Master's Thesis

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

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by

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Abstract

Contents

1 Introduction

Cancer represents a huge burden for health care systems worldwide and is one of the leading death causes. Scientific discoveries in the last decade have had an enormous impact on our understanding of the underlying causes of cancer. The development of omics techniques, in combination with advanced computational power, has lead to an explosion of biological data. It has become clear that cancer is an incredibly complex malignancy, which is affected by genetic, environmental and behavioural factors. The research community is trying to interprete this vast amount of data with the goal to get a deeper understanding of cancer and to cure it eventually. In recent years, several drugs have been approved, which target proteins needed for cancer development, proliferation or metastasis. Molecular testing is employed to check whether these targeted drugs would be of benefit. In that regard, Next-Generation Sequencing (NGS) is an interesting method to gain deep insights into the genetic information of a tumor and to guide personalized therapy.

1.1 The cancer genome

DNA undergoes continuous damage. In normal cells, this damage is repaired without without errors. In cancer cells, the equilibrium between DNA damage and repair systems is dysbalanced [1], leading to a mutator phenotype. The resulting genomic instability manifests itself in an accumulation of mutations.

The genetic diversity caused by this instability, the cardinal feature of cancer, in combination with several environmental factors, such as inflammation, enables the hallmarks of cancer [2]. These include replicative immortality, cell death resistance, ongoing proliferative signaling, invasion and metastasis, growth suppressor evasion, inducement of angiogenesis, energy metabolism reprogramming and immune destruction evasion.

1.1.1 Cancer: an evolutionary process

Cancer progression is a process that recognizes basic Darwinian evolution principles [3] [4] [5] [6]. Similarly as proposed in Darwins origins of species, cancer development and progression is based on two distinct processes. First, the population of cells has to harbor heritable genetic variation. These mutations may be of germline origin or may occur through somatic processes. If the occurring mutations are non–deleterious, they can be passed on to the next generation of cells. The second process, which has to take place in Darwinian evolution is natural selection. Each cell exhibits a

unique combination of genetic and environmental perturbations. Cells are in competition for a variety of resources in their microenvironment, which include space, oxygen and nutrients. Eventually, cells with the best fitness, e.g. with the highest proliferative potential and the lowest death rate, are then selected through natural selection principles. These cells will outlast less fit cells. This results in sequential waves of clonal expansion [3], leading to different subclones within the same tumor that differ in their proliferative, migrative and invasive potential.

1.1.2 Accumulation of somatic mutations

It is widely accepted that tumors accumulate somatic mutations during their progression in malignancy [8] [9]. Somatic mutations can have distinct origins [10]. DNA can be damaged by endogenous and environmental agents. Carcinogenic substances produced by industry [11] [12] or present in tobacco smoke [13] are known to increase cancer risk. Cellular metabolic processes also produce DNA-damaging products that induce cancer, such as reactive oxygen species [14] [15]. Several cellular DNA repair systems have emerged. DNA lesions can escape these repair mechanisms if the damage happens in an inaccessible region of the DNA or if the DNA repair system is defective [16]. Also, the repair systems cannot cope with the rate of mutation if the frequency at which they occur becomes to important. These DNA lesions, if not repaired, then induce errors in the replication by DNA polymerases.

The process of DNA replication is not free from errors. It has been estimated that DNA polymerase has error rates ranging from 10^{-4} to 10^{-6}. This is followed by mismatch repair, which corrects 90-99% of the replication errors, decreasing the overall error rate to 10^{-6} to 10^{-8} [10]. Additionally, several DNA polymerases exist, which differ in their error rates, which can be used interchangeabilly. DNA polymerase β has a much worse error rate than DNA polymerase δ or ϵ. There is evidence that that DNA β is increased in some tumors [17], resulting in increased mutagenesis.

Additionally, processes affecting chromosomal and microsatellite integrity instability contribute to genomic instability in cancer cells. **Chromosomal instability (CIN)** is the most common kind of instability in solid tumors [18]. Chromosome missegregation plays a crucial role in cancer adaptation [19]. Defects in proteins needed for chromosome segregation lead to chromosome missegregation. This leads to telomere dysfunction, faulty sister chromatid cohesion, loss of heterozygosity (LOH), hypo– or hyperactive spindle assembly checkpoint or defective centrosome duplication and aneuploidy. [18]. About 70% of solid tumors are aneuploid [22]. Another chromosomal instability process has been described recently: chromothripsis happens when chromosomes are fragmented [20] [21]

[21]. The cell tries to repair the chromosomes, but this process is far from being perfect, leading to massive chromosomal rearrangements. The question whether CIN is a cause or consequence of tumor development remains unanswered.

