

## References

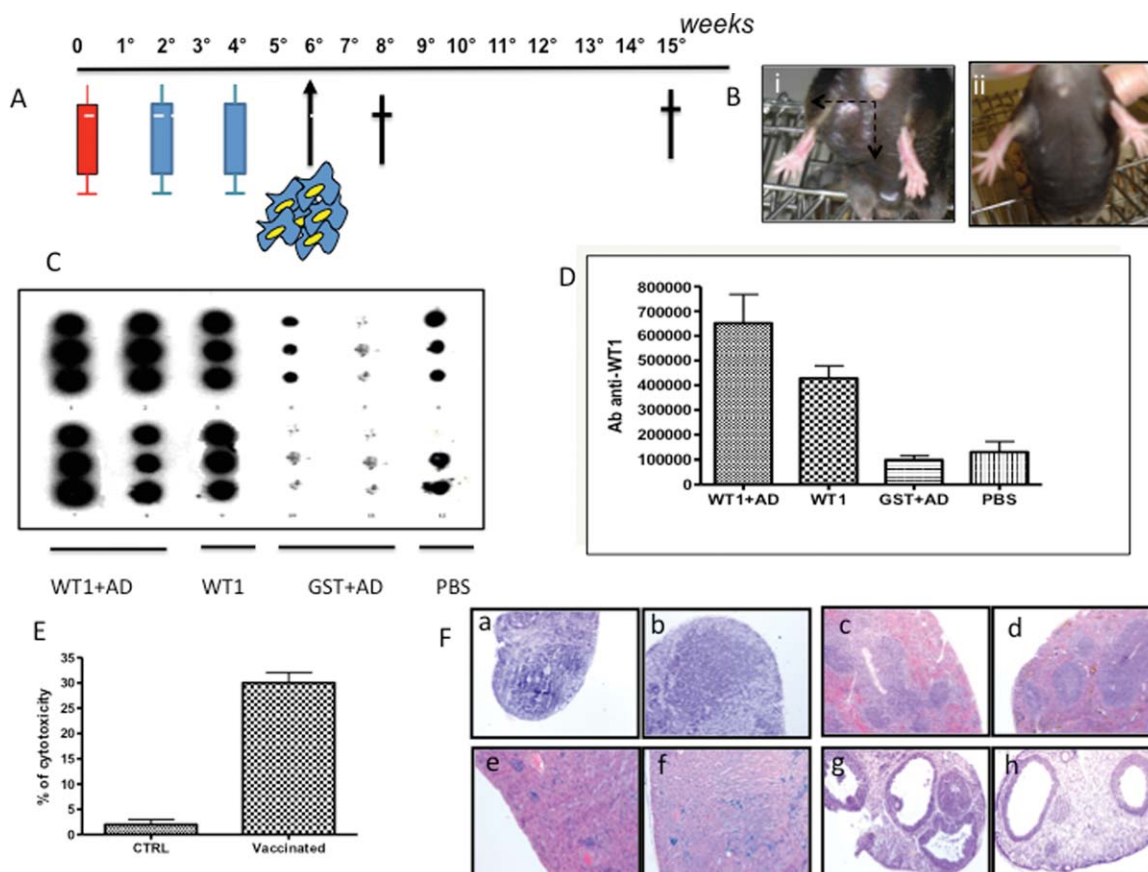
1. Calvaruso G, Vitrano A, Di Maggio R, et al. Deferiprone versus deferoxamine in thalassemia intermedia: Results from a 5-year long-term Italian multicenter randomized clinical trial. *Am J Hematol* 2015. doi: 10.1002/ajh.24024. [Epub ahead of print]
2. Taher AT, Porter J, Viprakasit V, et al. Deferasirox reduces iron overload significantly in nontransfusion-dependent thalassemia: 1-year results from a prospective, randomized, double-blind, placebo-controlled study. *Blood* 2012;120:970-977.
3. Taher AT, Musallam KM, Karimi M, et al. Overview on practices in thalassemia intermedia management aiming for lowering complication rates across a region of endemicity: The OPTIMAL CARE study. *Blood* 2010;115:1886-1892.
4. Musallam KM, Cappellini MD, Daar S, et al. Serum ferritin level and morbidity risk in transfusion-independent patients with beta-thalassemia intermedia: The ORIENT study. *Haematologica* 2014;99:e218-e221.
5. Taher AT, Porter JB, Viprakasit V, et al. Defining serum ferritin thresholds to predict clinically relevant liver iron concentrations for guiding deferasirox therapy when MRI is unavailable in patients with non-transfusion-dependent thalassaemia. *Br J Haematol* 2015;168:284-290.

