Towards new personalized treatment options for patients with genomically unstable tumors
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General introduction & scope of the thesis

1
GENERAL INTRODUCTION

Genome maintenance

DNA replication and transmission of the genome to daughter cells is an essential part of the life cycle of all living organisms and forms the basis of biological inheritance. Genetic variation normally occurs at a low frequency during replication and transmission of DNA and promotes biological variation and evolution. In contrast, excessive DNA damage can be caused by (inherited) DNA repair defects or by exposure to environmental agents, which impacts DNA integrity and threatens cellular viability\(^1,2\). Genomic instability refers to the phenomenon in which mutations, somatic focal copy number alterations (SCNAs), chromosomal rearrangements and gains or losses of whole chromosomes are continuously acquired, and is causally linked with various diseases, including cancer\(^3\) (Fig. 1). Normal cells are equipped with a number of DNA damage repair mechanisms directed at specific types of DNA damage. The various types of repair mechanisms that protect cells against genomic instability are described below\(^4\).

**Figure 1:** Consequences of loss of genomic integrity. (A) Types of DNA damage that – if not repaired or not accurately repaired- promote genomic instability. (B) Representative normal karyotype and that of a cancer cell. The cancer cell karyotype displays whole genome amplifications (aneuploidy), chromosomal rearrangements and somatic copy number alterations.

Multiple DNA repair pathways

Unrepaired mismatched base-pairs cause mutations due to erroneous or error-prone DNA synthesis. Mismatched base-pairs are detected and repaired during S-phase by mismatch repair (MMR), whereas mutations causing chemically altered bases are repaired throughout the cell cycle by base excision repair (BER)\(^5\). MMR and BER excise the mismatched base or
faulty nucleotide, respectively, both including some surrounding nucleotides and using the complementary DNA strand as a template for repair. More complex lesions such as intra-strand crosslinks and pyrimidine dimers are repaired by nucleotide excision repair (NER). NER functions essentially similar to MMR and BER, only a larger fragment of around 30 nucleotides is excised containing the damaged bases after which the site is repaired using homology-directed repair.

Besides repair pathways that only deal with one of the two strands of the DNA helix, double-strand repair pathways exist that protect cells against aberrant chromosomal rearrangements such as translocations, duplications, inversions and deletions. DNA double strand breaks (DSBs) are considered to be the most threatening type of DNA damage for genomic integrity as both strands are affected simultaneously. Depending on the phase of the cell cycle, DSBs can be repaired by two essentially different mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). When a sister chromatid is available during S and G2-phase of the cell cycle, DNA DSBs can be repaired through HR, whereas NHEJ can be employed regardless of cell cycle phase. The first stage of HR repair entails recognition of the damage by the kinase ATM in conjunction with the Mre11-Rad50-NBS1 (MRN) complex. After localization of the DSB, chromatin modification by the RNF8 and RNF168 ubiquitin ligases mediate recruitment of BRCA1 to the break site in a Ctp-dependent way. Subsequently, the DSB break is end-resected creating single-stranded DNA (ssDNA) which is coated by RPA and activates the ATR branch of the DNA damage response (DDR). The kinase ATR then stimulates the recruitment of a series of proteins including PALB2, BRCA2 and ultimately RAD51, which replaces RPA at ssDNA and facilitates HR repair.

Unlike HR, NHEJ (or classical NHEJ, c-NHEJ) joins DNA ends directly, in a sequence-independent fashion. The core pathway of NHEJ consists of the MRN complex, DNA-PKcs, Ku70 and Ku80, endonucleases and the XLF-XRCC4-LIG4 ligation complex. Together, these components detect, stabilize and repair broken ends directly through ligation. Of note, when classical NHEJ is not possible, alternative non-homologous end joining (alt-NHEJ) can be used, although usage of this pathway leads to increased mutation rates, when compared with classical NHEJ.

Inter-strand crosslinks (ICLs) are toxic lesions that can arise following treatment with chemotherapeutic agents or endogenous toxins (e.g. aldehydes produced by cellular metabolism) and produces covalent adducts between DNA bases on both DNA strands. The formation of an ICL prevents the separation of the covalently linked DNA strands during DNA replication, which can - if left unrepaired - lead to gross-chromosomal aberrations and DNA breaks. Although the exact mechanism of ICL repair is still unclear, the Fanconi anemia (FA) proteins, consisting of a network of more than 20 proteins, mediates the ubiquitylation of FANCD2 with the FANCD2/FANCI complex, which coordinates downstream DNA repair. Incisions around either side of the ICL allow subsequent repair involving interplay of NER, HR and translesion synthesis (TLS) polymerases. These combined DNA repair pathways need to be tightly controlled for ICL removal and subsequent repair.

