Cancer in the light of evolution – from cancer genomes towards novel treatment and prevention approaches

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Abstract

Cancer is one of the deadliest diseases and a major health burden for mankind. In the past, our conceptual understanding of cancer has largely been determined by the medical need to classify distinct stages of the disease to select the most appropriate therapy. This view created only a very limited understanding of the true processes governing the disease, as it only perceives a snapshot at the time point of operation or the initial therapy. Cancer, though, is the result of a long-lasting evolutionary process following exponential expansion rules. It usually takes years or even decades until an initially hidden transformed cell in one of our organs is being noticed through symptoms as a clinically detectable lesion. During this time of inapparency, multiple genomic alterations accumulate from generation to generation of replicating cells, that are all governed by the nature of the initial molecular hit that was responsible to push normal somatic cell into the state of a true cancer precursor. To develop better tools to diagnose, treat and prevent cancer, it becomes more and more clear that is essential to understand the dynamic genomic processes that allow pre-cancer cells to expand and continuously change their biological behavior. Recent advances in DNA sequencing techniques allowed to obtain an unprecedented amount of high-resolution data on genomic changes in cancer cells. This increasing knowledge

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helps to reconstruct the genomic history of individual cancer cells. However, it also indicates that pre-cancerous lesions in all of us seem to be substantially more common than previously anticipated, suggesting that we are well armed with a broad range of genetic and immunological weapons to control or defeat the vast majority of initial cancer cells clones. We will discuss the current understanding of these evolutionary processes using the paradigmatic example of DNA mismatch repair-deficient, microsatellite-unstable cancer. We will outline how a better understanding of cancer evolution can guide the development of tailored tumor diagnostics and treatments and retain the clues to effective cancer prevention.

1 General considerations – cancer as a genetic disease

The evolution of life is driven by replication of genomes, and by genetic variation during replication. Whereas increased cell division and proliferation capacity represents an evolutionary advantage in unicellular organisms, the evolution of multi-cellular organisms required coordination and governance mechanisms to control and limit the proliferative activity of individual cells within an organism. Cell replication and continuous renewal of tissues has emerged as a powerful concept of maintaining the homeostasis of multicellular organisms; however, the price is the risk of developing cancer. Therefore, increasingly potent and multi-layered tumor-suppressive mechanisms evolved in parallel with the increasing complexity and size of multicellular organisms (1).

Similar to the evolution of organisms within a population and a given ecosystem, tumors are the result of continuous evolution of somatic cells within an organism. The principle of variability and selection postulated by Charles Darwin (2) shapes tumor development at all stages from initial pre-cancerous stages up to late stages of invasive cancer, metastasis formation and development of resistance under systemic treatment.

Paramount to the understanding of cancer as the result of an evolutionary process is the concept that cancer is caused by alterations of the genome. In pioneering work of 1914, Theodor Boveri proposed abnormal chromosomal aggregation as a cause of cancer (3). Following this theory and the experimental observations by others, Karl Heinrich Bauer, later Head of the Surgery Clinic at Heidelberg University and one of the founders of the DKFZ, in 1928 published a seminal book titled “Mutationstheorie der Geschwulst-Entstehung—Übergang von Körperzellen

in Geschwulstzellen durch Gen-Änderungen (Mutation Theory of the Origin of Tumors—Transition of Somatic Cells into Tumor Cells by Gene Changes)" (4). In his publication, Bauer provides profound considerations on genome changes on the cancer cell phenotype and clinical implications such as invasive growth and metastasis. Together with other landmark publications (5) outlining the impact of genome-damaging agents on cancer risk, the foundation for understanding cancer as a disease of the genome was laid, long before the molecular structure of DNA and the sequence of specific genes were discovered (6).

With new information from epidemiological, cell biology, and gene sequencing studies becoming available, the multistep hypothesis of cancer was more widely accepted and acquired an increasingly well-defined shape (7). In 1990, Fearon and Vogelstein proposed a genetic model for colorectal tumorigenesis (8). This model has been the paradigm of genetic changes promoting the development of colorectal cancer; moreover, it has described the multi-step nature of solid tumor development in an exemplary way. The concept that solid tumor development is the result of stepwise progression from normal cells over pre-cancerous cells towards invasive cancer, a process driven by defined genetic alterations, has been summarized in the "Three Strikes" hypothesis proposed by Vogelstein in 2015 (9).

Although this model is of tremendous importance for understanding the genetic alterations giving rise to human cancers, it only develops its full power if it is embedded in a broader evolutionary context, accounting for the fact that most, i.e. almost all, precancerous cells acquiring somatic mutations are prone to cell death or senescence and will never grow out to manifest cancers. A schematic illustration of cancer evolution as a process of random mutation and subsequent selection is provided in Figure 1.

2 Molecular classification of cancers

Therapy selection for cancer patients has been based historically entirely on the organ, from which a tumor originated, the tumor stage, i.e. its local and systemic spread, and tumor histology as seen in light microscopy. Although still the majority of therapeutic decision making is based on this type of information, molecular information has become increasingly important for the selection of appropriate tumor therapies.