## Development of cellular and humoral response against WT1 protein vaccination in mice

*To the Editor:* Many anti-cancer vaccination strategies have been tested in mice and humans in the attempt to eradicate leukemia cells [1]. The vast majority of clinical trials are based on peptide vaccination which allows the induction of cellular response to specific tumor associated antigens [2]. *WT1* (Wilms tumor-1) gene is located on chromosome 11p13 and encodes a zinc finger transcription factor that plays an important role in cell growth and differentiation. *WT1* was originally described as a tumor suppressor gene although many evidences demonstrated that it plays an oncogenic function in the setting of leukemia. *WT1* protein represents an optimal tumor antigen since it is highly expressed in acute leukemias, myelodysplastic syndromes (MDS) and myeloproliferative neoplasms [3]. By contrast, it is expressed at very low levels in normal hematopoietic progenitors. Expression of the *WT1* protein is restricted to a limited set of tissues, including the gonads, uterus, kidney, and spleen.

The success of a particular peptide vaccine to elicit an immune response is influenced by many parameters, including the presence of helper T cell epitopes, processing and presentation by professional antigen presenting cells (APCs), biodistribution, peptide length, peptide affinity, and route of administration. Recently Brayer [4] and colleagues published in this journal the results of *WT1* peptide vaccination in AML and MDS. The conclusion from this study and many others based on *WT1* peptide vaccination is that this strategy is safe, feasible but, at least in this study, it is not able to induce a consistent and measurable *WT1* specific T cell response. In the majority of the clinical trials *WT1* peptide elicited  $CD3^+ CD8^+$  T cells. Additional trials showed that the combination of short and long peptides induced also  $CD3^+ CD4^+$  T cells. Interestingly, it was shown that long peptide elicited the strongest immunological response against *WT1*. The clinical results are overall encouraging, describing several patients obtaining molecular remission, partial responses or stable disease. The main limits are the immune tolerance and immune-evasion. Two main strategies have been tested to overcome these limits, the use of long-sequence peptides preferentially processed by APCs in the lymph node, circumventing some of the tolerance mechanisms, and the addition of adjuvant to stimulate APC. Here, we report the results of *WT1* protein vaccination in mice.

The complete *WT1* murine coding sequence cloned in an expression vector (pGEX-4T-1) together with GST protein has been amplified. The fusion protein GST-*WT1* has been transfected in *E.Coli* and purified. Thirty C57BL/6 mice have been utilized according to the scheme represented in Fig. 1 panel A. The first group (10 mice) was vaccinated performing a first injection with 30  $\mu$ g of GST-*WT1* protein + 50  $\mu$ g of complete Freund adjuvant (AD) at Week 0. After 2 and 4 weeks, a second and third dose 30  $\mu$ g of GST-*WT1* protein + 50  $\mu$ g of AD were injected. The second group (5 mice) was vaccinated with 30  $\mu$ g of GST-*WT1* protein only at week 0, 2 and 4. The third group (5 mice) was vaccinated with 30  $\mu$ g of GST only plus AD at week 0, 2, and 4. The fourth group (10 mice) was treated with PBS only and used as control. After 2 additional weeks (weeks 6) 200,000 TRAMP-C cells, a syngenic prostatic cancer cell line overexpressing *WT1*, were injected subcutaneously in all animals. After 8 weeks from the first injection half the mice were sacrificed to evaluate the immune response, both cytotoxic and humoral



**Figure 1.** Panel A: Scheme of vaccination. Panel B: Response in terms of tumor burden in vaccinated mice (ii) in which the tumor is undetectable compared to control mice (i) which developed a measurable tumor mass of 1.5 cm after 8 weeks from the first vaccination. Panel C: Dot blot analysis for the detection of specific antibodies against *WT1*. The analysis has been performed after 8 weeks from the first vaccination. Panel D: Quantification of the dot blot results. Panel E:  $^{51}\text{Cr}$  release test for the evaluation of cytotoxicity. Panel F: Evaluation of organ toxicity before and after vaccination, respectively, in lymph node (a,b), spleen (c,d), kidney (e, f), and ovary (g,h).

and the tumor burden, while half of them were sacrificed after 15 weeks to evaluate immune response, tumor burden, and organ toxicity.

