Exploiting genomic instability as an Achilles' heel in cancer

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Discussion and summary

Chapter 6

Summary

Over the last 50 years, our increased knowledge about tumor biology, the extensive genomic characterization of tumors and the development of innovative detection methods have resulted in a range of effective treatments to target tumor cells, and have been crucial in extending the life expectancy of certain groups of patients with cancer. For instance, breast cancers that express the estrogen (ER), progesterone (PR) or HER2 receptors can currently be targeted with hormonal therapy or molecularly targeted drugs.

For other tumor subtypes, however, similarly effective treatments have not been developed. For instance, the so-called triple-negative breast cancers (TNBCs), which lack expression of the ER/PR/HER2 receptors, cannot be targeted by hormonal or targeted agents, and TNBC patients therefore depend on treatments involving chemotherapy and/or radiotherapy.

A feature that is shared between many difficult-to-treat cancers, including TNBCs but also high-grade serous ovarian carcinomas (HGSOCs), is their elevated levels of replication stress. Replication stress (RS) is defined as the slowing or stalling of DNA duplication and can be fueled by overexpression of oncogenes, such as Cyclin E1, Cdc25A or c-Myc. The accumulation of DNA lesions induced by RS may lead to replication fork collapse, and can subsequently compromise genome stability, which drives tumor heterogeneity and treatment failure. To cope with DNA lesions during replication, cells have evolved a complex DNA-damage response (DDR) which halts cell cycle progression through checkpoint kinases and facilitates DNA repair. In order to deal with the DNA lesions induced by RS, such cancer cells may become dependent on adaptive mechanisms for their survival, including DDR pathways.

The discovery of drugs that inactivate key DDR checkpoint kinases (e.g. WEE1, ATR) opens a window of opportunity to treat tumors that present with elevated levels of RS. To develop treatments for tumors with high levels of RS and design optimal selection tools to identify these patients, we first require a precise and unified system to measure RS and to be able to identify tumors with high levels of RS.

The aims of the research described in this thesis were therefore to:

1.Identify oncogenic alterations that are associated with elevated levels of RS in tumors.

2.Unravel the cellular consequences of oncogene-induced RS, and the therapeutic opportunities that they offer.

3.Identify tumors with high levels of RS.

In **chapter 1**, we provided a general introduction and the aim of this thesis.

In **chapter 2**, we performed a literature study. Specifically, first we discussed how normal replication is regulated at the molecular level. Next, we summarized the oncogenic alterations that can generate replication stress and chromosomal instability, and we discussed the mechanisms that cells use to deal with replication stress within the cell cycle. To exploit the notion that genomically instable cancers suffer from elevated levels of replication stress and display alterations on the activation of DNA damage response, we discussed distinct strategies to target key cell cycle checkpoint kinases, oxidized nucleosides, DNA resolvases and immune-checkpoints.

Replication stress is commonly studied in experimental models. However, to develop therapeutic approaches to target tumors with aberrant levels of RS in patients, it is necessary to study whether oncogene expression is associated with RS in clinical samples. To this end, in **chapter 3** we addressed if and how oncogenic alterations are associated with levels of RS in breast cancer tumor samples from 384 patients taken prior to treatment. Therefore, we analyzed the relation between the immunohistochemical expression of oncogenes (Cyclin E1, Cdc25A and c-Myc) and RS markers (pRPA and γ-H2AX) in

distinct types of breast cancer tissues. As expected, we found that triple-negative breast cancer samples, which are the most genomically instable of all the breast cancer subtypes, displayed the highest levels of RS. In addition, nuclear Cyclin E1 expression was shown to be associated with RS, especially in TNBCs. Taken together, our findings point at Cyclin E1 as a potential biomarker to select patients for treatment with checkpoint inhibitors, such as WEE1 and ATR, that target RS.