**DNA damage-induced cell cycle arrest**

In order to provide time for repair of DNA damage, cells can arrest the cell cycle at different points during the cell cycle; at the G1/S-phase transition, during S-phase and at the G2/M-phase transition. DNA damage-induced cell cycle arrest is achieved by blocking the activity of specific Cyclin/Cyclin-dependent kinase (CDKs) complexes that arrest cell cycle progression until DNA damage is repaired. Although the Cyclin/CDK complexes that are targeted in each checkpoint differ, the upstream kinases ATM and ATR are central in relaying the DNA-damage response throughout the cell cycle. In the instance of DNA damage, phosphorylation of downstream kinases CHK1 and CHK2, result in phosphorylation of CDC25 phosphatases and subsequent dephosphorylation of Cyclin/CDK complexes. Dephosphorylation of Cyclin/CDK complexes block their activity resulting in G1/S-phase, S-phase or G2/M-phase arrest. Once DNA damage has been repaired, the activation block on Cyclin/CDK complexes is removed which allows cells to progress through the cell cycle again.

The G1/S checkpoint prevents cells from proceeding to S-phase in the event of DNA damage. Cells are arrested in G1 in the event of a DSB by activation of ATM and subsequent phosphorylation of CHK2. Notably, the G1 checkpoint is heavily dependent on the ATM target p53, which activates...
Chapter 1

a transcriptional program and leads, among others, to inhibition of Cyclin E/CDK2, which is required for maintained cell cycle arrest\textsuperscript{16}. The intra S-phase checkpoint can be activated in the event of replication stress caused by stalled replication forks or stalled repair processes. Persistent replication stress leads to abundant amounts of single-stranded DNA (ssDNA) that are coated by RPA, which in turn activates the checkpoint kinases ATR and CHK1 and inhibits Cyclin A/CDK2\textsuperscript{14,17,18}. Beyond regulating the intra S-phase checkpoint, ATR and CHK1 are also responsible for detecting and arresting the cell-cycle at the G2/M checkpoint to prevent cells with damaged DNA from entering mitosis. ATR inhibits activity of Cyclin B/CDK1 by activation of Wee1, which keeps the inhibitory block on CyclinB/CDK1 until damage is repaired\textsuperscript{16}. Alternatively, when the damage cannot be repaired, cells will be triggered to undergo apoptosis or cellular senescence\textsuperscript{19}. Thus, ATM and ATR are essential components in initiating a DNA repair response as described previously but are also essential components for halting cell cycle progression to allow time for DNA repair.

The spindle assembly checkpoint (SAC) operates fundamentally different than the above-mentioned DNA damage response mechanisms. Yet, also this checkpoint ultimately takes care of the integrity of the genomic content of cells. Whereas DNA damage response pathways detect aberrations of DNA structure, the SAC monitors whether all chromosomes are correctly attached to the mitotic spindle, and thereby prevents gains or losses of whole chromosomes, i.e. aneuploidy. The SAC pathway consists of an effector complex of proteins, the mitotic checkpoint complex (MCC) consisting of CDC20, MAD2, BUBR1 and BUB3. Together, these complex members inhibit the anaphase-promoting complex or cyclosome (APC/C) until all chromosomes are attached to kinetochores. Removal of the inhibitory block on APC/C\textsuperscript{20,21} leads to degradation of downstream targets Cyclin B and Securin. Securin in turn releases separase which triggers the cleavage of cohesion, the protein complex that holds sister chromatids together. The APC/C complex also degrades mitotic cyclins resulting in the inactivation of M-CDK (mitotic cyclin-dependent kinase), promoting mitotic exit and chromosome segregation\textsuperscript{20,21}. Reduced or hyperactive signaling of SAC components can lead to premature anaphase onset before all chromosomes are properly attached to the microtubules, or alternatively, prolonged metaphase arrest which can lead to loss of whole chromosomes\textsuperscript{22}.

In summary, the variety of DNA repair pathways, cell cycle checkpoints and the spindle assembly checkpoint mechanisms together protect the genome against genomic instability. Defects in these genome-protective mechanisms, conversely, cause genomic instability which can be manifested at the resolution of single nucleotides, up until whole chromosome scale, including structural as well as numerical aberrations at the chromosome level\textsuperscript{23}.

