Master's Thesis

Implementation and Comparative Assessment of Diagnostic Cancer Gene Panels in the Molecular Pathology Laboratory

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Master in Integrated Systems Biology

by

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Abstract

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List of Abbreviations

NGS	Next Generation Sequencing	LNS	Laboratoire National de Sante
SGMB	Service of Genetics and Molecular Biology	TST15	Illumina TruSight Tumor 15

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1 Introduction

The accumulation of DNA mutations is a characteristic of tumors. Mutations in a cell's genetic material enable the so-called cancer hallmarks, which include, amongst others, capabilities as activating invasion and metastasis, enabling replicative immortality, sustaining proliferative signaling or evading growth suppressors. Classical anti-cancer treatments are tailored for the average patient and not for the individual. Traditional cytotoxic chemotherapeutic drugs, for instance, are unspecific and have numerous adverse effects [1]: they attack rapidly dividing cells and make no difference between healthy and cancer cells. For a long time, there was no possibility to predict the success of a patient's cancer treatment. In consequence, the treating clinician had no way to personalize the treatment to the individual patient.

In oncology, the customized healthcare approach is designed to the individual patient by taking into account the genetic information from a patient's tumor biopsy. Targeting proteins that give cancer cells a proliferative advantage, instead of simply using cytotoxic agents, allows a more specific treatment and might decrease treatment-associated side-effects. In the past years, the Food and Drug Administration (FDA, USA) has approved several drugs that specifically target proteins needed for cancerogenesis [2]. Many of these drugs are now effective treatments for several common cancers. In many patients, however, these agents might lack any efficiency: cancers are known to be highly heterogenous and mutations in some subpopulations of cancer cells make them resistant to the treatment [3]. Also, some of these targeted drugs can only be prescribed if the targeted protein is not mutated, e.g. in wildtype status. Identifying these mutations is thereby an utmost necessity for tailoring a targeted and efficient cancer therapy. Amongst the many mutation detection techniques, Next Generation Sequencing (NGS) constitutes the most powerful method and allows deep insights into the underlying causes of diseases.

The completion of the human genome project and technical advances in the field of genetics, in combination with advanced computer performance, enabled geneticists and pharmacologists to adopt the personalization of cancer diagnostics and treatment. The demand has driven the development of high-throughput and faster sequencing: second-generation sequencing methods, or NGS. NGS is also called massively parallel sequencing as a massive amount of DNA fragments from a single sample are sequenced at the same time. In the last decade, several benchtop sequencers have been developed that provide high-throughput and cost-effective sequencing.

By identifying somatic mutations in cancer samples, NGS can guide personalized cancer therapy approaches. The high-quality data produced by NGS can reveal associations between several genes

implicated in cancer subtypes and is thereby of importance in drug development. In addition, it can be implemented in molecular diagnostic laboratories to identify patients that would potentially benefit from these targeted drugs.

Critical alterations in cancer cells include single nucleotide variations (SNVs), insertions and deletions of one or multiple nucleotides (INDELS), copy number variations (CNVs) and rearrangements. Some of these variants are known to provide increased sensitivity or resistance to targeted drugs. Whole-genome (WGS) and whole-exome sequencing (WES) can detect these variants. These experiments however are performed with low-coverage (100–250x in most laboratories) and have thereby limited ability to detect somatic cancer variants with the required confidence. In fact, cancers accumulate mutations and are highly heterogeneous. A given small cancer subpopulation might present variants that are absent to the rest of the cancer. This variant might be present at low-frequency and might thereby not be detected in WGS or WES experiments. The variant however might provide resistance to the treatment to cells in this subpopulation. The cancer would eventually relapse. It is thereby of utmost importance to gain deep insights into underlying genetic information to guide an effective personalized therapy. This lead to the development of targeted NGS.

Targeted NGS uses gene panels, e.g. only some regions in selected genes are sequenced. The reduction of the regions of interest (ROIs) allows to significantly increase the coverage. This allows a higher confidence and thereby enables the implementation of NGS in diagnostic laboratories.

¿¿¿ Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics

Personalized therapy by identification and targeting of tumor-specific molecular abnormalities is rapidly becoming an important component in the management of cancer patients. Consequently, algorithms for tumor diagnosis necessitate not only morphological and immunophenotypic assessment of tumors but also molecular mutational profiling. Among the solid tumors, mutational status is important to the clinical management of patients with thyroid carcinomas, non-small cell carcinomas of the lung, melanomas and colorectal carcinomas. For example, treatment of patients with non-small cell carcinoma of the lung depends on the histological type (adenocarcinoma versus squamous cell carcinoma) and the mutational status of the epidermal growth factor receptor (EGFR) and KRAS genes.1 Thus, adequate tissue sampling is essential not only for pathological diagnosis but also for molecular testing that is required to guide therapeutic decisions.1, 2, 3 2: Hirsch FR, Wynes MW, Gandara DR et al. The tissue is the issue: personalized medicine for non-small cell lung cancer. Clin Cancer Res 2010;16:4909–4911. 3: Cagle PT, Allen TC, Dacic S et al. Revolution in lung cancer: new challenges for the surgical pathologist. Arch Pathol Lab Med 2011;135:110–116.

In clinical practice, minimally invasive fine needle aspiration (FNA) is a helpful and convenient method for establishing the diagnosis of solid tumors. FNA procedures are included in the recommended guidelines for the diagnosis of thyroid carcinomas,4, 5 lung carcinomas6, 7 and sarcomas, such as Ewing Sarcoma/primitive neuroectodermal tumor.8 Although the DNA yield obtained by FNA is adequate for morphological diagnosis, FNA material is not routinely processed for molecular analysis.9 This is because mutational assessment of multiple genes by traditional sequencing platforms require a large quantity of DNA, which is often difficult to obtain by FNA. Fortunately, most FNA procedures routinely have either a concurrent biopsy or a follow-up surgical excision, which provide adequate tissue for molecular assessment.10, 11 6: Aisner DL, Deshpande C, Baloch Z et al. Evaluation of EGFR mutation status in cytology specimens: an institutional experience. Diagn Cytopathol 2013;41:316–323. 7: Ulivi P, Romagnoli M, Chiadini E et al. Assessment of EGFR and K-ras mutations in fixed and fresh specimens from transesophageal ultrasound-guided fine needle aspiration in non-small cell lung cancer patients. Int J Oncol 2012;41:147–152. 10: Santis G, Angell R, Nickless G et al. Screening for EGFR and KRAS mutations in endobronchial ultrasound derived transbronchial needle aspirates in non-small cell lung cancer using COLD-PCR. PloS One 2011;6:e25191.

