NKFIH (OTKA) K_124881 project final report

Project title: EXPLORING NOVEL ROLES OF HOMOLOGOUS RECOMBINATION FACTORS THROUGH GENETIC STUDIES OF MUTAGENESIS

The general objective of this project was to gain a better understanding of the mutagenic processes observed in homologous recombination deficient cells. We formulated the following three specific aims:

Aim 1: Find the mechanism of base substitution mutagenesis observed in HR deficient cells.
Aim 2: Conduct a mutagenesis-based study on the mechanism of HR.
Aim 3: Test the presence and relevance of HR-specific mutational profiles/signatures in human cancer.

To summarise, we fully achieved all three aims in the duration of the project. Aims 2 and 3 were achieved in the first two years, with the majority of the results published in a high impact paper (Póti et al., 2019). We spent the remaining time concentrating on Aim 1, the molecular mechanism of the mutagenic processes. We published two papers on related studies which helped our concurrent search for the molecular mechanism (Szeltner et al., 2021; Szikriszt et al., 2021). Using the skills and methods acquired during our studies, the main grant-funded scientist Dr. Eszter Németh also finished a related study on the mutagenic effect of mismatch repair deficiency in cell lines and cancer (Németh et al., 2020). We took part in an on-topic collaboration in a mouse cell line system (Hámori et al., 2020). Finally, we prepared two manuscripts on our findings on the mechanisms of HR deficiency related mutagenesis. One of these is currently under review at a quality journal (Póti et al.), the other has been provisionally accepted for publication (Chen et al., see below).

In the following, I present our results through the published papers and submitted manuscripts.

Póti Á, Gyergyák H, Németh E, Rusz O, Tóth S, Kovácsházi C, Chen D, Szikriszt B, Spisák S, Takeda S, Szakács G, Szallasi Z, Richardson AL, Szüts D. (2019). Correlation of homologous recombination deficiency induced mutational signatures with sensitivity to PARP inhibitors and cytotoxic agents. *Genome Biol.* 20, 240.

It was known from our previous publication (Zámborszky et al., 2017, Oncogene 36, 746-755) and from the analysis of cancer genomes that homologous recombination (HR) repair deficiency arising from defects in BRCA1 or BRCA2 is associated with characteristic patterns of somatic mutations. In this genetic study, we asked whether inactivating mutations in further genes of the HR pathway or the DNA damage checkpoint also give rise to somatic mutation patterns that can be used for treatment prediction.
Using whole genome sequencing of an isogenic knockout chicken DT40 cell line panel, we found a universal HR deficiency-specific base substitution signature that is similar to COSMIC signature 3 observed in BRCA-mutated cancers. In contrast, we detected different deletion phenotypes corresponding to specific HR mutants. The inactivation of BRCA2 or PALB2 leads to larger deletions, typically with microhomology, when compared to the disruption of BRCA1, RAD51 paralogs, or RAD54. Comparison with the deletion spectrum of Cas9 cut sites suggests that most spontaneously arising genomic deletions are not the consequence of double-strand breaks. Surprisingly, the inactivation of checkpoint kinases ATM and CHK2 has no

mutagenic consequences. Analysis of tumor exomes with biallelic inactivating mutations in the investigated genes confirmed the validity of the cell line models.
In addition to the genomic analyses, we also presented a comprehensive analysis of sensitivity of the investigated mutants to 13 therapeutic agents for the purpose of correlating genomic mutagenic phenotypes with drug sensitivity. Our results suggest that no single genomic mutational class shows perfect correlation with sensitivity to common treatments, but the contribution of COSMIC signature 3 to base substitutions, or a combined measure of different features, may be reasonably good at predicting platinum and PARP inhibitor sensitivity.

Németh E, Lovrics A, Gervai JZ, Seki M, Rospo G, Bardelli A, Szüts D. (2020). Two main mutational processes operate in the absence of DNA mismatch repair. *DNA Repair (Amst).* 89, 102827.

Using the methods and skills developed for the previous study, we extended our investigations to the mutagenic effect of loss of a different DNA repair pathway, DNA mismatch repair (MMR). This investigation, and the first author, were primarily supported by the present grant. The analysis of tumour genome sequences has demonstrated high rates of base substitution mutagenesis upon the inactivation of MMR, and the resulting somatic mutations in MMR deficient tumours appear to significantly enhance the response to immune therapy. A handful of different algorithmically derived base substitution mutation signatures have been attributed to MMR deficiency in tumour somatic mutation datasets. In contrast, mutation data obtained from whole genome sequences of isogenic wild type and MMR deficient cell lines in this study, as well as from published sources, show a more uniform experimental mutation spectrum of MMR deficiency. In order to resolve this discrepancy, we reanalysed mutation data from MMR deficient tumour whole exome and whole genome sequences. We derived two base substitution signatures using non-negative matrix factorisation, which together adequately describe mutagenesis in all tumour and cell line samples. The two new signatures broadly resemble COSMIC signatures 6 and 20, but perform better than existing COSMIC signatures at identifying MMR deficient tumours in mutation signature deconstruction. We showed that the contribution of the two identified signatures, one of which is dominated by C to T mutations at CpG sites, is biased by the different sequence composition of the exome and the whole genome. We further showed that the identity of the inactivated MMR gene, the tissue type, the mutational burden or the patient's age does not influence the mutation spectrum, but that a tendency for a greater contribution by the CpG mutational process is observed in tumours as compared to cultured cells. Our analysis suggest that two separable mutational processes operate in the genomes of MMR deficient cells.