In **chapter 4**, we aimed to target tumor cells with high levels of RS by the inhibition of checkpoint kinases. To do so, we first engineered experimental models with doxycycline-inducible oncogene-mediated replication stress. Specifically, we induced overexpression of Cyclin E1 or Cdc25A, which led to slower DNA replication speed, as measured by DNA fiber analyses. Next, we studied whether oncogene-induced RS negatively affected segregation of chromosomes during mitosis and the stability of the genome. Indeed, we found mitotic defects and genomic instability to be caused by the accumulation of DNA lesions derived from RS. Interestingly, the inhibition of ATR or WEE1 exacerbated these mitotic aberrancies, increased the genomic instability and caused cytotoxicity in our oncogene-induced RS models. In conclusion, our findings further support the adoption of Cyclin E1 overexpression as a criterion to facilitate the selection of patients for treatment with WEE1 and ATR inhibitors.

To measure the levels of RS in tumor material, it would be very useful to identify generic effects of oncogene-induced replication stress. An approach to accomplish this involves analysis of common oncogene-induced mRNA expression changes, and identify common effects of a large panel of oncogenes. Therefore, in **chapter 5**, we developed an mRNA expression-based signature for oncogene-induced replication-stress to identify and categorize tumors based on their levels of RS. We modeled a panel of TNBC and non-transformed cells to overexpress Cyclin E1, c-Myc or Cdc25A, which caused RS. We observed that the induction of RS in our experimental models resulted in 52 common upregulated genes, as measured by RNA sequencing analysis. Interestingly, 6 out of 52 upregulated genes were commonly expressed in a gene expression data from 10,592 patient derived tumor samples. Next, we utilized our six-gene signature to classify the levels of RS in patient derived tumors using 8,862 RNAseq samples from TCGA and 13,912 microarray samples from GEO. Finally, we validated NAT10, one of the genes from our RS-signature, in breast cancer clinical samples. Our immunohistochemical analyses indicated that NAT10 was associated with RS. In conclusion, our RS-signature is valid to categorize tumors based on their levels of RS and can help to find patients suitable for WEE1 and ATR inhibition treatment.

Discussion

Increasingly, replication stress (RS) has been recognized as a key feature of difficult-to-treat cancers. Moreover, a range of drugs have been developed to target RS, and some of these agents are undergoing clinical evaluation, including inhibitors of the replication checkpoint kinases WEE1, CHK1 and ATR. Treatment with such drugs may provide an alternative and hopefully more potent treatment to cancer patients that do not respond to non-personalized anti-cancer therapies such as chemotherapy and/or radiotherapy. Although these developments are promising, there are several questions that remain unanswered. Particularly, we need more insight into the biology of the various replication-stress inducing factors, and better tools to identify tumors with high levels of replication stress.

Replication stress in breast cancer subtypes

To gain insight into the relation between oncogene expression and RS, we analyzed clinical samples from breast cancer patients. The study population was categorized

according to the status of ER, PR and HER2 (1). To evaluate oncogene expression, Cdc25A, Cylin E1 and c-Myc were selected and revealed distinct levels of intensity across the breast cancer subgroups.

Although not statistically significant, the TNBC subgroup presented the highest levels of Cdc25A expression in our study population in chapter 3. Not surprisingly, large publicly available data from GEO revealed that the TNBC patients displayed increased mRNA expression for CDC25A (encoding for Cdc25A). Also, mRNA and protein expression of c-Myc and Cyclin E1 predominated in the TNBC samples. Whereas TNBCs are known to frequently contain *CCNE1* and/or *MYC* amplifications, *CDC25A* amplifications do not prevail in this breast cancer subgroup (2,3). Therefore, in the comparison between TNBC and the other breast cancer subgroups, it is logical to find stronger variations of oncogene expression of c-Myc and Cyclin E1 than Cdc25A in TNBC.

As expected, our analysis of RS using gene expression profiling in chapter 5, combining TCGA and GEO signature scores, confirmed that TNBCs are the breast cancer subtype with the highest levels of RS. Specifically, gene expression profiling revealed that across 27 cancer subtypes, TNBCs are a subclass of cancers among those with highest levels of RS, following ovarian carcinoma, colorectal carcinoma and diffuse large B-cell lymphoma.

Another breast cancer subgroup that displayed high levels of replication stress was the HER2-positive, ER/PR negative subgroup. Although this subgroup was relatively small in the study population in chapter 3 and as a consequence the statistical power was lower, these cancers showed elevated levels of γ-H2AX and pRPA. HER2 amplified breast cancers have indeed been shown to have increased levels of genomic instability as judged by elevated levels of CNAs (4). Moreover, *HER2*-amplified BCs were shown to have elevated levels of γ-H2AX and pRPA in a separate cohort (5). Possibly, this BC subgroup could be considered for RS-targeting therapeutic strategies, when HER2-directed therapeutics fail.