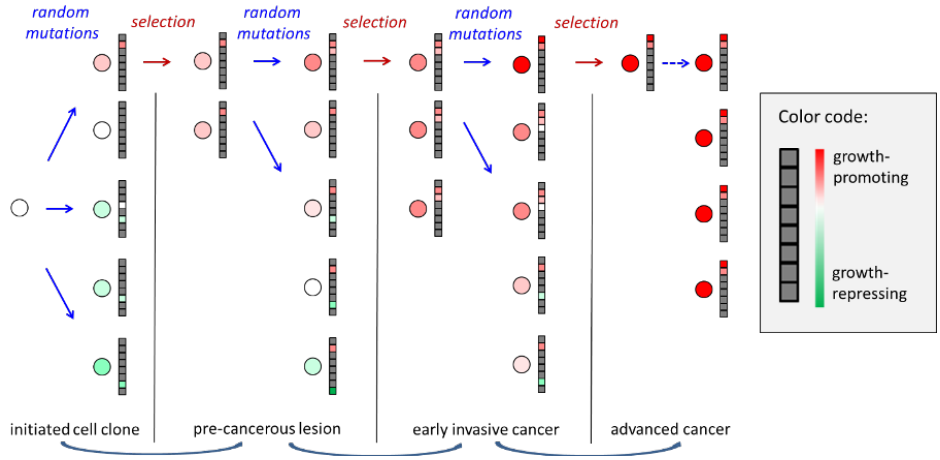

Figure 1: Schematic illustration of cancer evolution. Cancer evolution is a stepwise process that through the accumulation of somatic mutations leads to the malignant transformation of normal body cells (white circle on the right). This process, however, is not sequential and directed, but determined by the principles of random mutations and the subsequent selection of cell clones equipped to survive and grow out in the given environment. In contrast, after an initiation event, which crucially determines the subsequent evolutionary process (for example deficiency of the DNA mismatch repair system), random mutations occur and enhance the diversity in the cell pool, generating cells with enhanced (red) or reduced (green) proliferative capacity. Subsequently, diversity is reduced by selection, and survival of cells with high proliferative capacity is favored. For most cancer types, at least three crucial mutation events (driver mutations) are required to cause malignant transformation. The model implies that the majority of cells derived from the initiated cell will die and not grow out to the cancerous stage, because they acquired the wrong type or combinations of mutations. Conversely, cell clones in manifest cancers share characteristic combinations of mutations that occur significantly more frequently than expected by chance. The prevalence of a certain mutation in a group of manifest cancers is therefore a simple, but very helpful criterion for predicting its functional significance.

Therapeutic decisions based on molecular information were initially restricted to a few advanced stage cancers and a very limited number of specific gene mutations with well-characterized functional consequence. For example, the admission of targeted therapies using antibodies specifically blocking the epidermal growth factor receptor (EGFR) in colorectal cancer by the European Agency for the Evaluation of Medicinal Products (EMEA) (since 2009 European Medicines Agency, EMA) required the molecular determination of the presence or absence of a specific mutation activating the *RAS* (Rat sarcoma) oncogenes. The human

RAS gene family encompasses the *KRAS*, *NRAS*, and *HRAS* genes; activating mutations, which affect very specific positions of these genes, lead to a constitutive activation of the growth-promoting pathways that are usually triggered by binding of EGFR to activating ligands. Therefore, antibody blockade of the EGFR protein is ineffective in *RAS*-mutant tumors (10).

Similar to *RAS* mutation status, mutations of the *Breast cancer type 1* or *2* genes (*BRCA1* or *BRCA2*), which both code for repair enzymes involved in homologous recombination, are clinically relevant therapy predictors. Because homologous recombination is required for repairing double strand breaks in DNA, platinum salt-based chemotherapeutics that introduce such breaks, are effective in *BRCA1/2*-mutated cancers. Moreover, *BRCA1/2* mutations predict the effectiveness of novel tumor treatment modalities targeting the Poly-ADP-ribose polymerase (PARP inhibitors). PARP inhibitors impair tumor cells' capability of repairing treatment-induced DNA damage; therefore, they show activity in cells with homologous recombination deficiency such as *BRCA1/2*-mutant cancer cells (11).

The two examples of *RAS* mutations (as negative predictors) and *BRCA1/2* mutations (as positive predictors) illustrate a general principle, more and more molecular classifiers are used to guide clinical decision making in oncology. Interestingly, molecular characteristics such as *BRCA1/2* mutations often predict therapy response irrespective of the organ, from which the cancer has developed, and hence have significantly re-shaped the process of clinical decision making in oncology.

However, technically, these molecular tumor classifications are still end point classifications not directly accounting for the evolutionary history of a cancer. Nevertheless, they opened the path toward a profound reconsideration of the ways that can be used to classify tumors for clinical decision making: Although every individual cancer has a mutational landscape of its own, reflecting the randomness of single mutational events, computational neural network-based approaches were able to identify a few distinct common characteristics typical of certain evolutionary processes influencing tumor development, i.e. their *mutational signatures*.

3 Understanding cancer as the result of an evolutionary process – Mutational signatures

Mutational signatures mark a milestone on the road from an organ-based, phenotypical tumor classification to a more profound and comprehensive understanding of cancer as a dynamic process (12,13). This conceptual change represents a major paradigm shift, potentially comparable to the introduction of Darwinian evolution, which replaced the perception of species as discontinuous, constant entities by a non-discontinuous model of evolution, enabling the concept of phylogenesis.

A mutational signature is defined as a characteristic combination of specific types of mutations, which are associated with specific mutagenic processes underlying cancer formation. Such processes can be, as we will discuss in more depth, deficiencies of DNA repair systems or exogenous factors that cause certain types of DNA damage, e.g. UV light. For example, it has been known long before whole-genome sequencing techniques became available that certain tumor types were enriched for certain mutational base exchanges in the DNA: Smoking-induced lung cancer typically shows C/G to A/T transversions, whereas C/G to T/A transitions are commonly found in UV light-related skin tumors (14,15). The earliest alterations of a somatic cell's genome, if causing a deficiency of the machineries physiologically ensuring the fidelity of DNA replication, hence can define the mutational signature of the later evolving cancer cell clone. Its distinct molecular nature decides how the genome of this cell is being altered during the carcinogenic process and it critically also defines how this cell population will respond to individual therapy strategies.

For example, a deficiency of BRCA1/2, often observed in breast, prostate and ovarian cancers, leads to typical alterations, favoring certain types of basepair exchanges. Using computational approaches for detecting signatures of BRCA1/2 deficiency, recently, a classifier was developed that with high accuracy was able to identify tumors as *BRCA1/2*-mutant without having knowledge on the actual *BRCA1/2* mutational status (16). Notably, a substantial proportion of tumors, though not harboring detectable *BRCA1/2* mutations, showed mutational signatures indicative of BRCA1/2 deficiency and responded to platinum salt-based chemotherapy and PARP inhibitors, just as expected for *BRCA1/2*-mutant tumors.

This observation clearly indicates the power and superiority of using molecular processes of tumor evolution rather than actual snapshot mutational data for tumor classification. In terminology, the paradigm shift from mutational classification

to an evolution-based classification is represented by the introduction of the term "BRCAness", which instead of referring to the presence or absence of *BRCA1/2* mutations underlines that *BRCA1/2*-related homologous recombination was impaired during tumor evolution, leading to the mutational signature of BRCA1/2 deficiency.