Hámori L, Kudlik G, Szebényi K, Kucsma N, Szeder B, Póti Á, Uher F, Várady G, Szüts D, Tóvári J, Füredi A, Szakács G. (2020). Establishment and Characterization of a Brca1-/-, p53-/- Mouse Mammary Tumor Cell Line. *Int J Mol Sci.* 21, E1185.

In this work, our group contributed to the genomic analysis of a newly established BRCA1 mutant mouse cell line (CST). Genomic instability of CST cells was confirmed by whole genome sequencing, which also revealed the presence of COSMIC (Catalogue of Somatic Mutations in Cancer) mutation signatures 3 and 8 associated with homologous recombination deficiency.

Szeltner Z, Póti Á, Harami GM, Kovács M, Szüts D. (2021). Evaluation and modulation of DNA lesion bypass in an SV40 large T antigen-based in vitro replication system. *FEBS Open Bio.* 11, 1054-1075.

To complement the genomic methods for analysing mutagenic processes, we wished to employ in vitro assays for the replicative bypass of damaged DNA. DNA damage removal by nucleotide excision repair (NER) and replicative bypass via translesion synthesis (TLS) and template switch (TSw) are important in ensuring genome stability. In this study, we tested the applicability of an SV40 large T antigen-based replication system for the simultaneous examination of these damage tolerance processes.
Using both Sanger and next-generation sequencing combined with lesion-specific qPCR and replication efficiency studies, we demonstrated that this system works well for studying NER and TLS, especially its one-polymerase branch, while it is less suited to investigations of homology-related repair processes, such as TSw. Cis-syn cyclobutane pyrimidine dimer photoproducts were replicated with equal efficiency to lesion-free plasmids in vitro, and the majority of TLS on this lesion could be inhibited by a peptide (PIR) specific for the polη-PCNA interaction interface. TLS on 6-4 pyrimidine-pyrimidone photoproduct proved to be inefficient and was slightly facilitated by PIR as well as by a recombinant ubiquitin-binding zinc finger domain of polη in HeLa extract, possibly by promoting polymerase exchange. Supplementation of the extract with recombinant PCNA variants indicated the dependence of TLS on PCNA ubiquitylation. In contrast to active TLS and NER, we found no evidence of successful TSw in cellular extracts.
The established methods can promote in vitro investigations of replicative DNA damage bypass, and we employed these methods for our findings of increased TLS in the absence of HR (Chen et al., see below). Dr. Zoltán Szeltner was a participant of the project, and these studies were planned in the proposal.

Szikriszt B, Póti Á, Németh E, Kanu N, Swanton C, Szüts D. (2021). A comparative analysis of the mutagenicity of platinum-containing chemotherapeutic agents reveals direct and indirect mutagenic mechanisms. *Mutagenesis* 36, 75-86.

We frequently employed cisplatin treatments in our studies of the mutagenic effect of HR deficiency, with the reasoning that cisplatin generates DNA lesions at identifiable sequence motifs, thus the mutagenic consequences of the bypass in HR deficient cells can reveal whether the mutations occur directly at the lesion or via an indirect process. In this work, which was supported both by the PD_121381 postdoctoral grant to Dr. Bernadett Szikriszt and by the present grant, we conducted a thorough investigation of platinum-induced mutagenesis.
We coupled whole genome sequencing with phenotypic investigations on two cell line models to compare the magnitude and examine the mechanism of mutagenicity of cisplatin, carboplatin and oxaliplatin. Cisplatin induced significantly more base substitution mutations than carboplatin or oxaliplatin when used at equitoxic concentrations on human TK6 or chicken DT40 cells, and also induced the highest number of short insertions and deletions. The analysis of base substitution spectra revealed that all three tested platinum drugs elicit both a direct mutagenic effect at purine dinucleotides, and an indirect effect of accelerating endogenous mutagenic processes. Whereas the direct mutagenic effect appeared to correlate with the level of DNA damage caused as assessed through histone H2AX phosphorylation and single-cell agarose gel electrophoresis, the indirect mutagenic effects were equal. The different mutagenicity and DNA-damaging effect of equitoxic platinum drug treatments suggest that DNA damage independent mechanisms significantly contribute to their cytotoxicity. Thus, we suggest that the comparatively high mutagenicity of cisplatin should be taken into account in the design of chemotherapeutic regimens.

