Design of a Neo-Epitope Vaccine by a Combination of Prediction Tools & *in vitro* Peptide MHC Stability Binding Assays

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The recent progress of checkpoint inhibitors in cancer immunotherapy has led to remarkable clinical results in several tumor types. It is becoming increasingly evident that immune recognition of tumor specific cancer neo-epitopes plays a crucial role in successful tumor rejection [1]. The therapeutic targeting of cancer-specific neo-epitopes is being pursued as an opportunity for the generation of personalized cancer vaccines. A major obstacle for the successful implementation of this strategy remains to identify the immunogenic neo-epitopes among potentially thousands of tumor mutations. *In silico* epitope prediction tools such as netMHC and IEDB are often being used for this task, but the precision of these can be relatively low and in vitro confirmation would be desirable.

Introduction

In this work we use a combination of in silico prediction tools and in vitro MHC peptide stability assay to design a neo-epitope based vaccine for a patient with colorectal cancer. We used stability based assays since we and others have found that peptide/MHC stability is a better predictor of immunogenicity than affinity [2, 3]. The whole process from in silico prediction to *in vitro* stability testing was completed in less than 3 weeks.

Materials & Methods

Whole exome sequencing was performed on tumor biopsy and blood and more than 20,000 somatic mutations were identified through variant calling. The 120 best binding neo-epitopes to eight of the patient's MHC alleles (A*0101, A*2402, B*1301, B*3501, C*0401, C*0702, DRB1*0401, DRB1*1501) were predicted by netMHC and netMHCpan. Crude microscale **PepTrack™ Fast Track Peptide Libraries** from JPT were refolded with MHC for 24 hrs and transferred in quadruples to 384 plates where the complexes were subjected to urea denaturation at 0, 2, 4 and 6M. After incubation, plates were washed and developed using conformational specific antibodies that only recognizes mature peptide bound MHC complexes. The stability of the individual peptides was calculated relative to a known T cell reference peptide that was included in position H12 on the microscale peptide plates. To further reduce the number of neo-epitope vaccine candidates, peptides derived from known housekeeping genes was used as selection criteria. For some alleles and peptides a 5 point affinity measurement was done by doing peptide titration followed by addition of MHC and development by the alpha screen platform from Perkin Elmer [4]

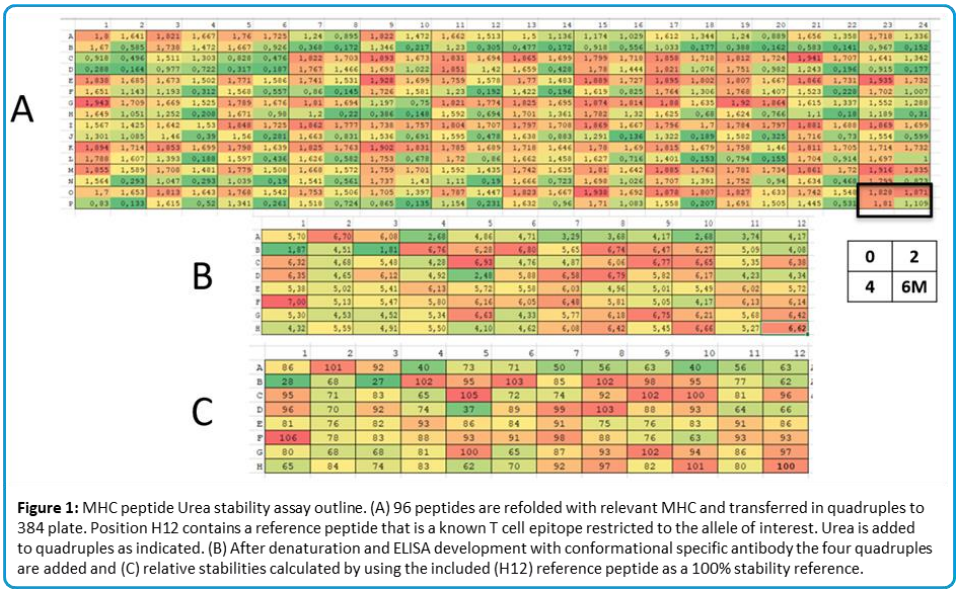

Figure 1: MHC peptide Urea stability assay outline. (A) 96 peptides are refolded with relevant MHC and transferred in quadruples to 384 plate. Position H12 contains a reference peptide that is a known T cell epitope restricted to the allele of interest. Urea is added to quadruples as indicated. (B) After denaturation and ELISA development with conformational specific antibody the four quadruples are added and (C) relative stabilities calculated by using the included (H12) reference peptide as a 100% stability reference.