Mutational signatures as fingerprints of evolutionary forces at work during tumorigenesis not only help in therapeutic decisions and drug selection in oncology. They also can be extremely helpful in the detective work, which is often required for the clarification of tumor etiology and the identification of cancers that arise in the context of a hereditary syndrome. Certain, otherwise rare base exchanges found in a tumor cell's genome can suggest possible culprits, for example deficiency of the base excision repair system, thus guiding human genetics diagnostic approaches.

4 Mismatch repair deficiency

The strength of using mutational signatures or, in general, tumor evolution-related parameters for therapy selection instead of anatomical tumor location or the mere presence of a certain mutation within a tumor, is strongly underlined by the great success of introducing DNA mismatch repair deficiency as a predictor of immune therapy response.

The DNA mismatch repair (MMR) machinery represents a very important system for maintaining genome integrity during the division of somatic cells. Therefore, MMR enzymes are highly conserved across different species. Tumors that develop as a consequence of MMR deficiency accumulate tremendous amounts of somatic mutations (17). The phenotype of MMR deficiency has first been detected by chance in 1993. When actually searching for larger chromosomal alterations in tumors, Ionov and coworkers noticed that some human tumors showed an unexpected pattern; instead of chromosomal losses or deletions of larger parts of genomic material, these tumors presented with additional, aberrant genome fragments indicating a previously unknown type of genomic instability in human cancer (18).

The high mutational load commonly found in MMR-deficient tumors causes a high load of mutational antigens. These antigens are highly immunogenic, due to mechanisms we will discuss in more detail below, and can therefore readily

be recognized by the host's immune system. Therefore, MMR-deficient cancer patients show pronounced responses towards treatment with immune checkpoint modulators (19). Immune checkpoints physiologically represent "brakes" that prevent overshooting immune responses and autoimmune reactions detrimental for the host. In the scenario of cancer, however, immune checkpoints can trigger tolerance of the immune system towards otherwise highly immunogenic tumors such as MMR-deficient cancers. Here, the application of antibodies targeting immune checkpoints can reactivate the host's immune response against the tumor and lead to immune-mediated tumor cell killing. This approach is only effective if a tumor presents sufficient amounts of sufficiently immunogenic antigens to the immune system.

Notably, a substantial proportion of patients with MMR-deficient cancers resistant to all other available treatment modalities such as chemotherapy, radiation, or oncogene-targeted therapies, showed pronounced tumor shrinkage and sometimes even complete tumor elimination upon immune checkpoint blockade. The overwhelming clinical success of immune checkpoint blockade led to the FDA approval of immune checkpoint-blocking antibodies for the treatment of metastasized MMR-deficient cancer. Interestingly, this was the first admission of an oncological drug entirely based on a molecular classification, accounting only for molecular mechanisms at play during tumor evolution, but irrespective of the tissue origin.

The 2018 Nobel Prize in Physiology or Medicine has been awarded jointly to James P. Allison and Tasuku Honjo, who pioneered research on immune checkpoints and identified two major targets of current immune checkpoint therapies, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell death protein 1) (20–22).

Notably, the remarkable success of immune checkpoint blockade has only become possible in an era that has already started embracing principles of tumor evolution for clinical decision making.

In the following, we will focus on mismatch repair-deficient cancers to highlight general principles, driving forces, and clinical implications of the evolutionary processes responsible for cancer formation.

5 Mismatch repair-deficient cancers – a model of tumor evolution

The human genome consists of more than 3.2 billion base pairs, every single one of which theoretically can be affected by multiple types of alterations, including point mutations, large chromosomal rearrangements, and epigenetic changes. In order to understand key principles of tumor evolution and identify specific forces that shape tumors over time, it is helpful to reduce complexity by focusing on certain tumor types with defined mechanisms of genomic instability.

Mismatch repair-deficient cancers represent a highly valuable model in this regard. They are driven mainly by one specific type of somatic alteration that affects the genome, namely single base pair insertion or deletion mutations (indels). MMR deficiency-related indel mutations affect a limited number of well-defined regions of the human genome, short repetitive sequences termed "microsatellites". In the scientific literature, MMR-deficient tumors are therefore also referred to as microsatellite-unstable (MSI) tumors, although the two terms are not exactly synonymous, the former referring to the forces shaping the evolutionary process and the latter to the resulting phenotype (17).

The likelihood of acquiring a mutation during the division of an MMR-deficient cell is elevated up to 100 times or more compared to other tumor cells. MMR-deficient tumors therefore not only represent a tumorigenesis model with a very well-defined type of somatic mutations, but also an extremely high mutational load, exposing the process of tumor evolution, in a figurative sense, under a magnifying glass.

6 Random mutations and selection in MMR-deficient cancers

As discussed above, the major type of mutation favored by MMR deficiency are indels, single nucleotide insertions or deletions. Indel mutations can affect all microsatellite sequences in the human genome. Most microsatellites are located in non-coding regions, and mutations affecting such non-coding microsatellites are mostly expected to have little or no effect on the phenotype or fitness of the cell. However, protein-encoding regions, which only represent about 1.5% of the entire human genome, are not entirely free from repetitive sequences. In fact, more than 1000 human genes encompass a repetitive microsatellite sequence of 8 base pairs or longer (for example an "AAAAAAAA" or A_8 sequence in the gene ACVR2A, or an "AAAAAAAAAA" or A_{10} sequence in the gene TGFBR2) (23).

Whenever indel mutations strike at such coding microsatellite sequences, the effects on the protein level can be dramatic. The insertion or deletion of a single base pair can disrupt the translation of a gene into a protein by shifting the translational reading frame and disrupting the functionality of the protein product.

Our group and others have studied hundreds of microsatellites in thousands of MMR-deficient cancers. When we look at all microsatellites in the human genome to identify parameters that can predict the likelihood, with which a certain microsatellite displays a mutation in MMR-deficient cancers, a clear pattern emerges: the longer a microsatellite, i.e. the more repetitive units it contains (for example 10 in *TGFBR2* and 8 in *ACVR2A*), the higher its average mutation frequency in MMR-deficient tumors (24). This correlation can be approximated by a sigmoid function. It is rooted in the fact that with increasing numbers of repetitive units the correct replication gets increasingly difficult for the enzymes involved in DNA replication.

The sigmoid function describing the correlation between microsatellite length and microsatellite mutation frequency sets a very useful baseline for studying MMR-deficient tumor evolution, as it provides information about average microsatellite mutation frequencies depending on microsatellite length. For example, a microsatellite consisting of 8 basepairs, such as the repeat in the coding region of the *ACVR2A* gene, has a predicted mutation frequency in MMR-deficient colorectal cancer of 9.7% (www.seltarbase.org) (25). In other words, if we examine 100 MMR-deficient tumors for mutations at a randomly selected microsatellite of 8 basepairs length, we expect a total of approximately 10 mutations.

