In vitro validation of the immunogenicity of the predicted neoepitopes from high-risk estrogen receptor-positive breast cancer

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Abstract

Whether estrogen receptor-positive (ER+) breast cancer (BC) can be a target for therapeutic neopeptide vaccination is not clear due to its low mutation burden.

We tested the immunogenicity of predicted neopeptides from exome and RNA-seq data from three ER+/luminal B subtype BC samples using IFN- γ ELISpot assays of HLA-matched donor PBMCs. As a control, three ER- BC and three lung cancers were tested. The ensemble of Neopepsee and pVACseq pipelines predicted 93 neopeptides from 299 SNVs in three ER+ BCs. Among them, 90 could be tested with ELISpot, and 14 (15.6%) were immunogenic (1, 5, and 10 for each tumor). In three ER- BC samples, 52 neopeptides were predicted from 271 SNVs, and 12 (25.0%) of 48 tested were immunogenic (2, 4, and 8 for each tumor). Of the three lung cancers, 53 of 72 predicted neopeptide candidates were tested, and 10 of them were immunogenic (18.9%) (0, 1, and 11 for each tumor). These differences were not statistically significant.

We conclude that luminal B subtype BCs express neopeptides and can be a candidate for therapeutic neopeptide vaccination.

Introduction

Cancer results from the accumulation of somatic mutations.¹ The Cancer Genome Atlas (TCGA) results demonstrated that most solid tumor cells have an average of 50 nonsynonymous mutations.² Minor fractions of peptides encoded by somatic mutations in cancer cells are presented by major histocompatibility complex (MHC) class I proteins and induce an antitumor immune response by T lymphocytes.³ Such mutant peptides are called “neopeptides”.

Neopeptides are thought to be the best targets for therapeutic cancer vaccines since, unlike nonmutated tumor-associated antigens, they are expected to induce a robust T-cell immune response due to a lack of central tolerance and are unlikely to induce autoimmunity to normal cells.^{4–6} Adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes enriched for neopeptide-specific T cells resulted in remarkable responses in some patients, especially when administered together with immune checkpoint inhibitors, demonstrating that mutated neopeptides are valid therapeutic targets.⁷

Early results from dendritic cell, mRNA, or peptide vaccination against neopeptides demonstrated induction of antitumor immunity and evidence of epitope spreading as well as a suggestion of clinical efficacy when used in combination with immune checkpoint inhibitors.^{8–11} The near absence of dose-limiting toxicities due to off-target immune responses against normal tissue in both therapeutic vaccine and adoptive cell transfer trials is reassuring.^{8–11} With the advancement of timely identification of neopeptide candidates, many clinical trials are now ongoing with a variety of vaccine platforms targeting neopeptides.^{4,5,12}

While multiple neopeptide vaccine trials are ongoing for solid tumors, ER+ BC patients are usually not targeted due to their lower mutation burden and hence lower predicted neopeptide burden.^{13–15} Among ER+ BC, the luminal B subtype is characterized by a high proliferation rate and poor outcome.¹⁶ Although they are sensitive to chemotherapy, their clinical outcome is still worse than that of other ER+ BC (luminal A subtype) even after chemotherapy.^{17,18} In the TAILORx trial for node-negative ER+ BC patients, 15% of patients with OncotypeDx recurrence scores over 25 (i.e., luminal B) experienced recurrence at a distant site within 9 years even after chemoendocrine therapy.¹⁹ Prognosis after distant recurrence events is dire with no curative therapy available.²⁰ Therefore, it is worthwhile to explore neopeptide-directed immunotherapy in this disease population. Although many studies have reported the neopeptide landscape of BC, most of them lack experimental validation of the immunogenicity of the predicted neopeptides.^{21–23} When considering the low positive predictive value of the prediction algorithms employed in those studies,²⁴ it is reasonable to conclude that the true immunogenicity of ER+ BC is largely unknown. In this study, we tested the immunogenicity of predicted neopeptides from 3 ER+ luminal B BCs using an ELISpot assay with HLA-matched PBMCs from three healthy donors.^{25,26} As a comparator, we included 3 ER- BC and 3 PDXs derived from lung cancers.