In some instances, however, cytological specimens may be the only material available for molecular testing. If the tumor is locally advanced or metastatic, surgical resection is not performed.1, 6 In some patients, tumor size, location or co-morbid conditions may preclude concurrent core needle or excisional biopsy.1

1.1 EGFR Signaling Cascade

======= Cancer is a major public health problem worldwide and one of the leading death causes. In 2012, there were an estimated 14.1 million new cancer cases with estimated 8.2 million cancer deaths [3]. Lung cancer is the most common cancer, both in terms of new cases (1.8 million) and deaths (1.6 million). Breast cancer is the second most common cancer (1.7 million cases) but only ranks 5th as cause of death (522,000 deaths). Colorectal cancer (1.4 million cases; 694,000 deaths), prostate cancer (1.1 million cases; 307,000 deaths), stomach cancer (951,000 cases; 723,000 deaths) and liver cancer (782,000 cases; 723,000 deaths) are following.

In Luxembourg, there were 1164 death cases caused by cancer in 2014, accounting for 30.6% of the number of deaths. Cancer is thereby the second most common death cause in Luxembourg, ranging behind cardiovascular diseases (1189 deaths; 31.3%). Amongst these cancer deaths, can-

cers of the digestive organs (340 deaths) and of the respiratory organs (272 deaths) were the most frequent ones. [4].

(Considerable effort is made by the research community to understand the underlying causes of cancer and its progression, but death rate related to cancer remains high. In Luxembourg, the death rate did not significantly drop from 1998 to 2014.)

In recent years, more targeted drugs. personalized medicine.

Next-Generation Sequencing (NGS) is a powerful method to analyze RNA or DNA molecules. Improved protocols and methods have been developed in recent years that allow to implement NGS for a variety of applications in both research and diagnostics. NGS is still hard to implement and is mainly used in research. Only a few laboratories are using this technique as a diagnostic technique.The SGMB is building expertise in NGS and is implementing targeted NGS in its molecular pathology laboratory. This implementation aims at gaining deep insights into the underlying causes of solid tumors and in guiding targeted cancer therapy.

The goal of this master's thesis was to compare commercially available cancer gene panels on samples of solid tumor patients by several parameters and by their ability to detect variants, which have previously been detected in the routine mutation detection workflow in the laboratory. As a second part, several freely available variant calling algorithms were compared for their potential implementation in the custom in-house variant detection bioinformatic pipeline.

1.2 EGFR Signaling in Cancer

Bla [1] ¿¿¿¿¿¿¿ a5147a619d3523c598b91a86e2c62e19a96fd26f

Describe pathway

Common mutations in this pathway

EGFR-targeted drugs: bevacizumab, cetuximab, panitumumab, ...

The samples analyzed in the SGMB originate from patients suffering from solid tumors, mainly melanoma, non-small cell lung carcinoma (NSCLC), and colorectal cancer. Evidence suggests that many solid tumors use and modify EGFR (Epithelial Growth Factor Receptor) signaling for their purposes [4]. Targeting this signaling pathway is thereby an attractive anti-cancer treatment. In this regard, anti-EGFR monoclonal antibodies (cetuximab (Erbitux®) and panitumumab (Vectibix®) and tyrosine kinase inhibitors (erlotinib (Tarceva®) and gefitinib (Iressa®)) have shown their usefulness

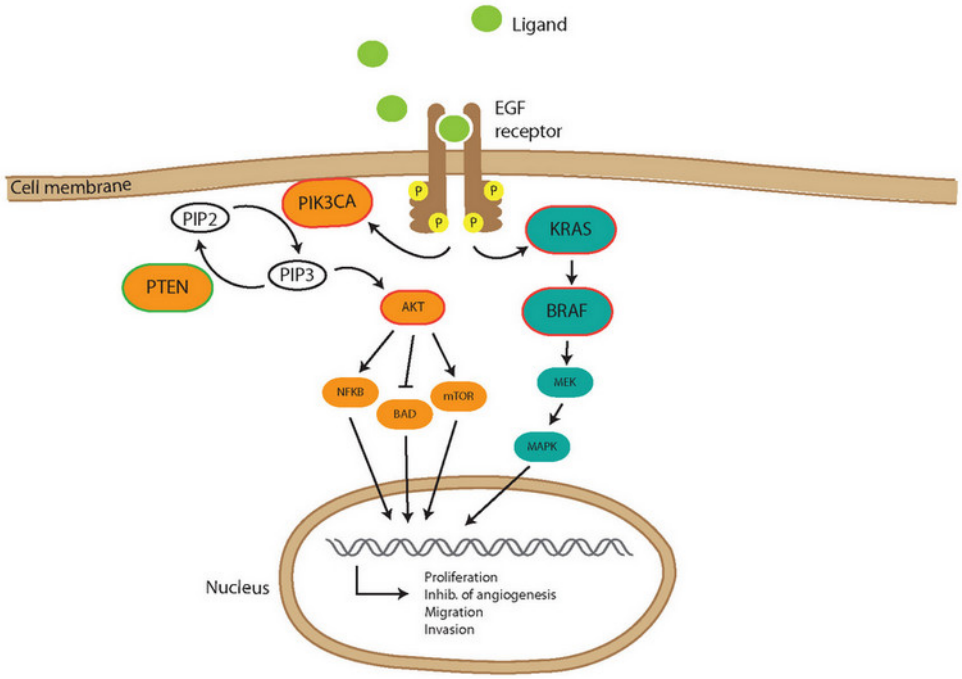


Figure 1: Schematic representation of the EGFR signaling cascade [1]

in cancer treatments [5]. EGFR and downstream proteins K-Ras / N- Ras and B-Raf are predictive biomarkers for the successfulness of the administration of the mentioned drugs [6]. Comprehensive information about these markers is thereby essential when choosing a suitable treatment in order to minimize treatment- associated side-effects and to maximize the benefits of the treatment.