The wealth of microsatellite mutation data collected for all kinds of microsatellites in the human genome does not only inform us about average mutation frequencies, it also provides a range of expected mutation frequencies. For example, 95% of microsatellites consisting of 8 basepairs have a mutation frequency lower than 30.5% in MMR-deficient colorectal cancer. Considering these numbers, how can a microsatellite such as the A8 repeat in the *ACVR2A* gene display an actual mutation frequency of more than 80%, according to some studies even more than 90%, “much higher than expected”?

Although limited information is available about the influence of certain other parameters such as the sequence context, in which the microsatellite appears, there is clear evidence that the observed mutation frequencies in MMR-deficient cancers mainly reflect one factor: selection during tumor evolution. We have good reasons

to assume that microsatellite length determines the likelihood of an indel mutation to form during the division of an MMR-deficient cell. However, microsatellite length is completely unrelated to the likelihood, with which a cell affected by the respective mutation will survive. A microsatellite mutation that inactivates a gene essential for cell survival (illustrated as green boxes in Figure 1) will induce cell death in mutant cells and therefore disappear from the pool of precancerous cells. On the other hand, a microsatellite mutation inactivating a gene coding for a growth-suppressive protein (red boxes in Figure 1) may enable survival and even favor proliferation of the affected cell, ultimately providing it with the means to overgrow other competing precancerous cell clones.

If this hypothesis is true one would expect a significant enrichment of microsatellite mutations inactivating growth-suppressive genes in MMR-deficient cancers. The genome-wide maps of microsatellite mutations faithfully follow the prediction of random mutations (determined by microsatellite length) and consecutive selection, showing microsatellites located in tumor suppressor genes significantly enriched among those with high observed mutation frequencies (overrepresentation of red boxes in early invasive and advanced cancers, Figure 1) (26). Taking a closer look, commonly detected microsatellite mutations can be made responsible for the functional breakdown of a broad spectrum of genes, indicating how a single mutational mechanism enabled by the deficiency of the DNA MMR system provides the framework for the step-wise acquisition of all the hallmarks required for a cell to become a transformed malignant tumor cell (27).

7 The high immunogenicity of MMR-deficient tumors

The history of cancer immunology in the modern era even goes back to the late 19th century, when the American surgeon William B. Coley observed several cases of end-stage tumor patients who showed dramatic improvement of their tumor condition upon severe bacterial infection (28). Similar to complete remission of some MMR-deficient tumor patients upon immune checkpoint blockade, Coley observed complete tumor elimination in a patient with recurrent and inoperable sarcoma shortly after contracting erysipelas, a severe bacterial infection affecting the skin and the underlying lymphatic tissue. He later developed a mixture of killed bacteria, “Coley’s toxin”, to treat patients with malignant tumors. Similar clinical histories were reported by the German physicians Friedrich Fehleisen and

Wilhelm Busch who independently from each other reported significant tumor shrinkage in patients suffering from erysipelas (29,30).

These clinical observations very early indicated that the immune system in principle is capable of targeting tumor cells and, under certain conditions, even eliminating them. However, anti-tumor immune responses and the success of modern-day immune therapy, seemed to be restricted to certain tumor types.

As mentioned above, patients with MMR-deficient cancers respond very well to treatment with immune checkpoint inhibitors. Pronounced local immune responses were among the first characteristics that were noted when this new molecular subtype of human cancer was discovered (31). In MMR-deficient cancers, we often encounter extreme amounts of lymphocytes, immune cells of the adaptive immune system, which are trained to recognize and fight specific structures that indicate a danger to the integrity of the organism. The phenotypical observation of massive lymphocyte infiltration in MMR-deficient cancers suggests that the immune system reacts particularly strongly against emerging MMR-deficient cell clones during their evolution. It has long been known that the strength of the host's local immune response influences prognosis, the more lymphocytes at the tumor site, the longer the patients' average survival time (32). The pronounced immune response of the host represents the most likely reason for the comparatively good prognosis associated with MMR-deficient cancers (33).

Why are particularly MMR-deficient cancers such an attractive target for the immune system? Why would immune cells be more capable of recognizing and attacking tumors that exhibit MMR deficiency than other tumors?

The answer is only partly related to the high number of mutations accumulating during the evolution of MMR-deficient cancers. More importantly, the type of mutation plays a key role: Indel mutations responsible for the malignant transformation of MMR-deficient cells are frameshift mutations, this means that the deletion or insertion of a single base pair within a gene causes the wrong translation of the entire part of the gene that follows this mutation Figure 2. Whereas point mutations, such as the ones activating the *RAS* oncogenes described above, generally only lead to one wrong amino acid, indel mutations in MMR-deficient cancers can trigger the generation of long non-functional protein stretches that are completely unknown to the host's immune system. In this sense, immune responses against frameshift peptides (also termed "neo"-peptides) can mimic immune

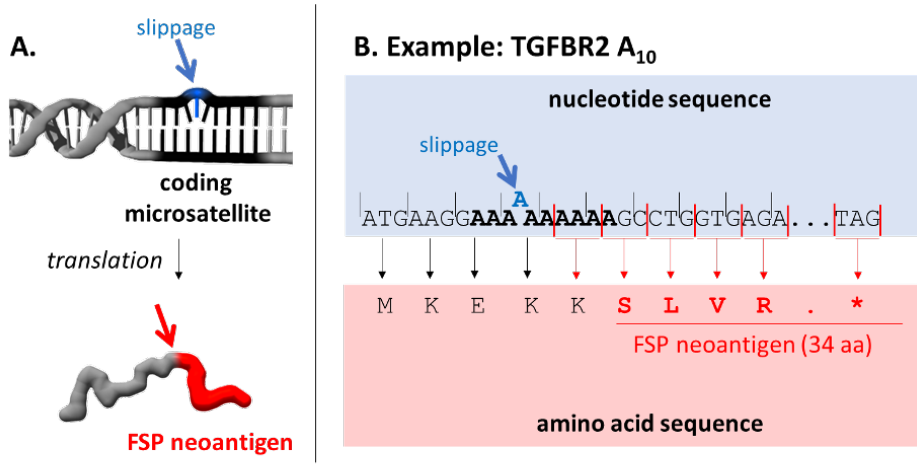

Figure 2: Mutations give rise to antigens that can be detected by the immune system. A. In MMR-deficient cells, indel mutations (i.e. losses or gains of single nucleotides, blue arrow) occur at repetitive microsatellite sequences located in genes that code for proteins. The translation of mutant nucleotide sequences results in the generation of a translational frameshift and protein sequences encompassing entirely novel amino acid stretches downstream of the mutation (red lines framing the shifted, novel nucleotide triplets in **B**). Key to this process is that the alteration of a single nucleotide, by causing a frameshift, affects the translation of the entire protein area that follows the mutation (red letters in **B**). Thus, a small alteration on the nucleotide level (blue) has big effects on the protein level (red) and for the immunological visibility of tumor cells.

responses directed against pathogen-related peptides, as there is no tolerance of the immune system.

