = \sqrt{NP_M(1 - P_M)}$ . The probability that a distance measurement falls within  $M$  standard deviations from the mean is given by the integral of the distribution function. By setting  $m$  the area limits can be defined. The smaller the number of SNPs the narrower the region of uncertainty and the higher the probability of making an incorrect call.