

ESMO Translational Research Fellowship (07/2017 – 04/19)

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FINAL REPORT





Host Institute: Vall d'Hebron Institute of Oncology, Barcelona (ES) Mentor: Violeta Serra

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Project title: Exploiting the DNA-damage response upon PARP inhibition in homologous recombination deficient tumors to maximize the activity of anti-PD-L1 therapy.

Home Institute: University Hospital of Parma, Parma (IT)

Introduction

Tumors with DNA repair deficiencies in the homologous recombination (HR) double strand break (DSB) repair pathway, such as those from germline BRCA1/2 (gBRCA) mutation carriers, are highly sensitive to DNA damaging agents, e.g. platinum salts, and to poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi).

Rationale and Aim

Rationale

HR-deficient tumors elicit an innate immunity signal (STING pathway), due to their intrinsic high levels of cytosolic DNA that encompasses the upregulation of interferon-related genes. This signal is associated with increased CD4+ and CD8+ lymphocytic infiltration, independent of neo-antigen presentation. Nonetheless, it also activates the expression of PD-L1 which may paradoxically prevent immune-mediated cytotoxicity. Based on these findings, we hypothesize that treatment of HR-deficient tumors with PARPi elicit an S-phase DNA damage response that results in upregulation of PD-L1, and may limit the autologous antitumor immune-mediated cytotoxicity but sensitize to anti-PD-L1 treatments.

Objectives

<u>Primary objective</u>: to evaluate the induction of PD-L1 expression upon PARPi-treatment in HRR-deficient PDXs and correlate it to their sensitivity to PARPi.

Secondary objectives: to analyze

a) the modulation of immune-mediated cytotoxicity in HRR-deficient tumor models treated with PARPi.

b) the modulation of immune-mediated cytotoxicity by PARPi and anti-PD-L1 treatments in PDCs coculture with autologous TILs.



Experimental design

Project Methodology:

The research question have been addressed in three different experimental contexts:

- a) Existing breast cancer (BC) patient-derived tumor xenografts (PDX) engrafted in NMRI mice (N=39). This mouse strain preserves innate immunity (NK cells and macrophages) and B-lymphocytes.
- b) PDCs co-culture with autologous TILs. The model has been prospectively developed for an N=2.
- c) BRCA1^{-/-} transgenic mouse models. Tumors derived from 1 BRCA1^{-/-} transgenic mouse will be implanted in 15 BRCA1^{+/-} syngenic mice with the same immunological background.
- d) FFPE patient tumor samples.

Variables (Figure 1):

We are quantifying the following variables:

In PDX:

- a) Baseline and treatment-induced levels of PD-L1 positive cancer cells by IHC, measured as the percentage of positive cancer cells.
- b) Tumor response rate defined as a Tumor Growth Inhibition (TGI) between -100% and -30%). This variable is readily available.
- c) Baseline and treatment-induced levels of S-phase DNA damage: percentage of cells exhibiting γH2AX foci (n≥5) and geminin staining (marker of S/G2-phase of cell cycle).
- d) Baseline and treatment-induced levels of cytosolic DNA: percentage of cells exhibiting cGas cytosolic foci.
- e) Baseline and treatment-induced levels of STING-pathway cytokines: interferons, CXCL10 and CCL5 by qRT-PCR, relative to at least two house-keeping genes.
- f) Baseline and treatment-induced levels of tumor immune markers: MHC-I by IHC/IF, measured as the percentage of positive cells.
- *g)* Recruitment of NK cells and macrophages to the tumor microenvironment by IHC, as the percentage of immune cells relative to tumor cells.

In PDCs co-culture with autologous TILs:

- a) HR-deficiency: percentage of cells exhibiting RAD51 foci (n≥5) and geminin staining (marker of S/G2phase of cell cycle).
- b) Baseline and treatment-induced activation of CD4+ and CD8+ T cells by FACS and Th1/Th2 cytokine expression by ELISPOT.
- c) Exome Seq and RNA Seq of the tumor and the T cell compartments.
- d) Baseline and treatment-induced levels of PD-L1 positive cancer cells by IHC, measured as the percentage of positive cells (more than 5 out 100 cancer cells).
- e) Baseline and treatment-induced levels of cytosolic DNA: percentage of cells exhibiting IDU cytosolic foci.
- *f*) Baseline and treatment-induced levels of STING pathway cytokines: interferons, CXCL10 and CCL5 by ELISA.