In a simplified picture, MMR-deficient cancers are composed of cells, which are full of non-self proteins that are exposed to the immune system. As the capacity of the human immune system to recognize tumor cells as foreign increases with the number of antigenic proteins present in tumor cells, MMR deficiency with the dramatically increased frequency of indel mutations and the resulting translational frameshifts are a prototype of immunogenic tumors.

8 Immune surveillance and immunoediting

Patients with MMR-deficient cancer show immune responses against frameshift peptides generated by tumor cells. Our group could demonstrate that lymphocytes specifically recognizing these neo-peptide targets were present not only in the peripheral blood of these patients, but also locally, infiltrating the tumor (34).

This raises a critical question: How can MMR-deficient cancers survive and even prosper in the presence of potentially hostile immune cells?

In principle, two explanations are possible: On the one hand, the immune system may transiently or permanently lose the ability of controlling the outgrowth of MMR-deficient cancer cells, either by exhaustion or by suppression of immune cell functionality. On the other hand, tumor cells may become invisible to the immune system by "hiding" immunogenic antigens to immune cells. Molecular studies of manifest MMR-deficient cancers indicate that the latter mechanism appears to be more frequent, as impairments of the cellular antigen presentation machinery can be found in up to 70% of MMR-deficient cancers, at least those originating in the colorectum.

Physiologically, all nucleated cells of the body continuously present their spectrum of intracellular proteins to the immune system. This process is mediated by the major histocompatibility complexes (MHC), which in humans are also referred to as human leukocyte antigens (HLA). The HLA system serves several purposes, including identifying a cell as "self", i.e. part of the organism. This is achieved by a high diversity of HLA genes between and among human populations. HLA diversity induces a high barrier between individuals; therefore, malignant tumor cells are not infectious and will not grow out to manifest lesions even if they are transferred to another host.

In addition to allowing for distinguishing self and foreign cells, HLA molecules also present intracellular protein fragments to circulating immune cells. This feature of the adaptive immune system, which apparently has evolved even before the separation of jawed and jawless vertebrates, approximately 500 million years ago (35), ensures that information about alterations of the intracellular protein content, which can indicate imminent danger e.g. in the context of a viral infection, is communicated to the immune system.

Similar to the situation in virally infected somatic cells, emerging tumor cell clones may accumulate non-self proteins that may be visible to the immune system upon HLA-mediated antigen presentation. Although rarely as prominent as in

MMR-deficient cancer, immune surveillance plays an important role in protecting humans from the development of many different types of tumors. The process of immune-mediated elimination of pre-cancerous cell clones and the outgrowth of cell clones capable of immune escape is often referred to as "immunoediting" (36).

The high frameshift mutation load and immunogenicity of MMR-deficient cell clones poses a serious threat for MMR-deficient cells to be eliminated by immune cells. Therefore, manifest MMR-deficient cancers show a wide spectrum of alterations impairing or completely abolishing the function of the physiological antigen presentation machinery.

The antigen presentation component most commonly inactivated by mutation in MMR-deficient cancer is *Beta2-microglobulin* (*B2M*), an essential part of the most important cellular HLA complex ("HLA class I"), if no B2M protein is present, HLA class I breaks down, and no antigens can be presented to the immune system.

Again, as almost all alterations shaping the phenotype of MMR-deficient cancers, MMR deficiency is the enabling mechanism: the *B2M* gene encompasses four short microsatellite sequences (a (CT)4 dinucleotide repeat in exon 1 and three five base pair repeats in exon 2), which represent mutational hot spots in MMR-deficient cancer (37). Using our model to estimate the average likelihood of mutations at a microsatellite of five base pairs length, we receive a prediction of 0.37%. In reality, however, more than 25% of MMR-deficient cancers harbor such hot spot mutations in *B2M*, underlining the massive positive selection pressure supporting the outgrowth of precancerous cell clones that have acquired one of these rare mutations.

The power of this selection pressure is supported by two additional observations: First, *B2M* mutations and other alterations that cause a breakdown of antigen presentation are mutually exclusive. This indicates that *B2M*-mutant cell clones only outcompete other cell clones if other clones still have an intact antigen presentation machinery and may therefore face the risk of elimination (38). Second, *B2M* mutations are significantly associated with the strength of the host's local immune response; if local infiltration with activating immune cells is absent, no *B2M* mutations are detected in MMR-deficient cancers (39).

The example of *B2M* mutations offers another insight into fundamental principles of tumor evolution: Surprisingly, patients affected by *B2M*-mutant cancers have a very good prognosis and long survival. In fact, B2M-mutant tumors very rarely

metastasize, so they can in almost all cases be cured by surgery. This first seems counterintuitive, regarding the fact that *B2M* mutations abrogate HLA-mediated presentation of tumor antigens to the immune system and thereby stops potential tumor cell elimination by lymphocytes, the main effector cells of the adaptive immune system.

What could be the reasons for an improved survival of patients with *B2M*-mutant cancer? Although data are not yet definitive, it seems reasonable to assume that *B2M* mutation-triggered loss of HLA molecules on the cell surface deprives tumor cells of their ability to identify themselves as "self", a process for which HLA molecules are essential. Loss of HLA on the surface may expose *B2M*-mutant tumor cells towards natural killer cells, effectors of the innate immune system, the most ancient defense system of multicellular organisms.