Previously, we reported a neopeptide prediction model, “Neopepsee”, which was trained with multiple features that may influence the immunogenicity of mutated peptides.²⁷ Although Neopepsee showed good performance compared to previously reported algorithms in a validation set *in silico*, experimental validation was lacking. Therefore, as an integral secondary aim, we compared the positive predictive value of Neopepsee in comparison to that of pVACseq.²⁸

Another secondary aim was to examine whether whole-exome and RNAseq-based HLA typing are reliable by comparing the results with two-digit clinical HLA genotyping and dedicated NGS-based HLA typing kits.

Methods

Subjects and ethics requirements

Fresh tumor and peripheral blood samples were obtained from six breast cancer patients undergoing surgery at Yonsei Cancer Center with written informed consent and institutional review board approval (Severance Hospital IRB approval number: 4-2017-0715). All patients except one had not received any systemic treatment or radiotherapy in the biopsied area before surgery.

Anonymized patient-derived xenografts (archived frozen at F4 generation) with paired peripheral blood samples were available for three lung cancer patients with informed consent (Severance Hospital IRB approval number 4-2016-0788). Leukoreduction system (LRS) chamber blood cells from 50 healthy donors were obtained from the central blood bank of Korea (Severance Hospital IRB approval number: 4-2018-0803).

All methods were performed in accordance with the relevant guidelines and regulations.

HLA genotyping

We used Sanger sequencing-based clinical HLA genotyping results with two-digit resolution (BIOWITHUS Inc. Seoul, Korea). In addition, we applied Optitype²⁹ and HLAMiner³⁰ and used the Omixon Holotype HLA™ kit (Omixon Biocomputing Ltd, Budapest, Hungary) to evaluate NGS-based HLA typing methods.

Neoepitope candidate selection

Paired whole-exome sequencing (WES) and RNA sequencing were performed at Yonsei Genome Center. A WES library was generated using SureSelect Exome V7 (Agilent Technologies, CA, USA) for breast cancer samples or V5 for lung cancer samples, and sequencing data were produced using Novaseq6000 (Illumina, CA, USA). Multiplexing for sequencing was designed to acquire more than 200X depth for tumor samples and 100X depth for matched normal samples. A total RNA sequencing library was generated using a TruSeq Stranded Total RNA Library Prep Kit (Illumina), and sequencing data were produced using NovaSeq 6000 (except for sample Neo 1, which was sequenced using NextSeq 550) to acquire more than 5 G data for each sample.

Tumor-normal paired WES reads were aligned to the human reference genome with BWA-MEM.³¹ To reduce mouse contamination from patient-derived xenograft (PDX) sample sequencing data, we made a concatenated reference of mice and humans as recommended and performed an alignment with concatRef on the PDX of 3 lung cancer patients.³² We used Picard (version 2.19) (<https://broadinstitute.github.io/picard/>) to sort the sequencing reads in order of genomic coordinates, to remove PCR duplicates and to fix the mate information of the paired-end sequence read.

Somatic mutations were identified via local assembly of haplotypes using Mutect2.³³ Following the GATK Best Practices recommendation, FilterMutectCalls GATK4 (version 4.0.8.0.1) was used for confident somatic calls, reads with base quality lower than 30 were removed, and germline variants were filtered with gnomAD.³⁴ We selected missense mutations with more than 20 depth and more than five alternative allele counts and manually reviewed calls with Integrative Genomics Viewer (IGV).