In syngenic mouse models:

- a) HR-deficiency (RAD51 foci formation as above).
- b) Baseline and treatment levels of CD3+, CD4+ and CD8+ T cells by IHC.
- c) Baseline and treatment levels of MHC-I and PD-L1 by FACS.
- *d*) Treatment response according to RECIST criteria.

In FFPE patient tumor samples (as part of an academic clinical study of PARPi in HR-deficient patients, PI comentor Dr. Judith Balmaña):

- a) HR-deficiency (RAD51 foci formation as above)
- b) Baseline levels of S-Phase DNA damage, cytokines, chemokines, MHC-I and PD-L1 (as above)
- c) Baseline levels of CD4+ and CD8+ T cells (as above)
- d) Treatment response according to RECIST criteria



<u>Data analysis</u>: this is an exploratory study with a limited small sample. In all aims of the proposal, descriptive analysis for main variables will be provided for each treatment group: continuous variables will be summarized using mean, standard deviation, median and range.

<u>Sample size:</u> The following assumptions are made:

- a) Percentage of PD-L1 positive tumors (<1%) in untreated HRD PDXs is assumed to be 20% by IHC
- b) Percentage of PD-L1 positive tumors (>1%) in PARP-inhibitor treated HRD PDXs is assumed to be 55% by IHC

This results in the following outcome table:

	PD-L1 pos	PD-L1 neg
Olap Arm – Olaparib-treated HRD PDXs	55%	45%
Control Arm – Untreated HRD PDXs	20%	80%
Treatment effect hazard ratio	HR+ = 0.4	HR- = 1.6

Assuming a one-sided test at 20% alpha and power=75% on the interaction term 28 mice are needed to reject the null hypothesis of no treatment-PDL-1 interaction (i.e. equal PD-L1 expression between the two arms; HR=1) in favour of the alternative hypothesis (increased PD-L1 expression in the treatment arm; HR=0.4). I will consider each PDXs as one experimental group. Taking into account 5 % drop-out, the total sample size is estimated to be 30 mice (Olap arm: 15; Control arm:15).

Results, Conclusions and Future Perspectives

Results

<u>In PDXs:</u>

- a) PD-L1 is equally expressed in sensitive and resistant untreated samples by RNAseq and RPPA. We performed PDL1 staining by IHC in 26 PDXs samples: in non-responders there was a marked increase in PD-L1 expression except for PDX196, 127 and JAL71OR that harbour ATM/ATR pathway alterations (Sato et al, Nat Com 2017), PDX270 that harbours a mutation in CD274 and PDX339 that have low PD-L1 expression by RNAseq (Figure 3). I compared PD-L1 expression between the correspondent patient and PDX and PDCs in a small cohort of model and it is quite similar.
- b) BrC and OvC PDXs show distinct PARPi olaparib sensitivity (n=5 CR, n=3 PR, n=3 SD and n=17 PD) that fully correlates with the HRR-status, as measured by RAD51 foci.
- c) All PDXs express high levels of S-phase DNA damage express as percentage of cells exhibiting γH2AX foci and geminin staining.
- d) cGAS expression is not induced upon PARPi treatment in most of the sensitive model. According to Gosh et al, this could probably due to the fact that cGAS often presents epigenetic alterations which are not solved upon PARPi.
- e) Interesting, 12 out of 20 non-responding models (SD+PD) harbor mutations in STING pathway genes (i.e. IFNA10C20* mutation and IFIT2 amplification in 5 and 3 resistant PDXs, respectively).
- f) HLA ABC expression increases in all sensitive models upon PARP inhibition.
- g) We performed CD45 staining (leucocytes) by IHC in 27 PDXs samples: intratumoral CD45+ cells statistically significantly increase after olaparib treatment, in particular in the responding samples. Interestingly, peritumoral CD45+ cells increase after olaparib treatment in resistant tumors (Figure 4). We performed the CD56 staining (NK cells) by IHC in 27 PDXs samples: peritumoral NK cells increase after olaparib treatment, no significant modification were observed at stromal and intratumoral levels. We performed the CD11b staining (myeloid cells) by IHC in 27 PDXs samples and in most of the sensitive cases, CD11b+ stromal cells increase after olaparib treatment, while decrease or slightly increase in the resistant models. CD45 positive cells are not mainly NK cells or myeloid cells, according to their IHC profile.