In an oversimplified analogy, emerging MMR-deficient cancer cells may face a situation resembling Odysseus between Scylla and Charybdis: either they fall prey to effectors of the adaptive immune system and die, or they through random mutation of *B2M* obtain the chance of sailing closer to Scylla, sacrificing the potential of metastatic spread by becoming targets of the innate immune system, but surviving for the time being.

The example of *B2M* mutations in MSI cancer that at the same time enable local tumor outgrowth and inhibit metastasis formation illustrates a principle common to the evolution of tumors and the evolution of species. Evolutionary adaptation allowing survival or increasing fitness under certain conditions may be unfavorable under other conditions or become unfavorable when environmental conditions change. This further extends the analogy between tumor and species evolution, underlining that tumor evolution may require massive adaptation under sudden environmental changes, including outgrowth in a completely different tissue context in the process of metastasis formation or when systemic cancer treatment is applied, often resulting in the generation of new tumor phenotypes mimicking new speciation (40).

9 Transmissible tumors – a special case of tumor evolution

Transmissible tumors represent a highly interesting group of malignant lesions that open a window into processes of cancer evolution and immune evasion that remain closed in clinically diagnosed human tumors. In contrast to virally induced

tumors, such as those caused by human papillomaviruses, transmissible tumors are caused by transmission of entire tumor cells from one host to another.

Conventionally, cancer cells are depending on the survival of the host, and the host's death marks an evolutionary dead-end. As described above, the diversity of MHC molecules between individuals of a species represent a strong inter-individual barrier that tumor cells cannot cross.

On some rare occasion, however, tumor cells have been documented to be transmitted from the original host to a recipient organism. Such clonally transmissible tumors are extremely rare in the human population. Tumor transmission in humans requires severe impairment of the immune system of the recipient organism, for example in immunodeficient individuals or after organ transplantation (41).

In animals, two examples of transmissible tumors have gained broader attention, the so-called "Devil facial tumour disease" (DFTD) in Tasmanian devils, and a transmissible venereal tumor (TVT, also termed Sticker sarcoma) in dogs. As immunosuppression is obviously not the reason for inter-individual spread of these tumors, the question is what is?

In Tasmanian devils', the genetic diversity is very low, probably due to rapidly declining population sizes during the last century (42). A low genetic diversity, accompanied by low diversity of the MHC gene loci, naturally favors transmission of tumor cells from one affected animal to another. On top of that, Tasmanian devils are involved in fighting, during which they bite each other, thereby supporting the spread of cells; in fact, aggressive male devils are more affected by transmissible tumors.

Obviously, low population diversity cannot explain the astonishing spread of TVT, a canine transmissible veneral tumor that affects dogs all over the world. However, recent genomic sequencing studies of more than 500 TVT lesions sampled over the whole world have revealed highly interesting facts about the early years of TVT evolution. At the time of its origination in the "founder dog", presumably between 4000 and 8500 years ago in Asia, the originating population was characterized by a very low genetic diversity, similar to the situation in Tasmanian devils. The fingerprint of this low genetic diversity, which has been conserved over millennia, is a low degree of heterozygosity at single nucleotide polymorphisms, indicating that the founder dog was considerably inbred (43).

According to genomic data, spreading of TVT around the world started around 2000 years ago, so at least hundreds of years after its origination. Hence, we can

assume that TVT could evolve over a long time span in the context of a largely inbred population. During this time, critical mutations could emerge that provided tumor cells with the prerequisites to survive under more "hostile" conditions in genetically further remote hosts, thus enabling world-wide propagation of TVT.

Notably, transmissible tumors in Tasmanian devils and canines, despite their ability to jump the barrier between individuals, both up to today have retained intact MHC genes. Instead, they downregulate MHC expression to very low levels. Assuming that there would have been ample time to irreversibly get rid of MHC molecules by random mutation, the retention of MHC further underlines that a complete loss of MHC is disadvantageous to tumor cells as exemplified by *B2M*-mutant MMR-deficient cancer, thus supporting the analogy to Scylla and Charybdis discussed above.

Due to the long history of transmissible tumors that in the case of TVT can be traced back thousands of years, these tumors represent a fascinating treasure chest that we have only started to explore. Current TVT tumors carry the memory of mutational signatures and processes at work during the past millennia. For example, mutations compatible with UV light signatures were found to be strongly associated with the country of sampling, showing an enrichment in areas with high sun exposure such as Mauritius (43). Notably, a previously unknown mutational signature ("signature A", characterized by CC>TC mutations) was identified that dominated the early evolution of TVT, an observation that may coincide with the accumulation of similar C>T mutations in the human germline postulated for a similar time span (44). Possibly, environmental factors that later vanished left their fingerprints in the human germline and in the mutational profile of TVT. This example demonstrates that mutational signatures and transmissible tumors hold great potential, providing a genomic analog to stratigraphy in archeology.

Some recent speculative publications take the idea of tumor cells abandoning the host and developing a life on their own even further. The SCANDAL ("speciated by cancer development animals") hypothesis postulates that some simple animals may have originated from tumors that had developed in more complex organisms (45). Conclusive proof for this intriguing idea is outstanding, although it may be in line with observations about the biochemistry and pathophysiology of tumor cells, such as their dramatically increased glucose consumption even under aerobic conditions (Warburg effect (46)) and the sensitivity of some tumor cells to antibiotic substances (47).

In comparison with transmissible tumors, the life span of "conventional", non-transmissible tumors is limited. This is reflected by the fact that the number of somatic mutations of TVT is more than hundred times higher than the average mutation frequency in human cancer. MMR-deficient tumors represent an exception among non-transmissible tumors, as the extremely high rate of mutations during their "accelerated" evolution compensates for the comparatively short life span. This accelerated evolution has direct implications on the clinical and histopathology presentation of these tumors, which will be discussed below.

10 Heterogeneity of MMR-deficient cancers

Histopathology classification is usually performed to inform clinical decision making and to estimate the prognosis of patients affected by cancer. In colorectal cancer, routine histopathology classification implies determining the differentiation state of the tumor, i.e. their "distance" from normal colon epithelial tissue, and describing the phenotype of cell growth, for example using categories such as "medullar" for tumors growing as a solid, cell-rich mass, or "mucinous" for tumors accumulating extracellular mucus. Typically, tumors diagnosed in pathology are characterized homogeneously by a dominant histopathology differentiation, i.e. they are, following this example, either medullar or mucinous.