\textbf{Consequences of loss of genomic integrity}

When repair factors or cell cycle checkpoint proteins are absent, malfunctioning or hyperactivated, genomic instability arises. For example, absence or malfunctioning of MMR components, can lead to somatic point mutations, deletions or insertions\textsuperscript{5}. This type of genomic instability, coined microsatellite instability (MSI), is characterized by an elevated frequency of short repeated DNA sequences. Since MMR-deficient cells are unable to correct for insertions of short DNA sequences, microsatellite instability is exacerbated\textsuperscript{24}. Individuals with Lynch syndrome, caused by germ-line mutations in genes involved in MMR, have a 90\% lifetime risk to develop colon cancer as a result of impaired MMR\textsuperscript{25}. Besides the notion that defective MMR causes colon cancer, other cancers such as endometrium, ovary, stomach, small intestine, hepatobiliary tract, urinary tract, brain and skin cancers can also be a consequence of defective MMR\textsuperscript{24}.

During replication, cells can encounter replication stress, which refers to the slowing or stalling of replication fork progression. Replication stress can occur when the nucleotide pool is insufficient as a consequence of premature S-phase progression\textsuperscript{17}. Subsequent activation of cell-cycle checkpoints by the DDR delays cell cycle progression and allows time for recovery and repair\textsuperscript{7}. Persistent amounts of replication stress in combination with inactivation of cell-cycle checkpoint kinases, however, can lead to progression of mitosis with under-replicated DNA. Replication forks may no longer be stabilized,
causing forks to collapse into DSBs. In the context of a defective HR pathway, DSBs are repaired by the error-prone non-homologous end joining (NHEJ), which can cause inaccurate ligation of DSBs and thus contribute to the accumulation of structural rearrangements of the genome\textsuperscript{6,7,8,26,27}. The impact of loss of genome maintenance is illustrated in individuals carrying mutations in DNA repair or cell cycle checkpoint genes\textsuperscript{7}. For example, women who carry a germline \textit{BRCA1} or \textit{BRCA2} mutation confer a lifetime risk of developing breast cancer of 69\% to 72\% by 80 years of age. Moreover, the lifetime risk to develop ovarian cancer is also increased in \textit{BRCA1} or \textit{BRCA2} mutation carriers and amounts to 44\% in \textit{BRCA1} mutation carriers and 17\% in \textit{BRCA2} mutation carriers\textsuperscript{28}. Whereas \textit{BRCA1} or \textit{BRCA2} mutations carriers invariably have heterozygous mutations, the tumors that arise have lost the remaining wild-type allele, and consequently these cancer cells have impaired HR repair\textsuperscript{29}.

In addition to an increase in cancer occurrence in cells deficient of DNA repair mechanisms, DNA repair deficiency is also tightly linked to neurodegenerative diseases. Ataxia telangiectasia (A-T) is one of the best studied neurodegenerative diseases caused by DNA damage response defects\textsuperscript{30}. Patients with A-T have germline homozygous mutations in \textit{ATM} and suffer from neurodegeneration, growth retardation, immunological defects and poor coordination\textsuperscript{31}. These symptoms are a consequence of loss of cerebellar and granular neurons in the cerebellum, and can be attributed to DSB processing defects during neural development\textsuperscript{31}. Other proteins involved in ATM activation, such as components of MRN complex which senses DSBs have also been linked to neurological diseases\textsuperscript{31}. Individuals with homozygous \textit{Mre11} mutations show symptoms similar to A-T patients, including neurodegeneration, and hence was called Ataxia Telangiectasia-Like Disorder (ATLD)\textsuperscript{32}. Likewise, germline homozygous hypomorphic mutations in \textit{NBS1} or \textit{RAD50} causes Nijmegen Breakage Syndrome and Nijmegen breakage-Syndrome-like Disease respectively, and is characterized by congenital brain development resulting in microcephaly\textsuperscript{31,32}. The differences in disease outcome when comparing mutation of individual MRN complex members - neurodegeneration versus microcephaly - are remarkable and are still studied. Nevertheless, the spectrum of clinical features shows striking overlap, and matches the biochemical requirement for Mre11, Nbs1 and RAD50 in regulation of ATM activity. Yet, these proteins may not be essential to promote ATM-mediated apoptosis, which may underlie critical differences between these syndromes\textsuperscript{32}.