In their article, Scaltriti and Baselga present the EGFR signaling pathway as a model for targeted therapy [7]. EGFR is part of the family of receptor tyrosine kinases. This transmembrane protein is composed of an intracytoplasmic tyrosine kinase domain, a short hydrophobic transmembrane region and an extracellular ligand-binding domain. Upon ligand (EGF, TGF) binding, EGFR becomes activated. This leads to homodimerization, which results in an auto- and cross-phosphorylation of key tyrosine residues on its cytoplasmic domain. This forms docking sites for cytoplasmic proteins that contain phosphotyrosine-binding and Src homology 2 domains. This allows, amongst others, signaling through the PTEN/PI3K/AKT and RAS-RAF-MAPK pathways. Activation of PTEN/PI3K/AKT leads to cell growth, proliferation and survival [8], while RAS-RAF-MAPK induces cell survival and cell cycle progression and proliferation [9]. In the RAS-Raf-MAPK pathway, Grb2 and Sos, two adaptor proteins, form a complex with the activated EGFR [10]. The resulting conformational change of Sos recruits Ras-GDP, which in turn becomes activated to form Ras-GTP. Ras-GTP activates Raf, which, in intermediate steps, phosphorylates a MAPK (mitogen-activated protein kinase). Activated MAPKs are then imported from the cytoplasm into the nucleus where they

act on target genes. The Ras and Raf subfamilies include several proteins, three of them are of interest in targeted cancer treatments: K-Ras, N-Ras and B-Raf.

Activating mutations of EGFR or its downstream proteins provide resistance to specific treatments. In that regard, the Institut National du Cancer (F) [11] provides recommendations about which mutations have to be searched to identify patients eligible for the administration of monoclonal antibodies or tyrosine kinase inhibitors. For instance, mutations on codon 12 and 13 in the KRAS gene provide resistance to the monoclonal antibody agents panitumumab and cetuximab. Gefinitinib, a tyrosine kinase inhibitor, can only be prescribed for patients, which show no activating mutations on EGFR.

1.3 Targeted Sequencing and Target Enrichment Methods

Targeted sequencing to a depth that allows detection of relatively low mutant allele frequency (MAF) may represent an alternative or a complement to WGS and WES to detect clinically relevant alterations. Additionally, in most clinical and research settings, the amount of DNA that can be isolated from tumor samples is limited and the DNA is often damaged owing to fixation and storage procedures such as those used with formalin-fixed paraffin-embedded (FFPE) samples. Therefore a multiplexed targeted platform that can generate reliable data with high sensitivity from limited amounts of DNA from FFPE samples is needed. Several targeted sequencing panels have been successfully implemented (6, 7). However, the details of a platform's design and parameterization will influence the precision and reliability of the molecular profiling results, impacting both translational research and clinical decision-making. Thus, it is of great value to explore multiple potential solutions in a real patient care environment until a community-wide solution is established, validated, and well accepted.

Benchtop sequencing devices: more labs can now do sequencing as it becomes faster & cheaper & more reliable.Several NGS bench-top devices have become available in the last decade. These instrumentations differ in their underlying chemistry that influences the instrument's performance, accuracy, output and time per run. Common sequencing principles include pyrosequencing (454), sequencing by ligation (SOLiD), ion semiconductor (Ion Torrent) and sequencing by synthesis (Illumina) [12]. Even though advances in sequencing technology and computational power and tools have decreased the time and cost of a sequencing experiment, NGS is still mainly used in research, with only a few laboratories using this technique in diagnostics.

In fact, validation of a NGS methodology requires careful assessment of methods and tools

[13]. Therefore, each step that is performed from the initial starting material to sample processing, sequencing library preparation, sequencing assay and bioinformatic processing has to be carefully checked for sources of potential errors or variability. Basically, in the validation process, it is checked whether the method measures what it claims to measure with the required sensitivity and sensibility.

With the success of NGS, many cancer genomes have been sequenced and made available to the worldwide research community. Companies, molecular diagnostics laboratories and academic centers are trying to use these big data for their purposes. A lot of mutations are described in these genomes, but only a small fraction of them are clinically actionable, e.g. can be targeted with specific drugs. Therefore, a molecular pathology laboratory does not need to perform whole-genome or -exome sequencing, but can employ targeted NGS to analyze some genes of interest, which include mutations for which there exists a clinical utility. Due to the low number of target regions, targeted NGS allows high coverage. In addition, it is a time- and cost- efficient alternative to whole-genome or -exome sequencing. Also, targeted NGS results in a significantly lower amount of produced data and thereby eases data storage and analysis time. Table 1 shows a selection of commercially available cancer gene panels, which all allow to analyze selected regions of genes implicated in cancerogenesis. Before implementing one of these panels for molecular diagnostics, it has to be ensured that the panel allows to study the genes of interest, e.g. genes that are clinically applicable, and a careful assessment of its analytical validity has to be performed.

More & more databases describing SNPs & known pathogenic hotspots

Bla [5]

Hybrid capture

Selective circularization

PCR amplification

1.4 Sequencing Chemistries

IonTorrent, etc

1.4.1 Illumina MiSeq

http://www.illumina.com/documents/products/techspotlights/techspotlight$_sequencing.pdf$

Illumina sequencing technology leverages clonal array formation and proprietary reversible terminator technology for rapid and accurate large-scale sequencing. The innovative and exible sequencing system enables a broad array of applications in genomics, transcriptomics, and epigenomics.

Sequencing templates are immobilized on a proprietary ow cell surface (Figure 1) designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface- bound template and low non-speci c binding of uorescently labeled nucleotides. Solid-phase ampli cation (Figures 2–7) creates up to 1,000 identical copies of each single template molecule in close prox- imity (diameter of one micron or less). Because this process does not involve photolithography, mechanical spotting, or positioning of beads into wells, densities on the order of ten million single-molecule clusters per square centimeter are achieved.

The Illumina sequencing approach is built around a massive quantity of sequence reads in parallel. Deep sampling and uniform coverage is used to generate a consensus and ensure high con dence in determination of genetic differences. Deep sampling allows the use of weighted majority voting and statistical analysis, similar to conventional methods, to identify homozygotes and heterozygotes and to distin- guish sequencing errors. Each raw read base has an assigned quality score so that the software can apply a weighting factor in calling differ- ences and generating con dence scores.