Microsatellite instability (MSI) is a phenotype caused by inactivation or loss of DNA mismatch repair [23]. Microsatellites are short DNA segments with tandem repeats. Microsatellite elongation or shortening is a consequence of defective or inactive DNA mismatch repair (MMR), which corrects base replication errors. Seven enzymes contribute to the MMR system (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, PMS2) [18]. Germline mutations in MMR genes cause the Lynch syndrome (hereditary nonpolypsos colorectal cancer) [25]. Patients have an 80% lifetime risk to develop colon cancer. Germline LOH of one allele with somatic inactivation on the other allele or double allelic inactivation by somatic mutations of these genes can cause MSI. The most common reason for MMR inactivation is through methylation of the promoter of the MLH1 gene [26]. DNA polymerase has a higher error rate in repetitive regions. When MMR genes are inactivated or defective, the replication mistakes in microsatellites cannot be corrected: MSI is the consequence. In some cancers, MSI can occur despite functional MMR through frameshift mutations at microsatellites. MSI is often associated with cancers harboring mutations in TGFβRII, EGFR, PTEN, and BAX, which contain such simple repeats [27].

Epigenetic changes

Blablabla

1.1.3 Driver and passenger mutations

Genomic instability in cancerous cells becomes a critical mechanism if it affects oncogenes or tumor suppressor genes, which have the potential to be causative tumor 'driver' mutations. These driver mutations are positively selected during cancer progression and confer a growth advantage to the cells harboring them. Many alterations found in cancer cells are passenger mutations, which occur subsequently or coincidentally to driver mutations. These mutations are defined to not contribute to the selective fitness of the cell, even though this conception has been challenged by stochastic tumor progression simulations [33]. Some studies have reported that cancer cells carry 40–80 somatic mutations, and only 5–15 of them are driver mutations [28].

Estimating the number of somatic driver and passenger mutations and the rate at which they occur is not well established [29]. Two tumors, even though histologically indistinguishable, might present different subsets of mutations [29] [30]. This observation has been defined as inter-tumor

heterogeneity. Additionally, tumors present heterogeneity at the intra-tumor level [31]. Subclones of the tumor might present different mutations.

As mentioned, cancer progression is an evolutionary process. Chemotherapy creates a selective environment [32]. Initially, patients often respond to the therapy, but might then become resistant to the treatment. This is due to intra-tumor heterogeneity: a subclone of the tumor might have acquired a driver mutation that confers resistance to the treatment. Chemotherapy might kill a large part of the tumor cells, but actively selects for this resistant clone. Eventually, this clone will be the origin of relapses and another treatment option is lost.

The identification of driver mutations has been a central aim of cancer research. Of the 20,000 protein coding genes, mutations in at least 350 human genes are found recursively in cancer genomes and are believed to contribute to cancerogenesis [34]. Studies in mice have demonstrated that mutations in a total of 2,000 genes might contribute to cancer development and progression. According to their functional importance, these genes can be divided into two classes: tumor suppressor genes and oncogenes.

Tumor suppressors genes protect a cell from entering the path to cancer. They comprise genes encoding for cell adhesion proteins, DNA repair proteins, proteins acting in apoptosis pathways, or cell cycle proteins. The action of these proteins inhibits metastasis, excessive cell survival or proliferation. Tumor suppressors mostly follow the two-hit hypothesis, which was first proposed by Knudson for the retinoblastoma protein (pRb): to inactivate the tumor-protecting role of tumor suppressors, two genetic events, often LOH in combination with silencing point mutations or silencing of both alleles by somatic events, are necessary to inactivate both alleles of the gene. Another possibility of tumor suppressor inactivation is methylation of the gene promoter. Compared to dominant oncogenes, tumor suppressor genes are often considered to be recessive. Alternatively, tumor progression can be influenced by functional haploinsufficiency of tumor suppressors. According to this conception, a disease state can emerge if a cell / organism has only one functional copy of a given gene and if it cannot produce enough of a gene product to establish a wild-type condition. APC, TP53 and the TGF-b pathway are amongst the most known tumor suppressors.

Adenomatous Polyposis Coli (APC) is a protein, which has binding sites for microtubules, cytoskeletal regulator proteins and Wnt signaling proteins (β–catenin, axin). Wnt signaling regulates cell migration, polarity, differentiation, adhesion and apoptosis. In the canonical Wnt signaling pathway, a destruction complex, including APC, leads to β–catenin phosphorylation, followed by ubiquitination, marking it for degradation in the proteasome. Once Wnt binds to the N-terminus of an

activated surface receptor of the Frizzled family and a co-receptor of the LRP5/6 family, the destruction complex is inhibited. Consequently, β–catenin is no longer marked for degradation and can translocate to the nucleus, where it acts on gene expression of target genes. Loss or dysfunction of APC leads to β–catenin accumulation in the nucleus even in the absence of an extracellular stimulus.