Defective DNA repair is also linked to premature aging and age-related neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis\textsuperscript{33}. Although elevated levels of DNA lesions and impaired DNA repair mechanisms were observed in Alzheimer’s disease patients, how repair defects exactly underlie age-related neurodegenerative diseases is not yet understood\textsuperscript{32}. Premature aging is commonly observed in diseases affected by mutations in NER such as Cockayne syndrome and xeroderma pigmentosum\textsuperscript{33,34}. Mutations in these NER genes hamper repair of oxidative DNA damage which contributes to premature aging and cancer\textsuperscript{33,34}.

In conclusion, the aforementioned observations underscore the notion that faithful repair is crucial for protecting the genome, and that inactivation of DNA repair genes predisposes individuals to the development of neurodegenerative disease, premature aging and cancer.

Genes involved in the spindle assembly checkpoint (SAC) arrest cell cycle progression to prevent anaphase onset until all chromosome are attached to spindle microtubules\textsuperscript{35}. Mutations that cause a defective spindle checkpoint force cells prematurely into anaphase, even when not all of the chromosomes are attached to spindle microtubules. Consequently, chromosome mis-segregation results in aneuploidy in the daughter cells, a phenomenon also described as chromosomal instability\textsuperscript{35-38}. For instance, mutations in the adenomatous polyposis coli gene (\textit{APC}) (which is distinct from the APC/C complex) causes aneuploidy and an increased risk for developing colorectal cancer\textsuperscript{37,38}. \textit{APC} regulates the Wnt singaling pathway which is responsible for proliferation of intestinal epithelial stem cells. Inactivation of \textit{APC} can lead to continuous Wnt signaling and ultimately unregulated cell proliferation. Additionally, \textit{APC} is also implicated downstream of the SAC by regulating chromosome segregation until all chromosomes have been attached to their spindle microtubules\textsuperscript{40,41}. Inactivation of \textit{APC} reduces the efficiency of correcting erroneous kinetochore-microtubule attachments leading to chromosomal instability\textsuperscript{42}.
The link between chromosomal instability and cancer is clearly demonstrated in individuals with mosaic variegated aneuploidy (MVA), also known as premature chromatid separation syndrome. Aneuploidy is a common feature in individuals with MVA, who suffer from cancer occurrence at an early age. Genetically, germline mutations in the BUBR1 gene underlie the high tumors incidence in MVA patients. The BUBR1 kinase forms an essential mitotic checkpoint component, which halts chromosome segregation until all chromosomes are attached to spindle microtubules. Inactivation of BUBR1 frequently leads to chromosome mis-segregations, causing aneuploidy and thereby promoting cancer.

Mice heterozygous for mutations of genes involved in the SAC such as Mad2l1 (encoding Mad2) or Bub1 develop spontaneous tumors. However, complete inactivation of the SAC appears lethal to (cancer) cells, due to massive chromosome mis-segregations. In line with this finding, it appears that the SAC is (at least partially) functional in most cancer cells, even though they show CIN. More likely, hypomorphic mutations in SAC genes, or minor protein imbalances of SAC genes cause mis-segregations and contribute to CIN.

Together, these examples illustrate that a well-functioning mitotic checkpoint is therefore crucial in preventing aneuploidy or gross chromosomal rearrangements, protecting cells from chromosomal instability.

Coping mechanisms of cancer cells harboring genomic instability
Genomic instability is a common feature of many cancers, however, the extent of genomic instability varies significantly across different tumor types. Some cancer subtypes display low levels of genomic instability (e.g. acute myeloid leukemia), whereas others display extraordinary high levels of genomic instability (e.g. subsets of head-and-neck squamous cell carcinoma (HNSCC), high-grade serous ovarian cancer (HGSOC) and triple-negative breast cancer (TNBC)). TNBCs – often with high levels of genomic instability – typically show aggressive behavior with higher grade, frequently have a greater size, metastasize earlier and respond poorly to conventional chemotherapy regimens compared to breast cancers with a lower level of genomic instability that express either the estrogen receptor (ER) and/or the progesterone receptor (PR), or are enriched for human epidermal growth factor receptor-2 (HER2). Up to 20% of the patients with TNBC harbor mutations in BRCA1 and/or BRCA2.