- h) To better understand the role of the immune system in PARPi response, I performed a differential expression analysis between resistant and sensitive samples by RnaSeq and, based on these data, olaparib resistant PDXs seem to have a negative regulation of leucocyte proliferation (Figure 2). Several pro-inflammatory genes (TNF, ILA1a, IL33, CXCL11) are statistically significant more expressed in the resistant untreated PDXs samples. To explore the role of the STING pathway in response to PARPi, I applied the 44-gene signature related to the STING pathway activation to my untreated PDXs samples but it does not correlate with RAD51 expression nor with olaparib response.
- i) To better characterized our series of PDXs, we classified them according to Lehman's classification and PAM50 subtypes. None Lehman's TNBC nor PAM50 subtype correlates with olaparib response.
- *j)* To confirm the predictive role of RAD51 beyond TNBC, I evaluated RAD51 in an independent series of Luminal-B like breast cancer, NSCLC and Bladder Cancer PDXs. RAD51 is successfully stained and the correlative analysis with PARPi response is ongoing.
- In PDCs co-culture with autologous TILs:
- b) In collaboration with the Breast Cancer (BC) group, we prospectively collected eleven tissue samples for establishing TILs co-cultures, in collaboration with Alena Gros, and I've been monitoring the growth of these cells. I compared the TILs' growth rate with TILs' infiltration of the related patients' sample but not statistically significant correlation was found. We performed two PDCs co-culture with autologous TILs. In both cases no TILs activation was seen neither in untreated nor in olaparib-treated samples with FACS.
- c) Since none of the autologous TILs isolated have reacted against the correspondent PDCs, we didn't perform the ExomeSeq and RNASeq of the T cell compartments.
- d) At different treatments' time points, I stored the supernatant and I fixed in formalin the pellet to perform PDL1 IHC. As already mentioned, PD-L1 expression is the same, in most cases, in PDCs and in the correspondent PDXs, both in untreated and treated samples (Figure 5).
- e) I have tried to performed PicoGreen as cytosolic DNA marker in PDCs, but the staining was not clear.
- f) In PDCs co-culture with autologous TILs, no modulation of the secretion of IFN gamma was seen by ELISA.
- g) To verify if the in PDCs in vitro response to PARPi mirror the PDXs' in vivo sensitivity, I set up the ex-vivo cultures of several olaparib sensitive and resistant PDXs. This set up included using two different culture conditions (cell suspension and matrigel-containing) and two time points. Results were compared with the known in vivo response for each model and, when available, with patients' response (Figure 5).
- h) To demonstrate that resistant PDCs have an impairment in the STING pathway activation compared to the sensitive models, we measured the IFN beta production by ELISA upon STING activation by STING agonist. Only PDCs derived from sensitive models seem to secrete IFN beta upon STING activation (ongoing experiments).
- *i*) Baseline and treatment-induced levels of cytosolic DNA: percentage of cells exhibiting IDU cytosolic foci.
- In syngenic BRCA1-mutant mouse models (they have a functional innate and adaptive immune system):
- a) I evaluated RAD51 in transgenic BRCA1 mouse and it was RAD51 negative.
- b) We performed the same IHC/IIF of PDXs samples plus staining for CD3, CD4 and CD8 by IHC. Olaparib treatment in *BRCA1*-mutated Tg mice significantly increases infiltration of intratumoral CD3+ immune cells and stromal myeloid cells.
- c) PD-L1 (evaluated by FACS) in Tg tumor cells is maintained upon PARPi. PD-L1 is also expressed in intratumoral CD3+ cells and increase upon PARPi (experiment to be repeated in the next weeks). MHC staining is pending.
- d) We evaluated olaparib response in vivo according to RECIST criteria and it experience a SD.