The patient-derived reliable somatic mutations and patient HLA genotyping information and RNA sequencing data were prepared as input for NoePepsee to predict patient-specific neoepitope candidates.²⁷ NoePepsee classifies potential neoantigens into three categories concerning predicted immunogenicity: high, medium, and low levels. Peptides at the high and medium levels were selected as candidates. The pVACseq pipeline was run at the Carreno laboratory at the University of Pennsylvania as reported with modified selection criteria (IC50 <100 nM instead of 500 nM, DNA VAF>20, and RNA VAF>0).²⁸

Processing of donor blood

Leukoreduction system (LRS) chambers from anonymous donors were obtained from the central bank. After removing 500 microliters for clinical HLA typing, a buffy coat was collected after Ficoll gradient centrifugation at 2,000 rpm for 25 minutes at room temperature. The buffy coat was washed in PBS and resuspended in CellBanker2 solution (AMS Biotechnology Ltd, Abingdon, U.K.) and stored in the deep freezer.

Clinical HLA genotyping of donor blood

DNA was extracted from 300 µL of whole blood using the Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany). Alternatively, DNA from PBMCs was isolated using the DNeasy Blood & Tissue Kit (QIAGEN). DNA was eluted in 30 µL nuclease-free water, and samples were stored in a -80°C deep freezer until analysis. Sequences were analyzed with BIOWITHUS Inc. (Seoul, Korea).

Peptide synthesis

All peptides were synthesized, HPLC purified to over 98% by AnyGen (Gwangju, Korea) and resolved in DMSO at a stock concentration of 10 mg/ml. A working concentration of 10 µg/ml was used for the ELISpot assay.

ELISpot assay

White blood cells from HLA-matched donors were subjected to an ELISpot assay. An IFN- γ precoated ELISpot assay kit (Cellular Technology Limited, Cleveland, Ohio) was used to determine the frequency of IFN- γ generating cells.²⁶ The ELISpot assay was calibrated with influenza or CMV viral peptides.

Briefly, frozen PBMCs isolated from LRS chambers were thawed and rested overnight in culture medium consisting of CTS optimizer T cell expansion medium SFM (Gibco), human serum (5%), and 20 Unit Interleukin-2 (Gibco). PBMCs were plated in U-bottom 96-well plates (10^5 cells per well) in culture medium. PBMCs in each well were incubated with the corresponding peptide (10 µg/ml) at three-day intervals for peptide stimulation. After three cycles of peptide stimulation, stimulated PBMCs were replated onto IFN- γ -coated ELISpot plates (2×10^5 cells per well) followed by peptide restimulation for 24 hours. After restimulation with the peptide (on day 10), IFN- γ production by the specific T-cell response to each peptide was estimated by ELISpot assay according to the manufacturer's instructions. Peptide-stimulated PBMCs were evaluated and compared with the media (no-peptide) control, and phytohemagglutinin (PHA) was used as a positive control. An ELISpot CTL reader was used for scanning the ELISpot assay plate, and Immunospot software was used to analyze spot information.

Since there were multiple alleles expressed in each donor blood, we used three donors for each test, and a peptide was called a true neoepitope only when more than two of the 3 HLA-matched donor PBMCs showed spot counts greater than 20% above the no peptide control upon rechallenge with the peptide after ten days of incubation with the peptide and cytokines. PHA was used as a positive control.

Result

Patient characteristics

Among six primary breast cancer patients, 3 were clinically triple-negative subtypes (Case ID. NEO 1,2,6; Table 1), a type of breast cancer with negative expression of estrogen, progesterone, and human epidermal growth factor receptor-2 (HER2), and 3 were hormone-receptor-positive breast cancer with luminal B subtype with high Ki67 labeling index (Case ID, NEO 3,4,5; Table 1). In hormone receptor-positive breast cancer patients, 2 showed invasive lobular carcinoma histology. At the time of sample collection, patients were not undergoing therapy except one patient (NEO2). NEO2 patients had received neoadjuvant chemotherapy (AC [doxorubicin/cyclophosphamide]) followed by a weekly paclitaxel regimen but withdrew neoadjuvant chemotherapy after one paclitaxel infusion due to early disease progression and underwent surgery.

Two PDXs established from surgical specimens from squamous cell carcinoma of the lung (AV10 and AV38) and one from non-small-cell lung cancer (AV10) were used to represent lung cancer (Table 1).