The observation that tumor cells continue proliferating with BRCA1 or BRCA2 mutations is contradictory with results from Brca1 and Brca2-deficient mouse models. Specifically, Brca1 or Brca2 mutant mice die early in embryonic lethality, showing that Brca1 and Brca2 are essential genes for proliferation and survival. Moreover, it was found that mouse embryonic fibroblasts (MEFs) derived from Brca1∆11/∆11 or Brca2∆11/∆11 embryos displayed poor proliferation in vitro, illustrating that the essential roles for BRCA1 and BRCA2 extend beyond embryogenesis. Specifically, Brca1∆11/∆11 or Brca2∆11/∆11 MEFs displayed exceptionally high levels of p53 and its downstream target p21. The increased levels of cell cycle checkpoint proteins p53 and p21 resulted from the accumulation of DNA damage, which trigger a DDR response and lead to a block in cell cycle progression, ultimately leading to senescence and lethality. These data show that the tumor-suppressive function of BRCA1 and BRCA2 require other genetic events to allow cell proliferation. Indeed, inactivation of the DDR response through Tp53 mutation accelerates tumor growth and is in good agreement with the invariable mutation of TP53 in BRCA1 and BRCA2 mutant cancers.

Even though embryonic lethality was rescued upon removal of Tp53 in homozygous Brca-mutant mice, these mice did not develop like their wild-type counterpart. Brca1 mutant mice developed tumors at multiple sites after 3 months and showed signs of premature aging. An incomplete rescue of the proliferation defect upon Tp53 loss and premature senescence, led others to search for additional genes - specifically in the DNA damage response (DDR) pathway - allowing for survival of HR-deficient cells. Using a candidate-gene approach, it was found that gene expression of Chk2, which was significantly decreased in Brca1∆11/∆11 Tp53−/− mice that survived up to 7.5 months, indicating that impaired Chk2 might have aided in survival. Similar results were seen for Brca1∆11/∆11 Atm−/− and Brca1∆11/∆11 Atm−/− mice which survived into adulthood. These examples illustrate that loss of CHK2/ATM likely attenuates the ATM-CHK2-TP53 pathway and thereby blocks p53-dependent apoptosis.
Deletion of other genes in the DDR pathway or cell cycle regulatory components such as Chk1, H2AX, p21, PTEN, Gadd45a, p19ARF and Parp1 failed to rescue the premature senescence in Brca1-deficient MEFs. Strikingly, Brca1-deficient MEFs lacking 53BP1 appeared resilient to signs of premature aging and Brca1-null cells that lacked 53BP1 continued proliferating. Continued proliferation was specifically observed upon 53BP1 loss in cells with reduced Brca1 activity, but not in other situations of DNA damage, including irradiation or oxidative stress. Brca1∆11/∆11 53BP1/− mice showed a near wild-type phenotype with a low tumor incidence, even at 20 months of age. Similarly, 53BP1 loss is a common aberration in BRCA-associated breast cancers, and may be a requirement for survival of these cancers. Mechanistically, 53BP1 is an activator of p53, and in conjunction with NHEJ promotes NHEJ, while it blocks HR through inactivation of the BRCA1-CtIP axis. In line with a role of 53BP1 in blocking HR, removal of 53BP1 circumvents the proliferation defect in Brca1-deficient/Brca1-null cells by partial restoration of HR. In other words, the choice for repair mechanisms used by cells upon DSBs are of crucial importance for maintaining genomic integrity. Factors influencing the choice for repair can impact genomic integrity and cellular viability.

The observation that tumor cells are apparently able to cope with loss of BRCA1 or BRCA2, whereas BRCA1 and BRCA2 are essential genes in normal cells was coined the “BRCA paradox.” The increased cell survival in homozygous Brca1 or Brca2 mutant mice after p21 or Tp53 loss is explained by failure to arrest cells via a Tp53-dependent cell cycle arrest when DNA damage cannot be properly repaired. Consistent with this notion, many BRCA-deficient cancers harbor mutations in p53 allowing these tumors to proliferate. The absence of HR repair allows for the accumulation of secondary mutations which may benefit survival of BRCA1 and BRCA2-mutant tumors. The additional secondary mutations (e.g. loss of Tp53 or loss of ATM or CHK2 function or amplification of ATR, CHK1 or MYC) may allow cells to bypass checkpoint controls, evade apoptosis and contribute to viability. Thus, the continuous random acquisition of mutations, SCNAS, chromosomal rearrangements or gains and losses of whole chromosomes across the genome in genomically unstable tumors, could lead to a survival benefit in these cells and thereby promote tumorigenesis.