In FFPE patient tumor samples (as part of an academic clinical study of PARPi in HR-deficient patients, PI comentor Dr. Judith Balmaña):

I didn't manage to have access to clinical samples.

Conclusions

- a) PD-L1 is expressed in some PARPi resistant models. According to exome-sequencing data, several sensitive models harbour mutations that impair PD-L1 expression.
- b) cGAS expression by IF is not induced upon PARPi treatment in most of the sensitive models.



- c) Olaparib recruits stromal CD45+ cells in PARPi sensitive tumors and peritumoral CD45+ cells in PARPi resistant models. CD45+ cells have been evaluated by IHC.
- d) Several HRR-proficient tumors: a) present an impairment in the IFN-type I production (i.e. IFNA10 C20*), i.e. primary resistant tumors (PDX270, 280, 274, 341); b) overexpress PD-L1, i.e. primary (PDX418) and acquired resistant models (PDX230OR). This impairment may interfere with the recruitment of intratumoral immune cells thus being relegated to the periphery of the tumor.
- e) PDCs co-culture with autologous TILs is a feasible method to study immune-checkpoint activity in vitro but the technique's failure rate is high.
- f) In *BRCA1*-mutated Tg mice that experience a SD upon olaparib, infiltration of intratumoral CD3+ immune cells and stromal myeloid cells significantly increases upon PARPi.
- g) PD-L1 (evaluated by FACS) in Tg tumor cells is maintained upon PARPi. PD-L1 is also expressed in intratumoral CD3+ cells and increase upon PARPi (experiment to be repeated in the next weeks). If confirmed, this data paves the way for a new rationale of PARPi and immunecheckpoint inhibitor combination. Indeed, the Impassion 070 trial demonstrated as most of the TNBC patients who responded to atezolizumab expressed PD-L1 in the immune cells compartment.

Future Perspectives

- a) To understand if the activation of the alternative STING pathway may have a role in PARPi resistance while the activation of the canonical STING pathway may be involved in PARPi sensitivity by western blot (experiments ongoing)
- b) Testing the in vivo efficacy of PARPi in combination with the recombinant IFN alpha agonist in IFNmutated PDX models.
- c) Testing the in vivo efficacy of PARPi in combination with anti-PDL1 in the transgenic mouse model



Figure 1. Material and Methods









List of Publications and Presentations Resulting from the Translational Research Project "Exploiting the DNA-damage response upon PARP inhibition in homologous recombination deficient tumors to maximize the activity of anti-PD-L1 therapy."

Publications

- 1) A RAD51 assay feasible in routine tumor samples calls PARP inhibitor response beyond BRCA mutation. M Castroviejo-Bermejo, C Cruz, A Llop-Guevara, et al. EMBO molecular medicine 10 (12), e9172
- 18P Dissecting the antitumor immune response upon PARP inhibition in homologous recombination repair (HRR)-deficient tumors. B Pellegrino, A Llop-Guevara, C Cruz, et al. Annals of Oncology 29 (suppl_10), mdy493.016
- Controversies in Oncology: homologous recombination repair deficiency (HRD) is useful for treatment decision making? B Pellegrino, J Mateo, V Serra, J Balmaña. ESMO Open (Epub ahead of print)



- 4) Homologous recombination repair deficiency and the immune response in breast cancer: a literature review. B Pellegrino, A Musolino, A LLop-Guevara, et al. Annals of Oncology (submitted)
- 5) PARP inhibition increases immune infiltration in homologous recombination repair (HRR)- deficient tumors. B Pellegrino, A LLop-Guevara, F Pedretti et al. ESMO 2019 (submitted abstract)
- 6) PARP inhibition increases immune infiltration in homologous recombination repair (HRR)- deficient tumors. B Pellegrino, A LLop-Guevara, F Pedretti et al. ESMO 2019 (in writing, to be submitted to Annals of Oncology)

Presentations

- 1) "Sliding doors. La terapia personalizzata nel carcinoma ovarico" (April 2018)
- 2) "ESMO Prostate Cancer Preceptoship" (Clinical case presentation; November 2018)