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BRCA1 deficiency specific base substitution mutagenesis is dependent on translesion synthesis and regulated by 53BP1

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In this work, we examined the mechanism of the elevated rate of mutagenesis seen in cells defective for *BRCA1*, *BRCA2* and other genes of the homology-dependent DNA repair (HR). The HR pathway is well known for its function in double strand break repair, and HR proteins also protect stalled replication forks from breakage. An increased use of non-homologous end joining (NHEJ) to repair DNA breaks in *BRCA1* mutants offers a straightforward explanation for the arising genomic deletions and rearrangements, but not the single base substitutions (SBSs). An important general cause of base substitution mutagenesis is the replication of damaged DNA by the translesion DNA synthesis (TLS). There also exists an error-free mode of damage bypass that uses the nascent strand of the sister chromatid as a lesion-free alternative template. This recombination-mediated template switching mechanism relies on the recombinase Rad51 and its paralogues or Rad52 in *S. cerevisiae*, and we earlier showed that the use of the sister chromatid as template is dependent on BRCA1 in vertebrate cells. We thus hypothesised that the majority of mutagenesis in *BRCA1* deficient cells, especially base substitution mutagenesis, is the consequence of an increased use of TLS that results from the loss of the template switching bypass pathway.

Using genome sequencing to detect spontaneous mutagenesis rates and patterns in knock-out cell lines we showed that Y family TLS polymerases contribute to the spontaneous generation of base substitution and short insertion/deletion mutations in *BRCA1* deficient cells. Specifically, SBS mutagenesis was lower in *BRCA1*$^{-/-}$ *POLH*$^{-/-}$ cells (p=0.004, t-test), *BRCA1*$^{-/-}$ *POLK*$^{-/-}$ cells (p=0.055), and *BRCA1*$^{-/-}$ *REV1*$^{-/-}$ cells (p=0.0001) than in *BRCA1*$^{-/-}$ controls. We further showed that mutagenesis at cisplatin-induced DNA adducts is increased in *BRCA1* and *BRCA2* mutants, indicating that replication-blocking lesions are more commonly bypassed by TLS in HR deficient cells.

We also created double mutant *BRCA1*$^{-/-}$ *KU70*$^{-/-}$ and *BRCA1*$^{-/-}$ *53BP1*$^{-/-}$ DT40 cell lines to assess the role of NHEJ in mutagenesis in *BRCA1* deficient cells. Surprisingly, the inactivation of *53BP1* but not *KU70* in *BRCA1* mutant cells markedly reduced TLS-specific mutagenesis, and rescued the deficiency of template switch–mediated gene conversions in the immunoglobulin V locus of *BRCA1* mutant chicken DT40 cells. Using the SV40 T antigen driven in vitro lesion bypass assay described above (Szeltner et al., 2021), we showed that 53BP1 also promotes TLS in human cellular extracts *in vitro*. Our results show that HR deficiency–specific mutagenesis is largely caused by TLS, and suggest a newly identified

function for 53BP1 in regulating the choice between TLS and error-free template switching in replicative DNA damage bypass.
In this work we achieved the third specific aim of the K_124881 project.

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Characterisation of the spectrum and genetic dependence of collateral mutations induced by translesion DNA synthesis
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The manuscript is under review.

This study is based on the observation from our genomic mutagenesis experiments that base substitution mutations sometimes occur in closely spaced pairs which cannot be explained by random chance. We investigated the hypothesis that misinsertions by low fidelity TLS polymerases may introduce additional mutations on undamaged DNA near the original lesion site, and these 'collateral mutations' are responsible for the phenomenon of close mutation pairs. The project is connected to our studies on HR deficiency related mutagenesis in two ways: (a) for the project, we reanalysed the genomic datasets generated for the above listed publications, (b) we found that close mutation pairs are especially enriched in HR deficient cells.
To briefly summarise the study, we used whole genome sequencing datasets of chicken DT40 and several human cell lines to obtain evidence for collateral mutagenesis in higher eukaryotes. We found that cisplatin and UVC radiation frequently induce close mutation pairs within 25 base pairs that consist of an adduct-associated primary and a downstream collateral mutation. By repeating the mutagenic treatments and genomic analyses in mutant cell lines, we genetically linked the formation of collateral mutations to TLS activity involving PCNA ubiquitylation and polymerase kappa. PCNA ubiquitylation was also indispensable for close mutation pairs observed amongst spontaneously arising base substitutions in cell lines with disrupted homologous recombination, further confirming our finding that increased base substitution mutagenesis in HR deficient cell lines is due to TLS activity. Collateral mutation pairs were also found in melanoma genomes with evidence of UV exposure. We showed that collateral mutations frequently copy the upstream base. We extracted a base substitution signature that describes collateral mutagenesis regardless of the primary mutagenic process, whether it is a DNA adduct-forming agent or HR deficiency. Using this mutation signature, we showed that collateral mutagenesis creates approximately 10-20% of non-paired substitutions as well, underscoring the importance and universality of the process.