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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, al 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 immunologial response against WT1. The clinical results are overall encouraging, describing several patients obtainig 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, cincumventing 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. Thirthy C57BL/6J 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 μg of GST-WT1 protein + 50 μg of complete Freund adjuvant (AD) at Week 0. After 2 and 4 weeks, a second and third dose 30 μg of GST-WT1 protein + 50 μg of AD were injected. The second group (5 mice) was vaccinated with 30 μg of GST-WT1 protein only at week 0, 2 and 4. The third group (5 mice) was vaccinated with 30 μg 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 singenic prostatic cancer cell line overexpressing WT1, were injected subcutaneously in all animals. After 8 weeks from the first injection half the mice were sacrified to evaluate the immune response, both cytotoxic and humoral

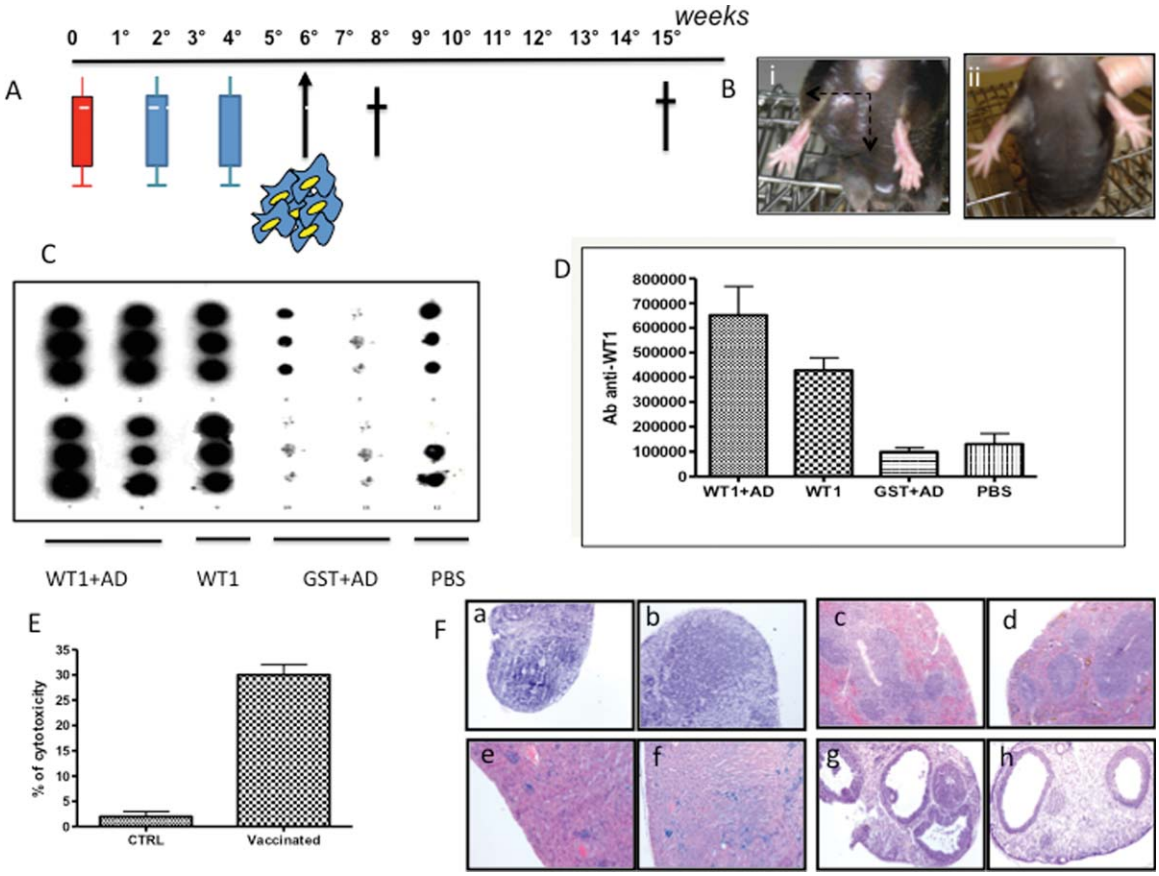

Figure 1. **Panel A**: Scheme of vaccination. **Panel B**: Response in terms of tumor bourden in vaccinated mice (ii) in which the tumor is undetectable compared to control mice (i) which developed a measurable tumor mass of 1.5 cm after 8 weeks from the first vaccination. **Panel C**: Dot blot analysis 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}Cr release test for the evaluation of cytotoxicity. **Panel F**: Evaluation of organ toxicity before and after vaccination, respectively, in lymphonode (a,b), spleen (c,d), kidney (e, f), and ovary (g,h).

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

Dot blot analysis on mice serum showed the presence of IgG antibodies againt 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}Cr release test. In mice injected with GST-WT1 protein + AD the level of cytotoxixity was 30% ± 2 compared to 2% ± 0.5 (background level) in control mice. Finally, we examined the toxixity in organs which physiologically express WT1 al low levels: lymphonode, spleen, ovary, and kidney in vaccinated mice and controls. No toxicity was observed (Fig. 1 panel F). Hemocromocytometric 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/μl in vaccinated mice and 6357//μl in controls ($P > 0.05$), the median platelet count was 1128000/μl in vaccinated mice and 1020000/μl 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 borden. The median reduction of the volume of the tumor after 8 weeks of vacciantion 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$^+$ response. This strategy allows to exploit the whole reactive potential of the immune system, both cytotoxic and humoral.