However, one characteristic feature of MMR-deficient cancer is the simultaneous presence of several distinct histopathology differentiation patterns within the same tumor (48). This intra-tumoral phenotypic diversity reflects mutational diversity, which itself is a direct consequence of the high mutation likelihood under MMR-deficient conditions Figure 3.

Hence, MMR-deficient tumors nicely illustrate through their morphology one of the most striking features of tumors, which result from their evolution: heterogeneity. Most solid human tumors show mutational heterogeneity (49), although rarely as obvious for the naked eye as MMR-deficient cancers. Heterogeneity is one of the most important obstacles for the success of tumor treatment using targeted drugs. Such drugs in theory can attack tumor cells with a high degree of specificity, sparing normal cells that lack the respective target. However, in practice, disease relapses under targeted therapy are very common, because tumor cells either develop resistance after being exposed to treatment, or, considered more likely for most tumors, subclones of the tumor have been resistant upfront before

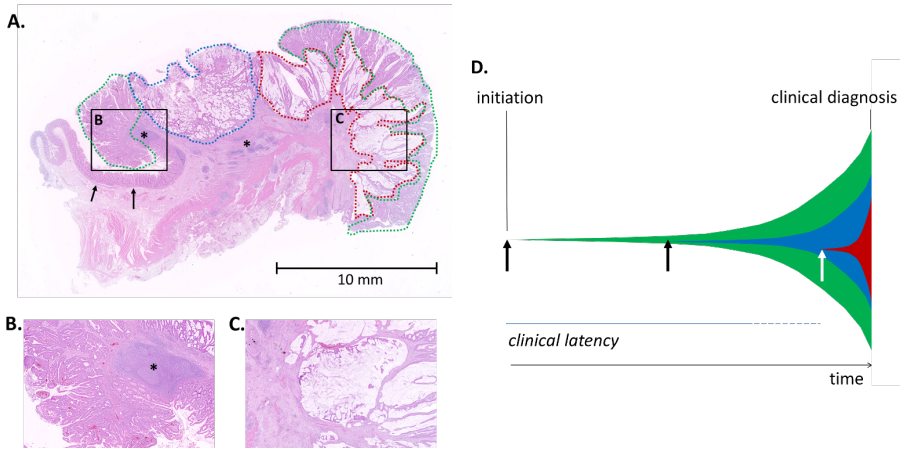

Figure 3: Cancer evolution and histology. A. Overview of a formalin-fixed, paraffin-embedded section (2 μm thickness) through an MMR-deficient colon cancer. The section is stained by hematoxylin (blue) and eosin (pink) to visualize cell nuclei and cytoplasm, respectively. Tumor areas are circled by dashed lines, normal colon mucosa is indicated by arrows. Different colors indicate well-differentiated tumor areas still resembling the normal tissue architecture (*green*, see **B** for higher magnification), and poorly differentiated tumor areas (*blue* – an area with mixed growth patterns encompassing so-called cribriform, i.e. sieve-like, areas; *red* – areas with pronounced extracellular mucus secretion, see **C** for higher magnification). In the center of the section and the transition from normal mucosa to cancer (marked by *asterisks*), signs of a pronounced reaction of the immune system are visible, represented by aggregation of lymphatic cells appearing as small bluish clouds (see also activated lymph follicles in **B**). **D**. Simplified schematic illustration of cancer evolution and its effect on tumor heterogeneity. After an initial transformation event, which enables subsequent steps of tumor evolution, precancerous cell clones may expand slowly, remaining clinically undetectable for a long time. Additional key mutation events (*black and white arrows*) can promote tumor growth by accelerating the proliferative activity of affected cells. Moreover, they can alter their biology and significantly shape the appearance of the manifest tumor in histopathology. In the given example, the clinically manifest tumor after surgical removal will consist of different parts schematically illustrated as *green*, *blue*, and *red*, in analogy to the histopathology section provided in **A.**

treatment. After treatment-induced shrinkage of the tumor mass, such resistant subclones can re-grow to large masses that are then completely therapy-resistant (50).

Compared to targeted therapy, which prolongs survival, but does not cure cancer, immunotherapy, as already reported by Coley, can induce complete and lasting responses in a subset of patients (for exemplary survival curves see (51)). The

direct comparison with oncogene-targeted therapy indicates the benefits of re-activating and supporting the immune system in its fight against cancer cells, using its capacities in tumor control that have evolved and improved in their effectiveness alongside the evolution of the host species.

11 Host factors – towards early steps of tumor formation

Expanding the analogy between the evolution of tumors and the evolution of species, tumors develop within a tissue, which is placed within an individual, similar to the evolution of species in an ecosystem such as an island.

The phenotype of manifest MMR-deficient cancers reflects the influence of the local environment or systemic host factors. MMR-deficient tumors developing in different organs show significant differences of their mutation spectrum. Referring to the examples mentioned above, MMR deficiency-induced mutations inactivating the genes ACVR2A, TGFBR2, and B2M, which are central to the development of MMR-deficient colorectal cancer, are comparatively rare in MMR-deficient endometrial tumors (25).

The reasons for the obvious differences of somatic mutation spectra have not yet been fully figured out. However, growth advantages conferred by somatic mutations depend on the tissue context and the environmental conditions determined by growth factor concentrations, immune cell infiltration and other factors typical of each individual organ site. Other organ-specific differences in the evolutionary process are detectable in the mutational landscape of MMR-deficient tumors: The overall mutation load is higher in MMR-deficient colorectal cancer than in their endometrial counterparts, suggesting that the duration of tumor evolution and the number of cell divisions occurring prior to tumor diagnosis is on average higher in the former.

Reconstructing the time course of tumor evolution by using mutation data as "molecular clocks" (52) provide more precise estimations about the time span of the precancerous evolution, i.e. the phase in which precancerous cells are present, but not yet clinically detectable. For kidney cancer, mathematical modeling based on genome sequencing data revealed the likely presence of hundreds of potential cancer precursor cells in every young adult, although in later life only about 2% of individuals in fact develop cancer, usually with a delay of 20 to 30 years or more (53).