Illumina data collection software enables users to align sequences to a reference in resequencing applications (Figure 13). Developed in collaboration with leading researchers, this software suite includes the full range of data collection, processing, and analysis modules to streamline collection and analysis of data with minimal user interven- tion. The open format of the software allows easy access to data at various stages of processing and analysis using simple application program interfaces.

https://en.wikipedia.org/wiki/Illumina$_dye_sequencing$

Illumina dye sequencing is a technique used to determine the series of base pairs in DNA, also known as DNA sequencing. The reversible terminated chemistry concept was invented by Bruno Canard and Simon Sarfati at the Pasteur Institute in Paris.[1][2] It was developed by Shankar Balasubramanian and David Klenerman of Cambridge University,[3] who subsequently founded Solexa, a company later acquired by Illumina. This sequencing method is based on reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands. It can also be used for whole-genome and region sequencing, transcriptome analysis, metagenomics, small RNA

discovery, methylation profiling, and genome-wide protein-nucleic acid interaction analysis.[4][5]

(amfong net :) The first step after DNA purification is tagmentation. Enzymes called transposomes randomly cut the DNA into short segments (“tags”). Adapters are added on either side of the cut points (ligation). Strands that fail to have adapters ligated are washed away.[6])

The next step is called reduced cycle amplification. During this step, sequences for primer binding, indices, and terminal sequences are added. Indices are usually six base pairs long and are used during DNA sequence analysis to identify samples. Indices allow for up to 96 different samples to be run together. During analysis, the computer will group all reads with the same index together.[7][8] The terminal sequences are used for attaching the DNA strand to the flow cell. Illumina uses a “sequence by synthesis” approach.[8] This process takes place inside of an acrylamide-coated glass flow cell.[9] The flow cell has oligonucleotides (short nucleotide sequences) coating the bottom of the cell, and they serve to hold the DNA strands in place during sequencing. The oligos match the two kinds of terminal sequences added to the DNA during reduced cycle amplification. As the DNA enters the flow cell, one of the adapters attaches to a complementary oligo.

Once attached, cluster generation can begin. The goal is to create hundreds of identical strands of DNA. Some will be the forward strand; the rest, the reverse. Clusters are generated through bridge amplification. Polymerases move along a strand of DNA, creating its complementary strand. The original strand is washed away, leaving only the reverse strand. At the top of the reverse strand there is an adapter sequence. The DNA strand bends and attaches to the oligo that is complementary to the top adapter sequence. Polymerases attach to the reverse strand, and its complementary strand (which is identical to the original) is made. The now double stranded DNA is denatured so that each strand can separately attach to an oligonucleotide sequence anchored to the flow cell. One will be the reverse strand; the other, the forward. This process is called bridge amplification, and it happens for thousands of clusters all over the flow cell at once.

Over and over again, DNA strands will bend and attach to oligos. Polymerases will synthesize a new strand to create a double stranded segment, and that will be denatured so that all of the DNA strands in one area are from a single source (clonal amplification). Clonal amplification is important for quality control purposes. If a strand is found to have an odd sequence, then scientists can check the reverse strand to make sure that it has the complement of the same oddity. The forward and reverse strands act as checks to guard against artifacts. Because Illumina sequencing uses polymerases, base substitution errors have been observed,[10] especially at the 3’ end.[11] Paired end reads combined with cluster generation can confirm an error took place. The reverse and forward strands should be complementary to each other, all reverse reads should match each other,

and all forward reads should match each other. If a read is not similar enough to its counterparts (with which it should be a clone), an error may have occurred. A minimum threshold of 97

At the end of bridge amplification, all of the reverse strands are washed off the flow cell, leaving only forward strands. Primers attach to the forward strands and add fluorescently tagged nucleotides to the DNA strand. Only one base is added per round. A reversible terminator is on every nucleotide to prevent multiple additions in one round. Each of the four bases has a unique emission, and after each round, the machine records which base was added. This process is "sequence by synthesis."

Once the DNA strand has been read, the strand that was just added is washed away. Then, the index 1 primer attaches, polymerizes the index 1 sequence, and is washed away. The strand forms a bridge again, and the 3' end of the DNA strand attaches to an oligo on the flow cell. The index 2 primer attaches, polymerizes the sequence, and is washed away.

A polymerase sequences the complementary strand on top of the arched strand. They separate, and the 3' end of each strand is blocked. The forward strand is washed away, and the process of sequence by synthesis repeats for the reverse strand.

The sequencing occurs for millions of clusters at once, and each cluster has 1,000 identical copies of a DNA insert.[10] The sequence data is analyzed by finding fragments with overlapping areas, called contigs, and lining them up. If a reference sequence is known, the contigs are then compared to it for variant identification.

This piecemeal process allows scientists to see the complete sequence even though an unfragmented sequence was never run; however, because Illumina read lengths are not very long [11] (HiSeq sequencing can produce read lengths around 90 bp long [7]), it can be a struggle to resolve short tandem repeat areas.[7][10] Also, if the sequence is de novo and so a reference doesn't exist, repeated areas can cause a lot of difficulty in sequence assembly.[10] Additional difficulties include base substitutions (especially at the 3' end of reads[11]) by inaccurate polymerases, chimeric sequences, and PCR-bias, all of which can contribute to generating an incorrect sequence.[11]

1.5 NGS Data Analysis

GATK best practices

1.6 Practical Implications in the Laboratory

FFPE : more details

¡¡¡¡¡¡¡ HEAD The quality of the genetic testing of the tumor is affected by several factors. These include the content of tumor cells in the sample, the quality of the tissue material, sequencing library preparation and the the bioinformatic pipeline.

The biopsy usually consists of healthy and cancer cells. The sensitivity of tumor variant detection is linked to the tumor cell content of the specimen. In addition, cancers are highly heterogenous, e.g. a small subpopulation might present mutations that provide resistance to targeted treatment. Detecting these low-frequency mutations and clearly separating them from eventual high-frequency fixation or sequencing artifacts presents a huge challenge [14].

Tumor biopsies usually yield a limited amount of tissue, therefore it is conceivable to use the same sample for more analyses. In Luxembourg, all relevant tumor biopsies are usually sent to the Laboratoire National de Sante (LNS) to the Service of Pathologic Anatomy where the biopsy is fixed in formalin and embedded in paraffin (FFPE). FFPE conserves the tissue morphology and thereby allows histological analysis. In addition, it allows to store specimens for decades. Sample quality, however, is influenced by this fixation method, but also by the size of the biopsy, and its fixation time [13]. DNA extraction from FFPE samples is difficult and yields low amounts of DNA [15]; formaldehyde leads to cross-linking of nucleic acids and proteins [16]; FFPE introduces fixation artifacts into DNA sequences [17—. These circumstances complicate sample processing as well as NGS data interpretation. Though, FFPE samples have been shown to be still suitable for downstream analyses [18].