TP53 is one of the master guardians of the genome. In normal situations, p53, the protein encoded by TP53, is targeted for ubiquitination and degradation in the proteasome. In case of cellular stress, p53 is no longer ubiquitinated. p53 can then stop the cell cycle at the G1/S and G2/M transitions, induce DNA repair, and induce apoptosis if the damage cannot be repaired. TP53 thereby maintains genomic stability. The importance of TP53 as tumor suppressor gene becomes evident in the autosomal dominant Li–Fraumeni syndrome. People suffering from this disorder inherit only one functional copy of TP53 and are likely to develop cancer in early ages. One mechanism by which p53 acts on cell-cycle arrest is by activating expression of p21. p21 binds to the G1/S transition complex and inhibits its activity, leading to cell-cycle arrest. Inactivation or mutation of TP53 is a crucial step in many cancers.

Oncogenes comprise several GTPases, transcription factors, receptor tyrosine kinases and growth factors. Overexpressed or overactive versions of these proteins lead to increased mitogenic signals, causing increased cell growth or proliferation. Mutations in proto–oncogenes can cause a loss of regulation or overactive proteins. Gene duplications or other chromosomal alterations lead to increased protein synthesis. Other mechanisms of importance include post-transcriptional mechanisms as misregulation of protein expression or increase of mRNA / protein stability. Two important oncogenic pathways include the RAS–RAF–MAPK and PTEN–PI3K–AKT pathways.

Signaling through the PI3K–AKT pathway leads to cell growth, proliferation and survival. The signaling cascade is initiated by integrins, cytokine receptors, T and B cell receptors, G–protein coupled receptors receptor tyrosine kinases, such as the Epithelial Growth Factor (EGFR). Ligand binding results in production of PIP3 (phosphatidylinositol–(3,4,5)) by activation of PI3K (phosphoinositide–3–kinase). PIP3 is anchored in the cell membrane and acts as docking site for proteins containing PH domains (pleckstrin– homology), such as PDK1. PIP3-bound PDK1 partially activates Akt by phosphorylation of its Thr308. Full activation of Akt is enabled by phosphorylation pf PDK1 at Ser473 by mTORC2. Activated Akt then acts on a variety of proteins necessary for protein synthesis, glucose metabolism, cell survival / death and proliferation. The phosphatases PP2A and PHLPP can dephosphorylate and thereby inactivate Akt. Additionally, PTEN dephosphorylates PIP3 and indi-

rectly also inactivates Akt. Dysregulation of the PI3K–AKT has been associated with several human diseases including neurological diseases, diabetes and cancer. In cancer, inactivation of PTEN and kinase activity activating mutations on PI3K and Akt are found recursively, leading to enhanced signaling, leading to inhibition of apoptosis and increased proliferation.

In the RAS–RAF–MEK–ERK pathway, ligand binding on cell surface receptor tyrosine kinases activates the receptor. One of these receptors is the EGFR. EGFR is a protein of the tyrosine kinase receptor family. It is anchored in the cytoplasmic membrane and is composed of an intracytoplasmic tyrosine kinase domain, a short hydrophobic transmembrane domain and an extracellular ligand-binding domain. Ligand binding causes a conformational change of the receptor, which leads to homo– or heterodimerization, followed by 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. GRB2 binds to Tyr1068 of EGFR through its SH2 domain and recruits SOS, a guanine nucleotide exchange factor. Grb2 and SOS then form a complex with the activated EGFR, which activates SOS. Activated SOS promotes recruition of Ras proteins to the activated EGFR. Through its GEF activity, SOS then induces GDP removal from Ras proteins, which can subsequently bind GTP and become active. Ras then recuits Raf proteins to the cell membrane and binds to their N-terminus. The activation of Raf, serine/threonine kinase proteins, is complex. In fact, Raf proteins are considered as gatekeepers of the RAS–RAF–MAPK pathway. In its inactive form, Raf is present in a 'closed' conformation, in which an autoinhibitory domain blocks the catalytic kinase domain. Recruitment to the cell membrane of Raf by Ras results in a conformational change, which disrupts the autoinhibitory interaction of Raf. Rafs then form homo– or heterodimers, which leads to partial activation by allostery. Transphorylative events, with optional phosphorylation by other kinases, such as PAK1, then fully activates Raf. Activated Raf can now bind to MEKS, which are tyrosine/threonine kinases. MEKs then phosphorylate ERKSs, which are also serine/threonine kinase enzymes. ERKs then translocate to the cell nucleus, where they influence expression of target genes. RAS–RAF–MEK–ERK signaling promotes cell-cycle progression, cell differentiation, growth and survival.