It could very well be that other oncogenes that were not included in our analysis in chapter 3 also contribute to the observed RS. Indeed, various other oncogenes that have been found to be activated in breast cancer have been previously associated with replication stress, including *CCND1*, *ARID1A*, *IDH1/2* and *PBRM1* (6–10). Also, many other factors could lead to RS that are independent on oncogene-overexpression, including DNA repair defects, and transcription-mediated R-loops in the DNA (11). These additional factors may explain the observed high levels of replication stress in tumor samples lacking overexpression of the indicated oncogenes. Yet, the fact that we find strong associations between the expression of the studied oncogenes and the markers of RS shows that the contribution of additional factors may not be as strong when compared to the effects of oncogene overexpression. To find relative contributions of individual factors and uncover contributions of other factors, a future study could employ the mRNA expression analysis in chapter 5 to identify TNBC samples with a high score of RS, but lacking amplifications of *MYC*, *CCNE1* to identify other oncogenes or DNA repair defects that could explain the high levels of replication stress in these samples.

Unique versus common effects of oncogene overexpression

A number of oncogenes has been described in literature to result in oncogene overexpression, including Cyclin E1, MYC, Cdc25A, Mos and EF21 (12–17). However, it remains unclear if the effects of these oncogenes on replication are similar. In this thesis, we have specifically analyzed the effects of overexpression of Cdc25A, MYC and Cyclin E1 to induce RS. For studying the cell biological effects of oncogene overexpression, we specifically analyzed Cdc25A and Cyclin E1, and analyzed their effects on mitotic fidelity and on the sensitivity to WEE1 and ATR inhibitors.

Our data indicates that Cyclin E1

and Cdc25A overexpression leads to RS, increased mitotic segregation defects and sensitizes cells to ATR and WEE1 inhibitors in our experimental models. Even though overexpression of both Cdc25A and Cyclin E1 caused RS, they produced distinct effects on chromosome segregation and sensitivity to ATR and WEE1 inhibitors depending on the *TP53* context (wild type or mutated) (18–20). Concretely, Cdc25A overexpression leads to more segregation defects and sensitivity to ATR and WEE1 inhibitors when compared to Cyclin E1, regardless of the *TP53* status. A possible reason for these observations is that Cdc25A is a phosphatase that activates both CDK1 and CDK2 to trigger cell cycle progression (21). Upon overexpression of Cdc25A, cells may bypass cell cycle checkpoints both at the G1/S and G2/M cell cycle transition, and accumulate more DNA lesions when entering mitosis (20). In contrast, Cyclin E1 specifically activates CDK2, which is required to promote the cell cycle at the G1/S transition (22–24). In addition, p53 activation prevents genomic instability under *CCNE1* amplification (encoding for Cyclin E1), which may explain why Cyclin E1 overexpression only promotes sensitivity to both ATR and WEE1 inhibitors under *TP53* mutation (14,25,26).

Similar differences may become apparent if other oncogenes are included in these functional analyses. When RNAseq analyses were performed on cells overexpression Cyclin E1, Cdc25A and MYC, besides common effects on gene expression many differences were identified. Likely these differences go along with different cell biological changes, and require further investigation. Although common effects are expected (elevated CDK2 activity), many differential effects can be expected as well. Development of isogenic models, in which each of these oncogenes are introduced combined with functional analysis will be required to acquire a comprehensive overview of the common and distinct consequences of each of these oncogenes. Of note, overexpression of Cdc25A and Cyclin E1 resulted in elevated expression of MYC targets (chapter 5), indicated that these oncogenes do not function as independent components, and that the observed effects may reflect indirect effects.

Analysis of mutations in tumors with high levels of replication stress also point towards differences in the effects of oncogene overexpression. For instance, *CCNE1* amplification is frequently observed in TNBC and HG-SOC, but is mutually exclusive with loss of BRCA1/2 (2,3,27–30). Likely, this observation points towards DNA lesions induced by Cyclin E1 overexpression that require BRCA1/2 for repair. In contrast, *MYC* amplification, that also leads to replication stress is commonly observed in tumors with *BRCA1/2* mutations (31,32). These data point further underscore that differences likely exist in the types of DNA lesions that arises in response to replication stress induced by the various oncogenes.