Sequencing library preparation also affects the final NGS result. Several technologies for target enrichment exist and are available for different sequencing instruments [19]. Essential for all these enrichment methods is the amplification of target regions and the introduction of multiplexing, which requires the incorporation of a unique index adaptor combination for each sample. Target enrichment methods can be separated into three basic groups: targeted circularization, hybrid capture of target fragments and PCR-based enrichment methods. PCR-driven methods happen on high-molecular DNA. In contrast to uniplex long-range PCR, short-range multiplex PCR produces short DNA fragments of target regions. There is thereby no need of DNA shearing. Hybridization-based methods require a so-called shotgun library construction before target regions can be captured. During this process, genomic DNA is sheared randomly into small fragments and an adapter- and index-linked library is produced. Biotinylated baits are added that bind to target regions. Target regions can then

be captured using streptadivin coated magnetic beads. Targeted circularization methods rely on a digestion of DNA by restriction enzymes. The produced DNA fragments are then circularized and uncircularized DNA fragments are removed by exonucleases. Only circularized target regions are then amplified by PCR.

The establishment and validation of a bioinformatic NGS data analysis pipeline still constitutes a challenge in diagnostics. After generation of FASTQ files of the sequencer, data generally undergo quality control, followed by trimming of low quality bases, alignment to the reference genome, variant calling and variant annotation. For each of these steps, several bioinformatic algorithms and tools exist [20]. The computational pipeline of the molecular pathology laboratory has to incorporate the tools that allow the most sensitive and sensible analysis of data. For instance, quality trimming influences the mapping to the reference genome. The mapping, in turn, strongly affects the variant calling. In fact, variant calling is a critical step in NGS data analysis. Several tool kits as SAMtools, SPLINTER, VarScan2 or GATK allow variant annotation, but vary in their false-positive and false-negative detection rates ([21], [22]). These tools have to be carefully assessed, as false-positives or false-negatives should absolutely be avoided when it comes to the subscription of a targeted chemotherapeutic agent.

To facilitate interpretation of NGS data, variants have to be annotated and their clinical actionability has to be identified. Several databases have emerged in this field (such as mycancergenome.org) and numerous tools allow to automatize variant annotation. Here again, the choice of the database and the variant annotator is important.

Finally, the sample-to-results time is a very pragmatic, but important factor. The time from the biopsy to the potential start of an administration of a targeted chemotherapeutic drug should be reduced to a minimum. For instance, in case of late-stage cancer patients, it would be unacceptable if analysis would take several weeks. To reduce the sample-to-results time to under two weeks, the sample processing workflow should be as short as possible, while still yielding high quality sequencing libraries. The bioinformatic pipeline should not only incorporate the best tools, but should also be automatized to further reduce the time of analysis. ======= Chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cell and of sub-cellular components such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). The most common fixative for light microscopy is 10phosphate buffered saline).

These fixatives preserve tissues or cells mainly by irreversibly cross-linking proteins. The main action of these aldehyde fixatives is to cross-link amino groups in proteins through the formation of methylene bridges (-CH2-), in the case of formaldehyde.

This process, while preserving the structural integrity of the cells and tissue can damage the biological functionality of proteins, particularly enzymes, and can also denature them to a certain extent. This can be detrimental to certain histological techniques.

Formalin fixation leads to degradation of mRNA, miRNA and DNA in tissues. However, extraction, amplification and analysis of these nucleic acids from formalin-fixed, paraffin-embedded tissues is possible using appropriate protocols.

The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 m (micrometres; 1000 micrometres = 1 mm) thick for light microscopy.

Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene. Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohistochemistry is required. Thicker sections (0.35m to 5m) of resin-embedded tissue can also be cut for light microscopy. Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine, or wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. The acrylic resins are polymerised by heat, ultraviolet light, or chemical catalysts. The hardened blocks containing the tissue samples are then ready to be sectioned.

Because Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, FFPE tissues are an important resource for historical studies in medicine.

For light microscopy, a steel knife mounted in a microtome is used to cut 4-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections

which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain.

quantitation

ffpe quality control necessary. FFPE are degraded. More C¿T

Target enrichment methods hun och vlt en effekt

choice vum sequencer, different error rates, different yield, analysis time.

choice vun den bioinformatic tools. t'gin der vill, vir- an nodeeler. zB variant calling algorithms: most are for haplotypes and not for somatic variants. many need matched tumor-normal samples, which we here in the lab do not have, only have tumor dna. after variant calling, they have to be annotated for their clinical relevance and potential actionability.

problem: many variants may be found, some of them may be harmless germline SNPs, many variants may be causative of cancer, but are not actionable.

time sample-to-result

how to report? all results or only those that are known to be actionable. ¿¿¿¿¿¿¿ a5147a619d3523c598b91a86e2c

1.7 Aims of the Thesis

Targeted NGS is still not widely used in diagnostics laboratories. The SGMB of the LNS is planning to build expertise with the aim to adopt NGS routinely in the laboratory, mainly in the context of diagnosis and therapy of cancer patients in Luxembourg.

The aim of this thesis project was to test commercially available cancer gene panels, e.g. Illumina Trusight Tumor 15 and Agilent Haloplex HS ClearSeq Cancer, for their potential use in the routine workflow of the laboratory. Several samples of cancer patients were prepared with both kits and were sequenced on the Illumina MiSeq device. Both kits vary in their sequencing library preparation principles: Illumina's tst15 uses the multiplex PCR approach while Agilent's Haloplex Enrichment System uses enzymatic DNA restriction followed by probe capture.

NGS data were analyzed with the respective recommended pipelines and a custom in-house pipeline.

Finally, several freely available variant calling algorithms were tested for their potential implementation in the custom in-house variant discovery bioinformatic pipeline.

2 Material and Methods

- How was the data analyzed ?
- Present econometric/statistical estimation method and give reasons why it is suitable to answer the given problem.
- Allows the reader to judge the validity of the study and its findings.
- Depending on the topic this section can also be split up into separate sections.

2.1 Library Preparation

2.1.1 Patients

Melanoma Non-Small Cell Lun Carcinoma (NSCLC) metastatic colorectal cancer (mCRC) Chronic lymphocytic leukemia (CLL) were extracted from blood and did not undergo FFPE treatment -¿ were used as some kind of good quality samples to see if there are really more C¿T variants in FFPE samples.