Results & Discussion

Generally, it is our experience that there is a reasonably good correlation between predicted and experimentally determined Kd values for well-studied alleles such as A*0101, A*0201, A*1101, and others, where an abundance of Kd measurement data exist, that can be used for training. This can be seen in **figure 2 (A)**, 95 peptides were predicted to have Kd values less than 100nM on A*0101, *in vitro* measurements confirmed that peptides bound with affinities lower than 100nM, most of them even lower than 10nM. For B*3501 (B) a reasonable correlation also seems to exist between predicted and determined values. This correlation is however lost for C*0401 (C), strangely the top 95 predicted Kd values were abnormally high (mean 1046nM) whereas in vitro measurements showed that app 20 of these peptides bound with Kd values lower than 100nM. The bad prediction for C*0401 confirms what we have seen for ather C alleles we have studied (C*0701, C*0702 and C*0102). Prediction for B*1301 was done using the netMHCpan, this type of predictor provides a predicted percentile rank rather than a Kd value. According to netMHCpan a rank below 0.5 is a good binder; however all our peptides ranked below 0,1 (most of them less than 0,01), and thus should all bind extremely well. This was not confirmed by in vitro analysis that showed a wide spectrum of affinities.

Approximately 960 peptides were analyzed for their stability of binding to 8 MHC alleles (120 per allele). The analysis identified

more than 150 peptides that bound with similar or higher stability to the intended MHC than the included reference peptide. To further reduce the number of peptides to a more suitable number for vaccination we further defined a vaccine candidate as a peptide originating from a globally expressed housekeeping gene. In doing so we limited the number of neo-epitope vaccine candidates to 46 peptides.

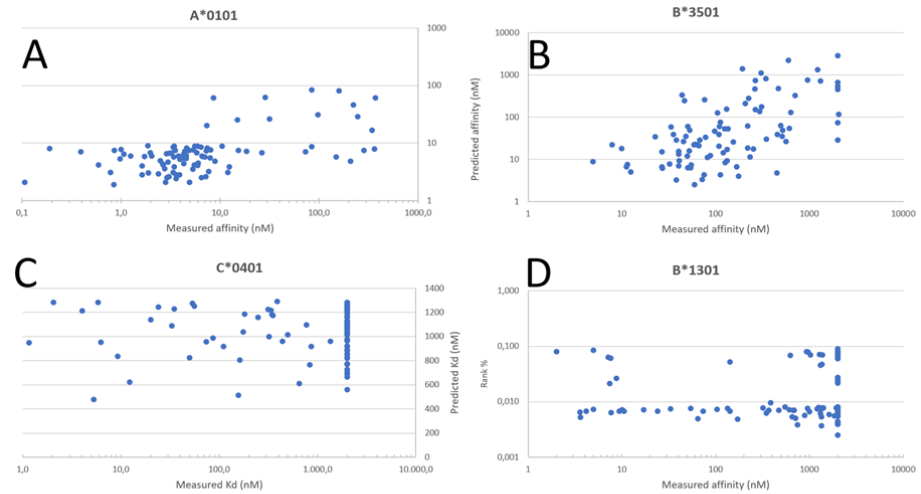

Figure 2:: Comparison of predicted and measured Kd for 96 peptides on 4 MHC I alleles. (A): A*0101, (B): B*3501, (C): C*0401 and (D): B*1301 netMHCpan was used to predict binding. A rank below 0,5 is accepted as a good binder. Kd values above 2000nM are normally considered NON BINDERS and are given the value 2000nM.

Concluding Remarks

This work clearly shows that prediction tools such as netMHC and in particularly netMHCpan should be used with caution and that confirmation by in vitro stability assay has the potential to increase the chance of success for neo-epitope vaccination. We further prove that this analysis, filtering and confirmation by *in vitro* assays can be done within a relatively narrow timeframe.

Table 1: Summary of work

Allele	Peptides selected for stability analysis	Average predicted affinity (nM,)/Rank*	>100% stability	Selected for vaccination
A*0101	120	9	18	5
A*2402	120	17	25	10
B*1301	120	0,1*	12	3
B*3501	120	115	25	7
C*0401	120	1043	8	3
C*0702	120	40	20	6
DRB1*0401	120	68	8	6
DRB1*1501	120	64	55	6
Total	960		171	46

References

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For more than a decade responsible for developing and maintaining and one of the largest collections of recombinant Major Histocompability Complex I and II molecules at the University of Copenhagen. In a decade long NIH and EU funded effort, these molecules were used in sensitive peptide binding assays to generate massive amounts of peptide binding data and later used to train artificial neural network driven epitope prediction tools. The free and accessible tools (netMHC I/II/pan, NN align etc) are recognized as best in class and used extensively: to identify neo-epitopes, study diseases, develop novel vaccines and create better and safer bio-therapeutics.

In 2014 the know-how gained from the netMHC project was spun-out into **Immunitrack**. Apart from developing PrdX a new best in class T cell epitope predicition tool based on stability measurements, Immunitrack offers highly sensitive peptide MHC affinity and stability assays. Our biotinylated MHC I and II molecules also work excellent as tetramer reagents and we have recently developed HTP production methods for both MHC I and II to address the unmet need for customized MHC/epitope complexes and reagents in the neo-epitope field.
The combination of our assay technology and supporting MHC I/II molecules provides a very strong platform for most vaccine projects.

The Company

JPT Peptide Technologies is an ISO 9001:2015 certified provider of innovative peptide solutions for immunotherapy development, cellular and humoral immune monitoring, epitope & target discovery, targeted proteomics, and enzyme profiling.

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