Technologies to measure replication stress in cancer samples.

In our research, a number of techniques are used to measure replication stress. All these techniques have their advantages and short-comings. Below, each of the techniques are discussed.

1. Immunohistochemistry of replication stress markers

Immunohistochemistry (IHC) is a widely used technique - both in research and diagnostics - to determine the distribution and expression of proteins in (tumor) tissue. Concerning the analysis of RS, antibodies against γ-H2AX and pRPA are commonly employed to measure RS both in experimental models and patient samples. However, both these stainings may capture additional biological events that could bias the interpretation of the results. For instance, the collapse of replication forks which leads to the formation of double-strand breaks (DSBs) leads to phosphorylation of histone H2AX at serine 139 (known as γ-H2AX) (33–35), which is the relevant source of γ-H2AX

staining in the context of replication stress. However, when cells undergo apoptosis, DNA breaks are induced which also leads to γ-H2AX positivity. To distinguish between DSBs that are unrelated to RS, and γ-H2AX related to RS, it is important to consider patterns of γ-H2AX staining, and the context of γ-H2AX staining. Whereas non-RS DSBs are considered to form γ-H2AX foci, RS leads to pan-nuclear expression of γ-H2AX. A second tool to look into RS, is to additionally analyze other markers to provide context. A well-established marker of RS is RPA, which is phosphorylated by ATR after coating stretches of single-strand DNA (ssDNA), as a result from the uncoupling of the DNA helicase from the DNA-polymerase machinery. Since elevated levels of ssDNA are considered a source of RS, pRPA is a considered a reliable marker of RS. However, pRPA may also reflect generation of ssDNA during DNA end resection, which is a crucial step in the repair of DSBs by homologous recombination (HR) (36,37). Again, to exclude potential ongoing DNA repair measured by pRPA, we could analyze RS by measuring the pan-nuclear staining of pRPA. Furthermore, the combined analysis of both γ-H2AX and pRPA to detect RS in (tumor) tissue should increase the change to measure replication stress. Additional measurements, including the activation status of replication stress response pathways (e.g. ATR, CHK1) could be considered, although these pathways also are activated during the DNA repair of DNA breaks that occur independently of DNA replication (38). In conclusion, IHC is a straightforward analysis platform for RS, providing a means to analyze the wealth of archival patient tissues that are present as paraffin-embedded tissues, although the specificity depends on the available antibodies, which is currently suboptimal.

2. mRNA expression signatures

Rather than analyzing the direct consequences at the protein level, RS could be measured by mapping the downstream consequences. Such an approach would also address the issue that RS could be caused by multiple oncogenes. However, a uniform approach to optimally detect and measure the consequences of oncogene-induced RS in tumors has not been established yet. Comparison of mRNA signatures induced by oncogene-induced RS samples with non-RS mRNA expression profiles could therefore be an effective solution to reveal common differential gene expression changes involved in RS. Upon analysis of mRNA signatures, we generated a robust approach to precisely map gene expression changes related to RS, which would circumvent the limitations of measuring single-protein abundance in a sample, including the binding specificity of the antibody. Moreover, mRNA signatures display the current phenotype of cancers, rather than events that may not necessarily translate into cellular consequences. The disadvantages of employing mRNA signatures are the increased costs of the analyses and the many technical and biological factors that may alter the gene expression profiles, including the age, the gender and the pathological condition of the patient (39). To accurately reduce the impact of these cohort-specific factors, we have integrated our oncogene-induced RS signature from experimental models with TCGA gene expression data of patient-derived tumor samples and built a six-gene signature that captures oncogene-induced RS in a platform-independent fashion. Our analysis showed that the observed signature was independent on platform, since we observed a strong correlation between RNAseq and microarray-based samples. However, our analysis was done on a large set of samples, and whether analysis of an individual new sample can be reliably measured requires follow-up analysis, including definition of control samples as references.