Table 1
Patient demographics.

Case ID	Age	Histologic Type	Histologic Grade	Ki67 (%)	Sample source	Subtype	Stage	HLA type
NEO1	60	IDC	3	77.2	Surgery	TNBC	Stage IIA (pT1N1M0)	A*24:02, A*33:03
NEO2	47	IDC	3	66.1	Surgery	TNBC	ypStage IA (ypT1N0M0)	A*24:02, A*33:03
NEO3	74	IDC	2	63.4	Surgery	Luminal B BC	Stage IIA (pT2N0M0)	A*02:06, A*11:01
NEO4	52	ILC	2	55.6	Surgery	Luminal B BC	Stage IIA (pT2N0M0)	A*02:06, A*11:01
NEO5	52	ILC	2	47.2	Surgery	Luminal B BC	Stage IIA (pT2N0M0)	A*02:01, A*02:01
NEO6	74	IDC	3	82.3	Surgery	TNBC	Stage IIA (pT2N0M0)	A*02:01, A*24:02
AV10	NA	Squamous cell carcinoma	NA	NA	PDX	SQCC	stage IIB (T2bN1M0)	A*32:01, A*33:03
AV13	NA	Non-small cell	NA	NA	PDX	NSCLC	stage IIIA (T2bN2 M)	A*02:01, A*30:04
AV38	NA	Squamous cell carcinoma	NA	NA	PDX	SQCC	stage 1b	A*01:01, A*31:01
IDC; invasive ductal carcinoma, ILC; invasive lobular carcinoma, TNBC; triple-negative breast cancer, BC; breast cancer, SQCC; squamous cell carcinoma, NSCLC; non-small cell carcinoma of the lung, PDX; patient-derived tumor xenograft								

Comparison of HLA genotyping methods

We used Sanger sequencing-based clinical HLA genotyping results with two-digit resolution (BIOWITHUS Inc. Seoul, Korea). In addition, we applied Optitype²⁹ and HLAMiner³⁰ and used the Omixon Holotype HLATM kit (Omixon Biocomputing Ltd, Budapest, Hungary) to evaluate NGS-based HLA typing methods (Table 2).

Table 2
HLA genotyping results comparison among three different methods.

Case ID	HLA-A genotype (clinical)	OptiType	HLAminer	Omixon Holotype
NEO1	A*24:02, A*33:03	A*24:02, A*33:03	A*24:02, A*33:03	A*24:02, A*33:03
NEO2	A*24:02, A*33:03	A*24:02, A*33:03	A*24:02, A*33:03	A*24:02, A*33:03
NEO3	A*02:06, A*11:01	A*02:06, A*11:01	A*02:06, A*11:01	A*02:06, A*11:01
NEO4	A*02:06, A*11:01	A*02:06, A*11:01	A*02:06, A*11:01	A*02:06, A*11:01
NEO5	A*02:01, A*02:01	A*02:01, A*02:01	A*02:01, A*02:01	A*02:01, A*02:01
NEO6	A*02:01, A*24:02	A*02:01, A*24:02	A*24:95, A*69:01	A*02:01, A*24:02
AV10	A*32:01, A*33:03	A*33:03 , A*33:03	A*33:147, NA	ND
AV13	A*02:01, A*30:04	A*02:01, A*30:04	A*30:04, A*69:01	ND
AV38	A*01:01, A*31:01	A*01:01, A*31:01	A*01:01, A*36:03	ND
Average accuracy	reference	17/18 (94%)	11/18 (61%)	12/12 (100%)
Note: bold words represent mismatches				
There was a high concordance between NGS-based OptiType and clinical HLA genotyping tests with 94% agreement (Supplementary Table 1). However, the concordance rate for HLAminer was low (61%). Omixon Holotype assay results were 100% concordant with clinical genotypes.				
These results suggest that HLA typing with the OptiType algorithm for exome or RNAseq data are good alternatives for clinical HLA typing tests when analyzing candidate neopeptide selection, without the need to use costly dedicated kits for HLA genotyping.				