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

- 1) Lung toxicity in non–small-cell lung cancer patients exposed to ALK inhibitors: report of a peculiar case and systematic review of the literature. B Pellegrino, F Facchinetti, P Bordi, et al. Clinical lung cancer 19 (2), e151-e161
- Abstract P1-14-05: Phase II study of eribulin in combination with gemcitabine for the treatment of patients with locally advanced or metastatic triple negative breast cancer ...A Musolino, L Cavanna, D Boggiani, et al. Cancer Research 79 (4 Supplement), P1-14-05-P1-14-05
- 3) How to become a breast cancer specialist in 2018: The point of view of the second cohort of the Certificate of Competence in Breast Cancer (CCB2). G Montagna, D Anderson, J Bochenek-Cibor, et al. The Breast 43, 18-21
- 4) Metronomic Chemotherapy (mCHT) in HER2-ve Advanced Breast Cancer (ABC) Patients (PTS): When Care Objectives Meet Patients' Need. Preliminary Results of the Victor-6 Study. ME Cazzaniga, K Cagossi, MR Valerio, et al. The Breast 36, S48-S49
- 5) [68Ga] Nivolumab: a novel PET tracer to detect PD-1 expressing tumors. S Migliari, A Sammartano, B Pellegrino, et al. European Journal Of Nuclear Medicine And Molecular Imaging 44, S520-S520
- 6) 301PMetronomic chemotherapy (mCHT) in HER2-ve advanced breast cancer (ABC) patients (pts): Old drugs, new opportunities Preliminary results of the VICTOR-6 study. M Cazzaniga, K Cagossi, et al. Annals of Oncology 28 (suppl_5)
- 7) My best choice after a round trip from illness. C Tommasi, B Pellegrino, F Quaini, A Musolino. Acta Bio Medica Atenei Parmensis 88 (2), 196-197
- 8) Role of innate and Adaptive Immunity in the Efficacy of anti-HER2 monoclonal antibodies for HER2-positive breast cancer. Musolino A, Boggiani D, Pellegrino B, et al. Cancer Treatment Reviews (to be submitted)
- 9) Clinical case of a 67-years old woman affected by HER-2 positive breast cancer and autoimmune dermatomyositis. Pellegrino B, Mazzaschi G, Mori C. (submitted to NEJM)
- 10) The role of microRNAs in Breast Cancer. State of art and future perspectives. (in writing)



Selection of Courses and Workshops Attended During the Fellowship

"ESMO Prostate Cancer Preceptoship" (November 2018) "EORTC Breast Cancer group meeting" (September 2018) "Certificate of Competence in Breast Cancer 2 by Ulm University and ESO" (March 2017-April 2018) "FELASA course" (July 2017 – November 2017)

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Personal Statement

I think the ESMO Translational Fellowship was the best opportunity I had in my career. Thanks to the visibility it gave me, I had the chance to meet the most important European researchers and clinicians in the field of breast cancer and HRD. Dr Serra with all members of her lab helped me to learn the basis of several laboratory techniques and taught me the milestones of the scientific method. Thanks to their constant supervision, I have also improved my presentation skills. Dr Musolino supported me from Italy with his suggestions and advice and, as my home mentor, he is now helping me to continue collaborating with my host institute, even beyond my ESMO fellowship period. The considerable economic support of ESMO fellowship allowed me to participate at several international congresses, including ESMO congress in 2017 and 2018, helping me to share ideas and opinions with other young oncologists at the same stage of career and to improve my knowledge in the field of immune-oncology. Living in Barcelona, I have learnt Spanish and Catalan discovering such an amazing European culture. During my ESMO fellowship training, I felt the support of other ESMO fellows, who, thanks to their advice, helped me to get the best from this great experience.

I can conclude that the ESMO fellowship program made me really feel part of a constantly growing European scientific community, it made me feel properly European.

References

Gosh et al. PlosOne 2018 Murai et al. Cancer Res 2012 Iglehart & Silver N Engl J Med 2009 Parkes et al, JNCI 2017 Erdal et al, Genes Dev 2017 Harding et al, Nature 2017





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