The power of cancer sequence data to inform about the history of an individual cancer sheds light on crucial crossroads, at which key evolutionary milestones were required to further propel tumor growth. From another perspective, we perceive with increasing clarity that cancer development is an exception, and not the cogent result of a sequential progression scenario. This implies that the number of precancerous cell clones that either persist or perish needs to be much higher than the number of manifest tumors. As shown for the example of kidney cancer, it also implies that every human being is host to a multitude of cells with certain genomic changes compatible with our classical concept of cancer. Recent studies using deep sequencing of phenotypically absolutely normal tissue microdissected from different organs including skin, esophagus, endometrium, colorectum and others revealed a surprisingly colorful diversity of mutations, which showed striking resemblance to cancer mutation patterns (54).

Precancerous cell clones also outnumber manifest MMR-deficient cancers by a factor of more than 1000 (55). Here, the observation is visually even more striking, as simple tissue staining techniques used in histopathology can identify potential cancer precursor cells, without the need for sophisticated sequencing data. We will discuss the early pathogenesis of MMR-deficient cancers and the lessons we can learn for cancer prevention in the following.

12 Hereditary cancer and cancer prevention

On some rare occasion, the initial mutation that lays the foundation for later cancer development goes back beyond the birth of an individual. Some individuals inherit cancer-predisposing mutations through the germline and consequently carry them in all body cells. This constellation is called hereditary cancer predisposition.

Hereditary tumor predisposition seems to be responsible for approximately 5 to 10% of the overall tumor burden. The estimation varies depending on the definition; most germline alterations have none or only moderate effects on lifetime cancer risk (polymorphisms or low-risk alleles); others, however, lead to a dramatic increase of the cancer incidence in carriers. The familial adenomatous polyposis (FAP) syndrome is a prominent example for a hereditary cancer syndrome with a tangible and impressive clinical phenotype. Individuals affected by FAP develop hundreds or thousands of precancerous lesions called polyps in the colorectum, and without intervention almost all will develop cancer, often

already in young adulthood. Therefore, preventive surgery with removal of the colorectum is recommended in order to reduce cancer risk. The young age of onset and the severe phenotype is the reason for the relative scarceness of FAP in most populations, as they reduce the likelihood that affected carriers pass on the genetic predisposition to the next generation. This principle leads to an inverse correlation of the frequency and severity of tumor syndromes in the population.

The two most common hereditary cancer predisposition syndromes are hereditary breast and ovarian cancer syndrome (caused by mutations of the *BRCA* genes, see also above) and Lynch syndrome. In Lynch syndrome, named after the visionary pioneer of hereditary cancer research Henry T. Lynch (56), one of four genes coding for proteins (*MLH1, MSH2, MSH6, PMS2*) essential for the functionality of the DNA MMR system is present only as one functional copy or allele. Lynch syndrome mutation carriers have a lifetime risk of developing manifest MMR-deficient cancer between 50 and 80% (57). Current estimations assume that 1/200 to 1/300 individuals carry this genetic predisposition, corresponding to 270,000 to 400,000 people affected in Germany.

The "diploid" nature of our genome is the most powerful factor reducing the likelihood that we develop malignant tumors, as two somatic mutation events are required before a tumor suppressor gene is inactivated ("loss of function"). In Lynch syndrome, one "hit" alone is sufficient for inducing MMR deficiency, because the backup allele is missing in all somatic cells of an affected organism ("two hit hypothesis" formulated by Alfred Knudson (58)). This significantly elevates the lifetime risk for developing MMR-deficient cancer and makes Lynch syndrome a highly valuable condition to test the effectiveness of cancer prevention approaches. Where otherwise huge study populations and observation times would be required to obtain statistically significant results, in Lynch syndrome even moderately effective cancer prevention strategies may show a measurable effect. For example, the cancer-preventive effect of aspirin and other non-steroidal anti-inflammatory drugs could clearly be shown in a limited-size population of Lynch syndrome carriers (59).

Another feature makes Lynch syndrome a prime target for innovative cancer prevention approaches: as outlined above, Darwinian selection during MMR-deficient tumor evolution causes a highly similar landscape of immunogenic antigens (Figures 1 and 2). The rare coincidence of high tumor risk and predictability of tumor antigens provide the setting required to test the feasibility of cancer-preventive

vaccines. We have developed a prototype vaccine consisting of three antigenic peptides occurring in exactly the same form in MMR-deficient cancers; a first clinical trial showed that frameshift peptides can be safely delivered to patients without severe systemic side effects, but consistently inducing immune responses (https://clinicaltrials.gov/ct2/show/NCT01461148) (17).

Future clinical trials have to examine the effectiveness of such a vaccination approach for preventing tumors in Lynch syndrome. The innovative approach of cancer-preventive vaccination holds potential also beyond the scenario of Lynch syndrome and hereditary cancer predisposition. In theory, all tumors are associated with a non-infinite number of antigens resulting from genomic alterations. In addition, the amount of genome-wide sequencing data that help to predict these antigens is rapidly growing. Therefore, there is hope that cancer-preventive vaccines may become tangible in the future.

13 Outlook

Modifying Theodosius Dobzhansky's famous dictum one can state that "nothing in *tumor* biology makes sense except in the light of evolution" (60). New technologies providing genome-wide molecular tumor landscapes have helped to overcome traditional classifications of tumors as distinct, unconnected entities. Breaking this "tyranny of the discontinuous mind" (61), that Richard Dawkins blamed as a major obstacle for scientific progress, and instead perceiving tumors as manifestations of an evolutionary continuum are essential pre-requisites to develop improved concepts for tumor therapy and prevention.

However, this paradigm shift, which has been aided significantly by the advent of affordable tools for comprehensive molecular characterization of tumors, per se does not lead to advances in clinical oncology. Making sense of the massive amounts of data about molecular phenotypes of tumors and their evolutionary history is one of the major challenges for cancer research in the next years. The example of MMR-deficient cancer highlights how classification of tumors according to their evolutionary history can provide significant improvements of patients' outcome. Knowledge about "druggable" mutations, i.e. those that give rise to potential therapeutic targets, will help to select promising treatment approaches based on molecular data. Considering the complexity of data and

clinical trial designs required for obtaining high evidence levels, the translation of such approaches into the clinical routine is certainly no small task.

Reconstructing evolutionary trajectories of tumors should also help to develop more effective prevention strategies. If we understand the key events responsible for transforming normal cells into potentially dangerous precancerous cells and at the same time the phenotype and potential "weakness" of these cell populations, we may be able to design new approaches to reduce tumor incidence. Possibly, being able to shift interventions from advanced stage tumor treatment towards elimination of precancerous cell clones is the biggest promise that understanding the principle of tumor evolution holds.

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