2.1.2 DNA Extraction, Quantification and Quality Control

DNA Extraction Kit from Qiagen

Quantification Qubit fluorometer, either High Sensitivity kit or Broad Range

Quality Control Illumina Infinium FFPE QC Assay kit

2.1.3 Agilent Haloplex ClearSeq Cancer

2.1.4 Illumina TruSight Tumor 15

2.2 Bioinformatic Analysis

2.2.1 Agilent SureCall

With alignment algorithms installed Windows System, 3.00GHz, 16GB RAM$ modifiable

2.2.2 Illumina BaseSpace TruSight Tumor 15 App

Cloud-based parameters not modifiable

2.2.3 Custom In-House Pipeline (Velona)

Linux System

2.2.4 Variant Calling Algorithms

Tested on Linux Ubuntu 14.04.4 LTS Ítrusty Tahrínstalled on VMware virtual box with of 12GB RAM

GATK HaplotypeCaller

VarScan 2

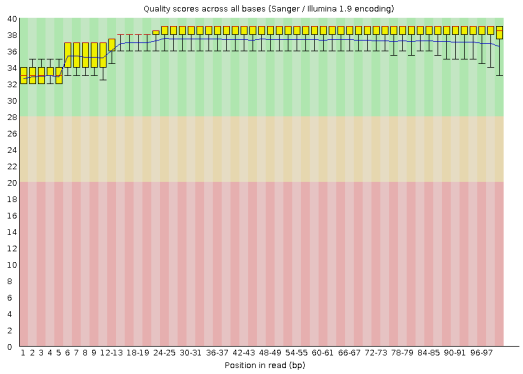
Mutect1.1.7 [2]

SomVarIUS

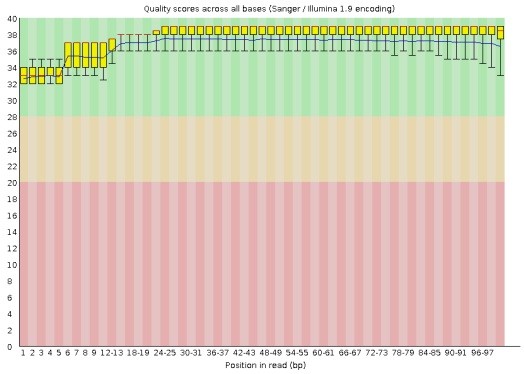
3 Results

3.1 Sample Preparation

Before pooling the adaptor-ligated and indexed sequencing libraries, the sucess of library preparation is validated using the Agilent Bioanalyzer instrument. 2a and 2b show representative electropherograms of a sample that has been processed using both kits. The expected DNA products should be detected at 175-600 bp for Haloplex CSC and 200-400 for TST15.



(a) Agilent Haloplex CSC (High Sensitivity DNA Chip)



(b) Illumina TST15 (MixA) (DNA 1000 DNA Chip)

Figure 2: Electropherograms of representative sequencing libraries prepared by Agilent Haloplex ClearSeq Cancer and Illumina TruSight Tumor 15. (*) represents the lower marker, (**) represents the upper marker

Using the blablabla software, the concentration, molarity and total peak area (TPA) of the expected sequencing libraries were calculated.

Maybe: relationship between dCt and TPA?

Maybe: the enrichment of one or the other kit is more affected by bad quality samples

3.2 NGS Data Quality

Sequencing run parameters were calculated by the Illumina Sequencing Viewer software. Table XXX shows the averaged run parameters of runs with Haloplex CSC and TST15 sample preparation.

TST15 has a higher cluster density and therefore a higher total yield, but has lower reads passing a phred-score threshold of Q30 than Haloplex. This is due to the different chemistries used. TST15 uses v3 chemistry, while Haloplex uses v2. v2 generally has lower cluster density and output, but

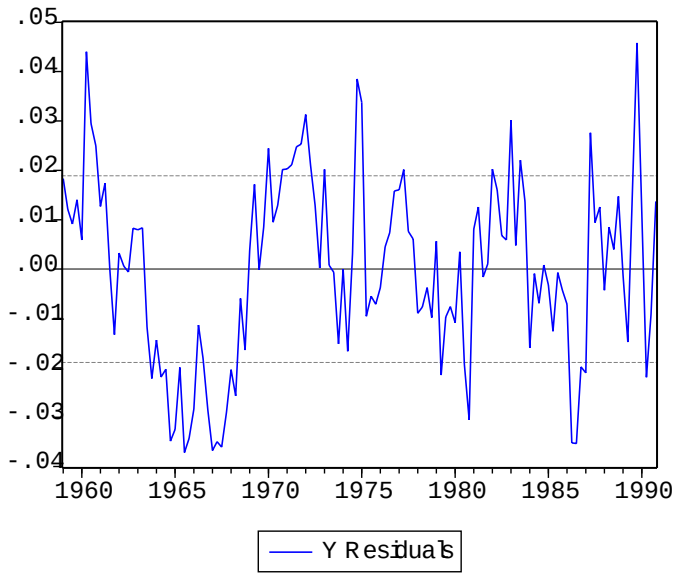
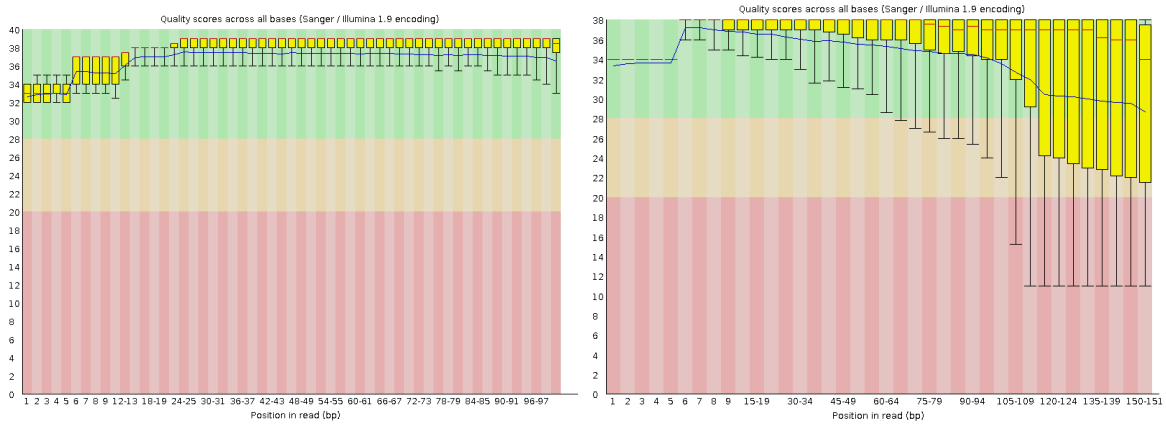


Figure 3: Scatter plot of the corrected peak area (X axis) of the regions corresponding to the sequencing libraries defined in the blabla software and the dCt (Y axis). Agilent Haloplex ClearSeq Cancer data are represented as blue dots, Illumina TruSight Tumor 15 data are represented as red dots.