In conclusion, these studies elucidate that the ‘rewiring’ of genomically unstable tumors acquired by primary and secondary mutations can promote survival and allow for clonal expansion. Primary and secondary mutations that lead to inactivation of apoptotic pathways, restoration of repair mechanisms or loss of DNA damage checkpoint proteins can allow tumors to survive high levels of genomic instability, and probably also lead to resistance to genotoxic chemotherapy. Unravelling upon which genes genomically unstable cancers have come dependent on for their survival, could result in the development of molecular treatment regimens tailored to patients with genomically unstable cancers.

With the discovery that BRCA1 and BRCA2 play an important role in DNA repair, the question arose how mutations in such a generic mechanism show such tissue specific spectrum of tumorigenesis (i.e. predominantly breast and ovary tissue, to a lesser extent prostate, colon, pancreas, cervical, laryngeal, stomach and fallopian tube). One explanation suggests that inactivation of BRCA1 or BRCA2 can impact ovarian steroid hormone signaling by altering hormone levels and stability, and levels of receptor expression. Increased breast and ovarian cancer risk in BRCA1 and BRCA2-mutation carriers can be caused by an increase in estradiol and progesterone levels in serum, which subsequently promote proliferation of breast epithelial cells. Another explanation focuses on the involvement of BRCA1 on transcription regulation. Although, only relevant for breast cancer occurrence, absence of functional BRCA1 can lead to unregulated activation of the estrogen receptor alpha and stimulate excess proliferation. Other explanations for tissue-specificity in cancer predisposition focuses on the ability of breast and ovary tissue to survive for a prolonged period without BRCA1. The absence of a proper DNA repair mechanisms allows time for other secondary mutations to accumulate such as genes that provide additional DNA repair capacities, genes that account for BRCA1 function or genes that increase anti-apoptotic signaling thereby suppressing DNA damage-induced cell death. The latter can also be induced by the microenvironment in which hormones such as estrogen can support anti-apoptotic signaling in these tissues. Apparently, cell type-specific or microenvironmental factors in the breasts and ovaries are favorable towards survival of HR-deficient cells, whereas HR-
deficient cells in other tissues are eliminated from the population before tumorigenesis can occur.

Methods for the detection and analysis of genomic instability

Measuring a tumor’s degree of genomic instability can give insight into the genetic alterations, heterogeneity and the defective mechanism(s) that led to genomic instability in a tumor. The mutational signature or “historic scars” that caused genomic instability can reveal the defective underlying DNA damage or DNA repair processes. Apart from that, identifying specific alterations in a tumor could reveal to which genes a tumor possibly has become dependent on for survival and could guide treatment choice. Thus, unravelling the underlying cause and degree of genomic instability may point to genomic alterations that can contribute to the development or choice of molecular treatment regimens that is tailored to a patient’s cancer. In addition, measuring the degree of genomic instability may be informative for determining the required dose of DNA damaging agents that could drive cancer cells into intolerable levels of genomic instability. A number of detection methods are available that assess the degree of genomic instability (Fig. 2). Their methods, shortcomings and type of genetic alterations that can be detected will be described below.

A method for detecting whole chromosome copy number gains and losses and large chromosomal rearrangements can be obtained by metaphase spread-based karyotyping in which chromosomes are arrested in metaphase and dropped on glass slides. Subsequent staining of slides using Giemsa or DAPI enables visualization of the chromosomes using a light microscope and allows for the detection of whole chromosome copy number gains and losses, large amplifications, insertions, deletions, inversions and translocations. A more advanced application on this technique uses fluorescence in situ hybridization (FISH) or spectral karyotyping (SKY) which allows for the detection of SCNAs. These methods rely on chromosome-specific probes that are fluorescently labelled and hybridize to chromosomes resulting in chromosome-specific color patterns. Detection based on karyotyping is a relatively fast method for determining structural rearrangements and gains and losses of whole chromosomes. Downsides of this technique, however, is that detection based on karyotyping requires dividing cells and can introduce technical artefacts.

Although distinct from metaphase spread-based karyotyping, flow cytometric analysis reveals cellular ploidy by labelling all the DNA with one fluorescent dye. This technique estimates fluorescence emittance as cells in suspension are passed through a laser and determines cell ploidy based on DNA content and stage of the cell cycle. While flow cytometry is very accurate in determining ploidy and can be performed in large numbers, other genetic alterations even segmental or whole chromosome aberrations cannot be detected.