Dot blot analysis on mice serum showed the presence of IgG antibodies against WT1 after vaccination with GST-WT1 protein + AD and GST-WT1 protein alone. By contrast, the antibodies were not present after injection of GST + AD and PBS. (Fig. 1 panel C and D). Furthermore, cytotoxicity of T cells was evaluated by  $^{51}\text{Cr}$  release test. In mice injected with GST-WT1 protein + AD the level of cytotoxicity was  $30\% \pm 2$  compared to  $2\% \pm 0.5$  (background level) in control mice. Finally, we examined the toxicity in organs which physiologically express WT1 at low levels: lymph node, spleen, ovary, and kidney in vaccinated mice and controls. No toxicity was observed (Fig. 1 panel F). Hemocytometric analysis as well as BM smears (data not shown) excluded any kind of hematological toxicity. The mean Hb level was 13.9 gr/dL in vaccinated mice and 14.2 gr/dL in controls ( $P > 0.05$ ), the median WBC count was 5135/ $\mu\text{L}$  in vaccinated mice and 6357/ $\mu\text{L}$  in controls ( $P > 0.05$ ), the median platelet count was 1128000/ $\mu\text{L}$  in vaccinated mice and 1020000/ $\mu\text{L}$  in controls ( $P > 0.05$ ). In conclusion, vaccination with WT1 protein induces a significant cytotoxic response and a potent antibody response. This results, at least in mice, in a significant reduction of the tumor burden. The median reduction of the volume of the tumor after 8 weeks of vaccination is 62%. (Fig. 1 panel B). This strategy may allow to overcome some of the limits associated with peptide vaccination including the restriction of the HLA typing of the patient and the prevalent T CD8<sup>+</sup> response. This strategy allows to exploit the whole reactive potential of the immune system, both cytotoxic and humoral.

PAOLO NICOLI,<sup>1</sup> CHIARA CALABRESE,<sup>1</sup> ROSA MARIA PELLEGRINO,<sup>1</sup> VALENTINA ROSSO,<sup>1</sup> ENRICO BRACCO,<sup>2</sup> ELISABETTA SIGNORINO,<sup>1</sup> SONIA CARTURAN,<sup>1</sup> JESSICA PETITI,<sup>1</sup> DANIELA GALLO,<sup>1</sup> VALENTINA GAIDANO,<sup>1</sup> MARCO DE GOBBI,<sup>1</sup> ANTONELLA ROETTO,<sup>1</sup> GIUSEPPE SAGLIO,<sup>1</sup> and DANIELA CILLONI<sup>1\*</sup>

<sup>1</sup>Department of Clinical and Biological Sciences, University of Turin, Turin, Italy;

<sup>2</sup>Department of Oncology, University of Turin, Turin, Italy

Conflict of interest: Nothing to report.

\*Correspondence to: Daniela Cilloni MD, Department of Clinical and Biological Sciences, University of Turin, Turin, Italy. E-mail: daniela.cilloni@unito.it

This is an open access article under the terms of the Creative Commons Attribution NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Received for publication: 12 June 2015; Accepted: 17 June 2015

Published online: 19 June 2015 in Wiley Online Library

(wileyonlinelibrary.com)

DOI: 10.1002/ajh.24092

## References

- Oka Y, Tsuboi A, Kawakami M, et al. Development of WT1 peptide cancer vaccine against hematopoietic malignancies and solid cancers. *Curr Med Chem* 2006;13:2345–2352.
- Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA*. 2004;101:13885–13890.
- Cilloni D, Gottardi E, De Micheli D, et al. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia* 2002;16:2115–2121.
- Brayer J, Lancet JE, Powers J, et al. WT1 vaccination in AML and MDS: A pilot trial with synthetic analog peptides. *Am J Hematol*, in press. doi: 10.1002/ajh.24014.