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Conflict of interest: Nothing to report.

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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 α-thalassemia, with estimated annual affected births of 14,000 globally [1,2]. Within Asia, both the -$\alpha^{3.7}$ and -$\alpha^{4.2}$ α^+-thalassemia deletions are prevalent, as are the --^{SEA}, --^{FIL}, --^{THAI}, and --^{SA} α^0-thalassemia deletions, whereas -$\alpha^{3.7}$, --^{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 intradeletion 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 intradeletion 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 microsatellite markers (Fig. 1A). Multiplex PCR amplification was performed essentially as described [8], but with the addition of 0.05 μM of primers Y1-F (5′-GACCTGATGCA CTCCTCAAAG-3′) and Y1-R (5′-AAGGATATGTATTAGGTGGAGGAGGT-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 (αα/αα) or silent carrier (αα/-$\alpha^{3.7}$) embryo. Embryo 3 displayed one allele each for *16PTEL05* and *16PTEL06* and was positive for *Y1*, indicative of α-thal-1 carrier genotype (αα/--^{SEA}). Flanking marker diplotypes were consistent with the mutation genotypes, identifying Embryo 2 to be a silent carrier (αα/-$\alpha^{3.7}$). 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 (αα/αα), and silent carrier (αα/-$\alpha^{4.2}$), 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 αα/--^{SEA} carrier spouse. One embryo was diagnosed as a silent carrier (αα/-$\alpha^{3.7}$), three were α-thal-1 carriers (αα/--^{SEA}), one had Hb H disease (-$\alpha^{3.7}$/--^{SEA}), and three had Hb Bart's hydrops fetalis syndrome (--^{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 (--^{SEA}/--^{SEA}), five were carriers (αα/--^{SEA}), and two were unaffected (αα/αα). 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 α-thalassemia PGD. This single-tube decaplex PCR assay should be applicable to PGD involving other common α-thalassemia 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[1,2] Seong Feei Loh[3] Su Ling Yu[4] Suresh Nair[5] Heng Hao Tan[6] Sadhana Nadarajah[6] Peng-Cheang Wong[7] Soon Chye Ng[8] Ethiraj B. Prasath[3] Arnold S.C. Tan[1,9] Felicia S.H. Cheah[9] Eugene E.L. Saw[9] Samuel S. Chong[1,9,10]*

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