A method for the detection of SCNAs and whole chromosomal aberrations can be performed by array comparative genomic hybridization (aCGH). This method relies on fragmenting and labelling DNA of interest and a reference DNA sample with fluorescent dyes and competitively hybridizing to an array with thousands of cDNA fragments. After hybridization, the relative fluorescence intensities to each labelled DNA probe are quantified using a microarray scanner. Even though, aCGH can detect SCNAs and whole chromosomal aberrations in bulk samples, it is unable to detect translocations and inversions as chromosomal content is not altered with these alterations.

DNA sequencing quickly revolutionized the field of molecular biology with the ability to determine DNA sequences of many samples in a short time. DNA sequencing determines the precise order of nucleotides by ligating fragments of DNA to a slide and amplifying each read with fluorescently
labelled bases. Computer processing of continuous images taken of the slide unravel which base was incorporated and elucidate the sequence of bases in a DNA molecule. Next-generation sequencing (NGS) further improved the rate of samples that could be sequenced. Other big advancements in DNA sequencing came with the development of single cell sequencing. Single cell sequencing relies on DNA sequencing only for single cell sequencing, samples are single cell sorted prior to sequencing. Single cell sequencing enables the construction of mutational landscapes within a tumor which gives insight into tumor evolution. In summary, the highest resolution for the detection of SCNAs can be achieved by NGS (using deep sequencing). SNP arrays can reach a comparable high resolution but are more reliable than NGS since SNP arrays only detect polymorphisms which means that less DNA is required compared to NGS that detects the whole genome and requires many reads. Consequently, the amount of false-positives introduced in NGS is higher, making it less reliable compared to SNP arrays.

Besides DNA sequencing techniques, the detection of point mutations can also be detected using a polymerase chain reaction (PCR) whereby lengths of the PCR products of the microsatellite of interest are compared to PCR products of normal DNA gel electrophoresis. Alternatively, instead of running the PCR products on a gel, the PCR products can be injected in a capillary electrophoresis apparatus which separates the fluorescent DNA fragments by size. These techniques are specifically useful in the identification of the degree of microsatellite instability manifested as point mutations. The establishment of five reference microsatellite markers further standardized the degree of microsatellite instability based on the amount of altered microsatellite markers.

Besides the development of detection techniques, functional assays can also give an insight into genomic instability underlying for example impaired DNA repair. A classic example is the formation of RAD51 foci after ionizing radiation in cells that can repair the damage using HR. In parallel, cells that are HR-deficient e.g. after inactivation of BRCA1 or BRCA2, show almost no irradiation-induced RAD51 foci. Advantages of the RAD51 assay is the accurate prediction of BRCA status based on the presence or absence of RAD51 irradiation-induced foci (IRIF), making it a useful assay in discriminating HR-deficient and HR-proficient tumors. Also, acquired secondary mutations could allow restoration of HR components in some tumors which will easily be distinguished using the RAD51 assay. Down sides on the other hand are that is requires fresh ex vivo material and an irradiator for the induction of breaks.

Replication stress, another inducer of genomic instability, can be measured with DNA fiber fluoro graphy. In this technique, cells are labelled consecutively with two thymidine analogs.
and spread on a glass surface as DNA fibers. The use of two different thymidine analogs can be useful when comparing the initial replication speed of cells and the replication speed after a treatment. The length of the fibers can be visualized by immunofluorescence which represents the speed of the replication fork. Shorter fiber lengths represent slow fork speeds likely caused by stalled or collapsed replication forks. Apart from DNA fiber fluorography, electron microscopy can also form a useful technique that provides insight on replication fork dynamics.

Lastly, mutational signatures give insight into the degree of genomic instability as they provide an indication of operative mutational processes to which cancer cells have been exposed. Although more a predictive tool rather than a functional test, one of these efforts included the design of an algorithm that can detect BRCA1 and BRCA2-deficient cancers in a cohort of breast cancers and successfully led to the HRDetect algorithm. HRDetect appears to be a good tool to predict BRCA1 or BRCA2 defects, which will be advantageous in selection of patients for therapy.

**Targeting genomically unstable cancers**

Identifying specific genomic alterations in the tumors of patients and identifying the underlying defective DNA repair mechanism(s) can enhance the development of targeted strategies. Targeting vulnerabilities of tumors, such as defective HR repair, offers the unique opportunity to exploit molecular differences between normal and tumor cells. The increased sensitivity of HR-deficient tumors to the treatment of platinum-based chemotherapy is an example of a targetable vulnerability in these tumors. More precisely, the absence of HR-repair makes tumors more sensitive to platinum analogues and irradiation that induce intrastrand and interstrand cross-links and eventually cause cell death due to the accumulation of DNA damage.