Table 1: Comparison of Run Parameters (Averaged) of Sequencing Runs with Haloplex CSC & TST15 Sample Preparation

Parameter	Halo CSC	TST15
Yield total (Gb)	3.7	7.37
% >Q30	93.8	82.355
Cluster Density PF (k/mm2)	1084	1180
Cluster Density PF (%)	85.95	79.95



(a) Agilent Haloplex ClearSeq Cancer

(b) Illumina TruSight Tumor 15

Figure 4: Comparison of coverage distributions per amplicon as reported by FastQC

therefore better quality.

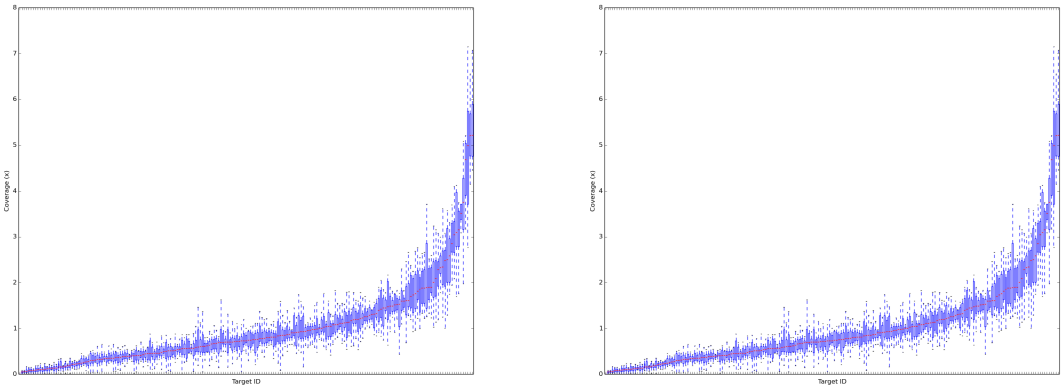
Table XXX shows the boxplot representations of the read qualities per position of two representative FASTQ files as reported by FastQC. Both workflows yield high quality data, yet Haloplex CSC data have more narrow distributions and are of higher quality. This is in direct relationship with the sequencing chemistry kit used.

Table 2: Blablabla

Parameter	Haloplex SureCall A	Haloplex SureCall B	Haloplex Velona	TST15 BaseSpace	TST15 Velona
% mapped	–	–	–	62.6	–
% paired	–	–	–	58.7	–
% singletons	–	–	–	3.8	–

The Samtools Flagstat command was used to determine some basic BAM statistics of BAM files of samples prepared with the respective library preparations and processed with the mentioned bioinformatic pipelines. **??** shows the averaged result of these statistics.

Considering the recommended pipelines, Haloplex CSC data, analyzed with Agilent's SureCall software, has a higher percentage (91%) of mapped reads when compared to Illumina's BaseSpace TruSight Tumor 15 App (62.9%). Data analysis with the recommended SureCall design includes a steps where mates are fixed, but they are not stitched together. Therefore no reads are considered as being paired. The TST15 app in contrast includes a read stitching step and 58% are considered as properly paired. This means that of the 62.6% of mapped reads, 4.2% are not properly paired.



(a) Agilent Haloplex ClearSeq Cancer

(b) Illumina TruSight Tumor 15

Figure 5: Comparison of Coverage Distributions per Amplicon

3.8% of reads processed with the TST15 online App are considered to be singletons, whereas only 1.8% of reads processed with the SureCall software are considered as singletons.

3.3 Coverage Analysis

Coverage Distribution TST15 vs Haloplex

Coverage Distribution per Patient (check if correlation IQR with dCt)

Coverage Distribution per Amplicon (check if some have always lower coverage, check if some failed)

Failed Amplicon Counter

Table 3: Blablabla

Amplicon	1x	50x	250x	500x	1000x
ATM_14	12	0	0	0	0
MAP2K1_2	1	0	0	1	4
ATM_5	0	1	1	0	2
ATM_11	0	1	1	2	3
PTEN_1	0	1	1	0	2
KIT_6	0	1	0	1	2
FGFR3_1	0	0	1	2	2

Table 4: Blablabla

Amplicon	1x	50x	250x	500x	1000x
1METxxxE16TF031SR031	0	11	0	1	3
2KITxxxE09TF003SR003	0	5	6	0	0
2KITxxxE09TF003SR003	0	5	6	0	0
27qxxxxExxTF034SR034	0	0	2	4	5
17qxxxxExxTF018SR018	0	1	1	4	4
1KRASxxE04TF002SR002	0	1	1	3	6
1TP53xxE02TF034SR034	0	0	3	5	9
1EGFRxxE19TF032SR032	0	0	1	5	5
1EGFRxxE21TF035SR035	0	0	1	2	2
2TP53xxE02TF033SR033	0	0	0	1	3

Table XXX and table XXX show how often a given amplicon failed a given coverage threshold. The number of failed amplicons is low for Haloplex CSC as wel las for TST15. Most amplicons were amplified efficiently in most samples, which is also confirmed by figure XXX (amplicon distributions). Some amplicons however fail coverage thresholds in several samples.

- Amplicon ATM_14 in Haloplex CSC was never amplified. This amplicon is defined in the BED file but obviously is not part of the kit. (negative control?)
- Amplicons ATM_5, ATM_10, MAP2K1, PTEN_1, KIT_6 and FGFR3_1 in Haloplex CSC data failed a coverage threshold of 1000x in several samples
- In TST15 data, more amplicons fail the respective thresholds. Several amplicons in genes MET, KIT, TP53, KRAS and EGFR fail the 1000x coverage threshold, and often even the required threshold of 500x, which is required by the TruSight Tumor 15 App.

The fact that several amplicons in the genes EGFR and KRAS in TST15 data repetitively fail the required coverage thresholds is problematic. This is especially the case for amplicon 1EGFRxxE21TF035SR035 as it includes the well-known EGFR L858R variant, which confers increases sensitivity for EGFR tyrosine kinase inhibitors.

Fragmentation ¡-¿ Coverage?

(GATK CallableLoci) (GATK CountLoci???) (GATK FindCoveredIntervals)

On-off target; Enrichment Efficiency TST15 vs Haloplex

Coverage across genome, check where there is coverage

Strandedness?