## Rapid and reliable preimplantation genetic diagnosis of common hemoglobin Bart's hydrops fetalis syndrome and hemoglobin H disease determinants using an enhanced single-tube decaplex polymerase chain reaction assay

To the Editor: Hemoglobin (Hb) Bart's hydrops fetalis syndrome and Hb H disease are the most severe forms of  $\alpha$ -thalassaemia, with estimated annual affected births of 14,000 globally [1,2]. Within Asia, both the  $-\alpha^{3,7}$  and  $-\alpha^{4,2}$   $\alpha^+$ -thalassaemia deletions are prevalent, as are the  $-\alpha^{SEA}$ ,  $-\alpha^{FIL}$ ,  $-\alpha^{THAI}$ , and  $-\alpha^0$   $\alpha^0$ -thalassaemia deletions, whereas  $-\alpha^{3,7}$ ,  $-\alpha^{MED}$ , and  $-\alpha^{(20,5)}$  are common in the Mediterranean region [3]. Preimplantation genetic diagnosis (PGD) for Hb Bart's hydrops fetalis syndrome can be achieved by gap polymerase chain reaction (PCR) [4,5], which requires customized primer sets for different combinations of deletion types, or by PCR of intradeflection microsatellite markers [6]. Indirect linkage-based PGD can be offered in cases where one or both members of the couple carry a nondeletional or undetermined mutation [7].

We recently described a general strategy for PGD of deletional Hb Bart's hydrops fetalis syndrome through multiplex PCR of intradeflection markers 16PTEL05 and 16PTEL06, supplemented by haplotype analysis of seven flanking microsatellite markers [8]. A significant deficiency of the marker panel, however, was its inability to detect the most common Hb H disease genotype ( $-\alpha^{3,7/-}$ ). We now describe an enhanced single-tube assay that can be used for PGD of all common deletional determinants of Hb Bart's hydrops fetalis syndrome and Hb H disease. We did this by adding an amplicon from the Y1 box region between the HBA2 and HBA1 genes to the nine closely linked microsa-

tellite markers (Fig. 1A). Multiplex PCR amplification was performed essentially as described [8], but with the addition of 0.05  $\mu\text{M}$  of primers Y1-F (5'-GACCTGATGCACTCTCAAAG-3') and Y1-R (5'-AAGGATATGTATTAGTGGAGGAGGT-3').

With this enhanced assay, deletional Hb Bart's hydrops fetalis syndrome will now be characterized by null amplification of 16PTEL05, 16PTEL06, and Y1 products. More importantly, Hb H disease involving the common  $-\alpha^{3,7/-}$  genotype can now be detected, based on null amplification of the Y1 box fragment, whereas Hb H disease involving the less common  $-\alpha^{4,2/-}$  genotype is detected by null amplification of 16PTEL05 (Fig. 1B).

The optimized assay was applied to six clinical PGD cases. The first case involved PGD for Hb H disease due to  $-\alpha^{3,7/-SEA}$  genotype. Embryo 1 displayed one allele each for 16PTEL05 and 16PTEL06 whereas Y1 was absent, indicative of affected genotype ( $-\alpha^{3,7/-SEA}$ ) (Fig. 1C). Embryo 2 displayed two alleles each for 16PTEL05 and 16PTEL06, and positive amplification of Y1, indicating either an unaffected ( $\alpha\alpha/\alpha\alpha$ ) or silent carrier ( $\alpha\alpha/\alpha^{3,7}$ ) embryo. Embryo 3 displayed one allele each for 16PTEL05 and 16PTEL06 and was positive for Y1, indicative of  $\alpha$ -thal-1 carrier genotype ( $\alpha\alpha/-SEA$ ). Flanking marker diplotypes were consistent with the mutation genotypes, identifying Embryo 2 to be a silent carrier ( $\alpha\alpha/\alpha^{3,7}$ ). Both carrier embryos were transferred, a singleton pregnancy ensued, and a healthy baby girl was delivered. The second PGD case involved Hb H disease due to  $-\alpha^{4,2/-SEA}$  genotype. Three embryos were analyzed and diagnosed as affected ( $-\alpha^{4,2/-SEA}$ ), unaffected ( $\alpha\alpha/\alpha\alpha$ ), and silent carrier ( $\alpha\alpha/\alpha^{4,2}$ ), respectively (Fig. 1D). The unaffected and silent carrier embryos were transferred without pregnancy success.