Another explored vulnerability of HR-defective tumors focuses on inhibition of PARP which is involved in single-strand break (SSB) repair. Inhibition of PARP in HR-defective cells causes synthetic lethality as these cells are unable to effectively repair DSBs induced by PARP inhibition. The PARP inhibitors olaparib, niraparib and rucaparib are now approved in the treatment of patients with advanced ovarian cancer and in patients with germline BRCA-mutated metastatic breast cancer.

Other vulnerabilities of tumor cells involve exploitation of replication stress. Inhibition of checkpoint kinases such as CHK1, ATR, or WEE1, exploit the level of replication stress in tumor cells by overriding cell-cycle checkpoints with intolerable levels of replication stress. Inhibitors for these checkpoint kinases are currently still under clinical development. Despite improved therapeutic strategies for patients with genomically unstable cancers, resistance to therapies remain challenging. Therefore, unravelling how genomically unstable cancer cells are rewired or, e.g. which escape routes cancer cells use to circumvent survival or resistance mechanisms, could potentially lead to the discovery of new targeted therapies. Hence, the main aim of this thesis is to identify and pre-clinically validate potential therapeutic targets in genomically unstable cancer cells that could result in new treatment strategies for patients with genomically unstable cancers.

**SCOPE of the THESIS:**

In chapter 1, we provided a general introduction focusing on the types of genomic instability, how genomic instability can be detected and how cancer cells cope with high levels of genomic instability. In chapter 2, we aimed to identify genes on which genomically unstable cancer cells might depend for their survival using previously ranked FGmRNA profiles of 16,172 patients on their degree of genomic instability. A transcriptome-wide association study was performed to predict the association of expression of individual genes with the degree of genomic instability. Eleven genes, that were in the top 250 genes that showed the highest association to a high degree of genomic instability, appeared to share similar predicted biological function. Next, we tested the relevance of these 11 genes, including our candidate gene TPX2. We then examined whether genomically unstable cancer cells, induced by BRCA2 inactivation, depended on the TPX2/Aurora-A signaling axis for their viability, and assessed how progression through mitosis was affected upon combined inactivation of BRCA2 and TPX2/Aurora-A.
Based on results described in chapter 2, we explored in chapter 3 whether genomically unstable TNBCs have elevated levels of TPX2 which could point to an actionable target for these tumors. To this end, we analyzed the levels of TPX2 protein expression in a large set of breast cancers. Furthermore, we associated the expression levels of TPX2 with other clinicopathological parameters and two other markers indicative of a high degree of genomic instability (phospho-RPA32-Ser33 and γH2AX) and a driver of genomic instability (Cyclin E). By associating TPX2 levels with levels of these proteins, we aimed to determine whether TPX2 levels are indeed associated with the degree of genomic instability.

The identification of actionable dependencies of genomically unstable cancer cells can lead to new therapeutic targets for patients with genomically unstable tumors. In chapter 4, we used FigmRNA profiling of HNSCC samples to identify commonly elevated genes which could lead to potential therapeutic targets. One of our top candidates was the DNA damage checkpoint kinase, ATR kinase, which was positively associated to a high degree of genomic instability and is commonly overexpressed in HNSCCs. Since genomic instability can be induced by increased levels of replication stress in HNSCCs, we aimed to gain insight into the levels of replication stress in HNSCCs. To this end, we measured replication fork speed, a readout of replication stress, in HNSCC cell lines and in ex vivo head-and-neck tumor material, in the presence or absence of ATR inhibitors. Furthermore, we investigated ATR activity by analysis of phosphorylation of its substrate RPA in a cohort of HNSCCs.

Antibody-drug conjugates (ADCs) constitute a novel class of molecularly targeted anticancer agents. These therapeutic agents combine an antibody bound to a cytotoxic agent. Important for an ADC to be effective in vivo, is the presence (and ideally elevated expression of) the target protein of the antibody moiety within the ADC on tumor cells compared to normal tissue. In chapter 5, we used FigmRNA profiling of HNSCC samples, to identify potential ADC targets. Glycoprotein nmb (GPNMB) was predicted to be the most elevated gene in HNSCCs in comparison to normal mucosa from the oral cavity, for which an ADC exists. We then assessed whether protein expression of GPNMB was indeed elevated in a cohort of HNSCC samples using tissue microarrays. Finally, in chapter 6, we summarize our findings and discuss future perspectives of personalized treatment options for patients with genomically unstable tumors.

REFERENCES

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