GATK DepthOfCoverage???

3.4 Variant Calling Algorithm Comparison

3.4.1 Detection of Known Single Nucleotide Variants and Deletions

Table XXX shows known variants in the analyzed samples and the variant frequency reported by the recommended pipelines. There is a high concordance between the results of both kits and previously known variants.

TST15 could not detect EGFR del19 in patient F due to low coverage. The corresponding region was inspected in IGV and the deletion was present. The amplicon was not amplified efficiently and has only a coverage of 167x. The TST15 BaseSpace App however applies a coverage threshold at 500x. Variants with a lower depth are not reported. The same sample was sequenced twice and the deletion was never detected. This is probably due to the fragmentation induced by the FFPE fixation.

Haloplex CSC did not find KRAS p.Gly12Val in patient G, also due to low coverage for this amplicon. The corresponding region was inspected in IGV: the region has a coverage of only 180x. Only one read showed the expected C¿A variant. Sample G is also the sample with the worst dCt (2.85). The hig fragmentation in this sample is probably responsible for the bad amplification. The same sample will be re-sequenced in a later run to check is the problem is related to the fragmentation of the sample or if library preparation was bad.

Additional previously unknown variants were found (TODO: put a table into the appendix)

3.4.2 Sensitivity Analysis

BRAF Mut and WT samples from Horizon were analyzed. The BRAF Mut sample was sequenced purely, as well as in a 1/3 and 2/3 dilution with the BRAF WT sample.

The observed variant frequencies as detected by the TST15 BaseSpace App are in line with the expected variant frequencies. D816V variant in cKIT could not be observed as the position of this

Table 5: Blablabla

Sample	Context	Tissue	Known variant	Halo CSC Freq (%)	TST15 Freq (%)
A	NSCLC	FFPE	EGFR L858R	24.7	21.4
B	NSCLC	FFPE	EGFR L858R	24.3	13.2
C	NSCLC	FFPE	EGFR L858R	32.7	29.8
D	Melanoma	FFPE	BRAF V600E	44.4	47.6
E	Melanoma	FFPE	BRAF V600E	18.4	21.4
F	NSCLC	FFPE	EGFR del19	not found	50
G	mCRC	FFPE	KRAS CD 12_13	p.Gly12Val (3.4)	not found
H	mCRC	FFPE	KRAS CD 12_13	p.Gly12Asp (37.4)	G12D (31.4)
I	mCRC	FFPE	KRAS CD 12_13	p.Gly13Asp (9.7)	G13D (8.7)
J	mCRC	FFPE	NRAS p.Gly12Asp	25.9	27.2
K	Melanoma	FFPE	BRAF V600E	66.2	59.5
L	mCRC	FFPE	NRAS p.Gly13Val	6.6	5
M	mCRC	FFPE	KRAS and NRAS WT	WT	WT
N	mCRC	FFPE	KRAS and NRAS WT	WT	WT
O	Melanoma	FFPE	BRAF V600E	37.4	

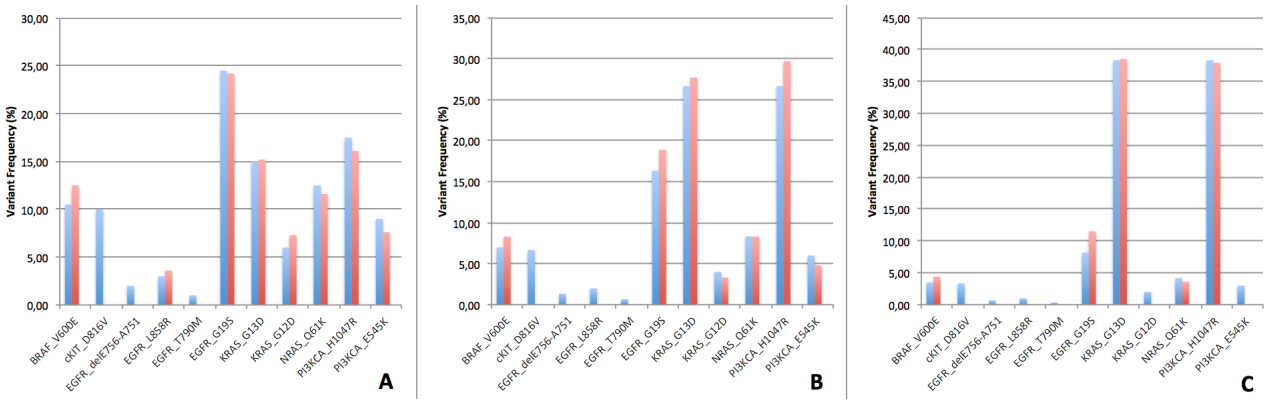


Figure 6: Blablabla

Table 6: Blablabla

Gene	Variant	Exp 100%	Obs Halo CSC	Obs TST 15	Exp at 66%	Obs Halo CSC	Obs TST15	Exp at 33%	Obs Halo CSC	Obs TST15
BRAF	V600E	10.5	–	12.5	7	–	8.3	3.5	–	4.4
cKIT	D816V	10	–	–	6.67	–	–	3.33	–	–
EGFR	delE756-A751	2	–	–	1.33	–	–	0.67	–	–
EGFR	L858R	3	–	3.6	2	–	–	1	–	–
EGFR	T790M	1	–	–	0.67	–	–	0.33	–	–
EGFR	G719S	24.5	–	24.2	16.33	–	18.9	8.17	–	11.5
KRAS	G13D	15	–	15.2	26.67	–	27.7	38.33	–	38.5
KRAS	G12D	6	–	7.3	4	–	3.3	2	–	–
NRAS	Q61K	12.5	–	11.6	8.33	–	8.3	4.17	–	3.6
PIK3CA	H1047R	17.5	–	16.1	26.67	–	29.7	38.33	–	37.9
PIK3CA	E545K	9	–	7.6	6	–	4.8	3	–	–

variant is not covered by the TST15 kit.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3986649/

TODO: do the same with Haloplex CSC

TODO: call variants with MuTect 1.1.7, VarScan 2, GATK HaplotypeCaller, SomVarIUS?, Freebayes?, Vardict??????? TODO: compare results

Among the variants detected by MuTect, 40–50 % are C¿T variants. This has been reported in several studies. TODO: do this for all samples, check if this is really statistically significant or only happened in a few samples

Tools that may be of use somehow: GATK SelectVariants; GATK VariantFiltration; GATK VariantEval; GATK ValidateVariants

4 Conclusions

References

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