The third PGD case involved a woman affected with  $-\alpha^{3,7/-SEA}$  type Hb H disease and her  $\alpha\alpha/-SEA$  carrier spouse. One embryo was diagnosed as a silent carrier ( $\alpha\alpha/\alpha^{3,7}$ ), three were  $\alpha$ -thal-1 carriers ( $\alpha\alpha/-SEA$ ), one had Hb H disease ( $-\alpha^{3,7/-SEA}$ ), and three had Hb Bart's hydrops fetalis syndrome ( $-\alpha^{SEA}/-SEA$ ) (data not shown). Two carrier embryos were transferred, resulting in a biochemical pregnancy (elevated maternal human chorionic gonadotropin 2 weeks after embryo transfer), which was subsequently lost.

The remaining three PGD cases involved Hb Bart's hydrops fetalis syndrome. Altogether, five embryos were affected ( $-\alpha^{SEA}/-SEA$ ), five were carriers ( $\alpha\alpha/-SEA$ ), and two were unaffected ( $\alpha\alpha/\alpha\alpha$ ). Carrier embryos were transferred in two cases, but without pregnancy success.

Although allele drop-out, which is the random failure to detect either one of two expected target alleles, was observed at 1–3 marker loci in some samples, the remaining markers in the panel enabled unambiguous haplotype phasing of unaffected and mutant chromosomes to be established in all instances. This enabled unambiguous diagnoses for all embryos in all six clinical PGD cases, underlining the utility of mutation detection plus haplotype analysis in  $\alpha$ -thalassaemia PGD. This single-tube decaplex PCR assay should be applicable to PGD involving other common  $\alpha$ -thalassaemia deletional determinants and should also be useful for PGD of nondeletional disease determinants when an index affected family member is available to establish disease haplotype phase.

## Author Contribution

S.S.C. conceptualized the assay design and revised the manuscript. M.C. performed the assay optimization and validation and wrote the manuscript. S.L.Y., Sa.N., S.C.N., H.H.T., Su.N., P.C.W., and S.F.L. co-ordinated patient care, and E.B.P. the IVF/ICSI and embryo transfer procedures, as well as reviewed and approved the manuscript. A.S.C.T., F.S.H.C., and E.E.L.S. performed the clinical PGD testing and revised the manuscript.

MIN CHEN<sup>1,2</sup> SEONG FEEI LOH<sup>3</sup> SU LING YU<sup>4</sup> SURESH NAIR<sup>5</sup> HENG HAO TAN<sup>6</sup> SADHANA NADARAJAH<sup>6</sup> PENG-CHEANG WONG<sup>7</sup> SOON CHYE NG<sup>8</sup> ETHIRAJ B. PRASATH<sup>3</sup> ARNOLD S.C. TAN<sup>1,9</sup> FELICIA S.H. CHEAH<sup>9</sup> EUGENE E.L. SAW<sup>9</sup> SAMUEL S. CHONG<sup>1,9,10\*</sup>  
<sup>1</sup>Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; <sup>2</sup>Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore; <sup>3</sup>Thomson Fertility Centre, Thomson Medical Centre, Singapore; <sup>4</sup>Centre for Assisted Reproduction, Department of Obstetrics & Gynaecology, Singapore General Hospital, Singapore; <sup>5</sup>Gynecology Consultants Clinic and Surgery, Singapore; <sup>6</sup>KKIVF, Department of Reproductive Medicine, KK Women's and Children's Hospital, Singapore; <sup>7</sup>Clinic for Human Reproduction, Department of Obstetrics and Gynecology, National University Hospital, Singapore; <sup>8</sup>Sincere IVF Center, Sincere Healthcare Group, Singapore; <sup>9</sup>Preimplantation Genetic Diagnosis Center, Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore; <sup>10</sup>Department of Laboratory Medicine, National University Hospital, Singapore  
 Contract grant sponsor: Health Services Development Program, Singapore Ministry of Health; Contract grant number: HSDP 05/X01.

Conflict of interest: All authors declare no competing financial interests  
 \*Correspondence to: Samuel S. Chong, Ph.D., Preimplantation Genetic Diagnosis Center, Level 3, Main Building, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074, Singapore. E-mail: paecs@nus.edu.sg

Received for publication: 6 May 2015; Revised: 22 May 2015; Accepted: 27 May 2015

Published online: 4 June 2015 in Wiley Online Library

(wileyonlinelibrary.com)

DOI: 10.1002/ajh.24077