3. DNA mutational signatures

Various processes in cancer have been associated to distinct mutational profiles (40). DNA mutational signatures provide a strong quantitative readout and have been reported to identify the genomic

scars caused by defective DNA damage repair, including DNA mismatch repair and homologous recombination (41,42). Since high levels of replication stress also impact genome integrity, it is assumed that RS will also lead to the accumulation of somatic mutations41. Additionally, the increased accumulation of DNA mutations may lead to secondary mutations, including in DDR mutations, that lead to mutational signatures. Therefore, it would be very interesting to search for DNA mutation signatures in cancers with confirmed RS. Although such signatures are expected to exist, they are currently elusive (43).

A first step towards mutational signatures of RS depends on isogenic models in which oncogene-induced RS is induced. Secondly, whole genome sequencing (WGS) needs to be performed at relatively high (30x) coverage. Besides the high costs associated with 30x WGS analysis, another limitation of this technique is that the presence of somatic mutations and DNA scars represent a historical signature and may not result in an ongoing RS phenotype. On the bright side, DNA is a very stable molecule for bioanalysis, and limited sample-to-sample variation is expected.

4. Mutational, amplification or expression analysis of specific genes

A straightforward procedure to identify tumors with high levels of RS is to analyze on a individual basis if a tumor contains mutations, amplifications and/or expression changes of a specific gene or gene panel associated with RS. The implementation of this strategy could serve as a selection tool to find cancer patients characterized by RS that could respond to replication checkpoint kinase inhibition, including WEE1. Therefore, a logical step is to investigate the prevalence of mutations and/or amplifications of genes in cancers that display RS, such as TNBCs. Even though around 80% of TNBCs exhibit TP53 mutations, the response to WEE1 inhibition treatment was shown to be independent of *TP53* status (2,44). Conversely, since Cyclin E1 overexpression sensitize TNBCs to WEE1 kinase inhibition (45), testing *CCNE1* (encoding for Cyclin E1) amplification status could be a first step to improve the patient response to this treatment (19), as is also currently being done in a clinical study involving WEE1 inhibitor treatment, using *CCNE1* amplification as a biomarker for patient inclusion (clinical trial identifier: NCT03253679). The prevalence of *CCNE1* amplifications and/or RNA upregulation in TNBC is relatively high (40% in METABRIC and 52% in TCGA) and is associated with worse outcomes and higher recurrence rate (2,3,28). The analysis of specific genes is relatively simple and facilitates data interpretation. However, the sources of RS are diverse and the study of one or few genes does not represent the full genetic landscape of RS, and eligible patients may be missed using this approach. In addition, gene amplification does not per se lead to protein overexpression, but can be considered as a starting point to find patients suitable for checkpoint kinase inhibition treatment.

Taken together, multiple approaches are being studied for the identification of tumors with replication stress. for some of these approaches validation in patient cohorts is required (IHC, RNA expression), whereas DNA mutational signatures related to RS still need to be uncovered using genetically-defined models. For all these platforms, the real test will be to assess patients that are treated with drugs that target RS, including ongoing trails using inhibitors of WEE1, ATR and CHK1.

Future perspectives

For future studies, it would be very interesting to further validate the ability of the identified 6-gene signature to identify tumors with RS (chapter 5). Such validation studies could initially be performed in retrospective clinical trials in which cell cycle checkpoints are evaluated. If successful, such tests -preferentially along with other candidate markers of RS – could be tested in prospective studies in which RS-targeting agents are tested, including inhibitors of the

WEE1, CHK1 and ATR kinases.

Additionally, it would be worthwhile to study if the identified genes in the mRNA signature of oncogene-induced replication stress encode proteins that are functionally involved in DNA replication, or reflect a compensatory mechanism that these tumors depend on for their survival. For one the components, NAT10, we confirmed its upregulation in tumors with elevated levels of the oncogenes MYC or Cyclin E1. Moreover, NAT10 expression appears to be induced by DNA damage (46), and is required for proper DNA replication (47). Interestingly, based on functional genome-wide screens with DNA damaging agents, NAT10, MOCS3 and DDX27 cluster together, with NAT10 and MOCS3 having profound and similar responses to a group of DNA damaging agents, including calicheamicin, trabectedin, duocarmycin (48). These observations suggest that these components may be involved in the cellular response to DNA damage and warrants investigation of their functions.

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