1.2 Targeting the EGFR signaling pathway

1.2.1 Molecular Profiling of Solid Tumors

Lung cancer is the most common cancer worldwide, both in terms of new cases (1.8 million) and deaths (1.6 million). Smoking is a widely accepted risk factor, as chemical carcinogens in tobacco

smoke induce several genetic mutations. Classic diagnosis and treatment decisions have relied on histological analysis of the tumor. Lung cancer can be divided into two histological subtypes: small-cell lung cancer (SCLL) and non-small cell lung cancer (NSCLC). Over the last decade, it has become clear that these subtypes can be classified into additional classes by the mutational status of recurrent driver mutations in genes that are frequently mutated in this type of cancer. Mutations in several oncogenes can be found at each stage of NSCLC and in all histological types, e.g. large cell carcinoma, adenocarcinoma, squamous cell carcinoma (SCC), in smokers and never-smokers as well.

A combination of oncogenic triggers cause cells of the normal bronchial epithelium to proliferate, giving rise to meta–, hyper– and dysplastic epithelial lesions. Genomic events in early stages of lung cancer giving rise to atypical adenomatous hperplasia include LOH of 3p (80%), $p16^{INK4a}$ (70%) or RB inactivation (15%), as well as mutations in KRAS (15–25%) or in β–catenin (10%). TP53 inactivation (50%) and LOH at 13q are believed to favor progression into the primary adenocarcinoma stage. After that stage, major chromosomic instability is often detected, giving rise to metastatic adenocarcinoma. These chromosomic events include LOH of 2q (70%), 9p (80%), 18q (85%), and 22q (75%). Additionally, the oncogene c-myc is amplified in 10%.

Frequent mutations in NSCLC, which are of potential interest in a targeted anti-tumor chemotherapy, affect EGFR (10–35%), KRAS (15–25%), PTEN (4–8%), HER2 (2–4%), DDR2 (4%), PIK3CA (1–3%), BRAF (1–3%), AKT1 (1%), MEK (1%) and NRAS (1%). Additionally, rearrangement of ALK (3–7%), RET (1%) and ROS1 (1%) and amplifications of FGFR1 (20%) and MET (2–4%) are found recursively. These mutations are rarely observed together in the same tumor.

Melanoma develops from the malignant transformation of melanocytes in the basal epidermal layer of the skin. Melanoma incidence has exploded over the last four decades, with a 15-fold increase in the United States. Both genetic predisposition and environmental factors influence the risk of getting melanoma. Skin cancer often affects fair-skinned individuals. Exposure to UV light, immunosuppression and multiple nevi are risk factors. UV radiation causes cyclobutane pyrimidine dimers (CPDs). By joining adjacent pyrimidine bases, T–T, C–C or C–T dimers (UV fingerprints) are formed, leading to direct DNA damage. People diagnosed with rare genetic disorders like xeroderma pigmentosum are at great risk. Traditionally, melanoma has been classified based on histological and pathological properties, such as the thickness of the tumor, ulceration or the anatomic location of the tumor.

Several mutations in oncogenes are recurrently found in melanoma, but are rarely found together in the same tumor. The occurrence of the different mutations differs by the anatomic location

of the tumor, e.g. whether the specific body part is chronically exposed to the sun. Mutations frequently found in melanoma occur on BRAF (37–50%), NRAS (13–25%), MEK (6–7%), NF1 (11.9%), CTNNB1 (2-4%), GNAQ (1.3%) and GNA11 (1.2%).

Colorectal cancer (CRC) 1.4 million cases are detected yearly with 694,000 deaths. CRC is one of the best studied cancers. The development of colorectal adenocarcinomas occurs over many years. Caused by the acquisition and accumulation of driver mutations, a normal colorectal epithelium can progress to adenoma, which develops into carcinoma, which can eventually mestastasize.

Mutations recursively detected in CRC occur on KRAS (36–40%), SMAD4 (10–35%), PIK3CA (10–30%), BRAF (8–15%), PTEN (5–14%), NRAS (1–6%), and AKT1 (1–6%).

The mentioned molecular progression profiles are likely to be an oversimplification. Due to tumor heterogeneity, these alterations do not always have to be observed in the tumor, and the chronological appearance of these alterations may vary from one tumor to another.

1.2.2 Biological Role of EGFR in Solid Tumors

EGFR expression and mutations

Cell survival enabled by EGFR

Cell death induced by EGFR blockade

1.2.3 EGFR-targeted drugs

1.2.4 Predictive markers

1.3 Tumor DNA Sequencing

1.3.1 Targeted NGS

Target enrichment methods

1.3.2 Practical implications in the laboratory

1.4 Aims of the Thesis

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