Candidate neopeptide selection

Paired whole-exome sequencing of tumor and PBMC DNA yielded an average of 95 (range 70 to 107) nonsynonymous somatic mutations in breast cancer tumors and an average of 109 (range 76 to 143) nonsynonymous mutations in lung cancer PDX samples.

In this study, we focused only on HLA-A allele-restricted neopeptides for experimental validation. For each tumor sample, all candidate epitopes predicted by Neopepsee and pVACseq are listed in Supplementary Table 1, with summarized ELISpot results presented in Table 3. Neopepsee considers only SNVs and does not consider fusion genes or intron retention. Neopepsee selected an average of 19 candidates (range 7 to 33) with breast cancer mutations and 17 candidates (range 11 to 21) with lung cancer mutations. For pVACseq analysis with a modified threshold of IC50 below 100 nM, DNA VAF >20 and RNA VAF >0 yielded an average of 9 best candidates for breast cancer (range 3 to 26) and 15 for lung cancer PDX samples (range 8 to 21).

In total, Neopepsee predicted 159 neopeptide candidates from 898 mutations (17.7%), and pVACseq predicted 84 (9.4%), with only 26 (2.9%) shared between the two prediction algorithms. Thus, the ensemble of Neopepsee and pVACseq identified 217 candidate neopeptides (24.2%) from 898 SNVs.

In vitro validation of candidate neopeptides

Based on a report by Stronen et al,^{25,26} we used HLA-matched donor blood for an ELISpot assay of IFN- γ secretion from neoantigen-specific T cell populations to validate candidate neopeptides.^{25,26} Among 217 candidate neopeptides, 22 Neopepsee candidate peptides and 9 pVACseq candidate peptides could not be tested either due to synthesis or purification failure or due to lack of donor blood for the specific HLA alleles. In total, 36 of 191 tested candidates (18.8%) were positive by ELISpot. The results summarized by case and prediction algorithms are provided in Table 3, with detailed information for the immunogenic peptides provided in Table 4. For some candidate peptides, although no memory response could be demonstrated, there were higher numbers of spots compared to the wells not stimulated with the peptide from day 0. We did not consider those peptides to be positive, although some reports in the literature considered such results to be immunogenic. For some peptides, there were strong responses in only one of the three donor blood samples, suggesting that the response could be directed toward the allele other than the predicted allele.

For our primary aim, we tested whether we could identify immunogenic neopeptides from ER+ BC with ER- BC and lung cancers as positive controls.

The Ensemble of Neopepsee and pVACseq predicted 93 neopeptides from 299 somatic mutations in three ER+ BC patients. Among them, 90 could be tested with ELISpot, and 14 (15.6%) were immunogenic (1, 5, and 10 for each tumor). In three ER- BC patients, 52 neopeptides were predicted from 271 mutations, and 12 (25.0%) of 48 tested were immunogenic (2, 4, and 8 for each tumor). From three lung cancer PDXs, 53 from 72 predicted neopeptide candidates were tested, and 10 of them were immunogenic (18.9%) (0, 1, and 11 for each tumor). These differences were not statistically significant. Therefore, we conclude that ER+ luminal B BCs express immunogenic neopeptides, although their numbers vary widely between individual tumors.

Table 3
Summary of ELISpot assays of IFN- γ secretion from neoantigen-specific T cell populations from donor PMBCs.

	HLA genotype	N of Mutations	NeoPepsee				pVACseq (modified)*				Common to both		
			predicted	tested	ELISpotpositive	PPV	predicted	tested	ELISPOT positive	PPV	predicted	tested	ELISpot positive
NEO1	A*24:02, A*33:03	70	9	9	3	0.33	4	4	1	0.25	3	3	1
NEO2	A*24:02, A*33:03	94	7	6	2	0.33	7	6	0	0.00	1	0	0
NEO3	A*02:06, A*11:01	103	28	27	6	0.22	26	25	4	0.16	11	10	2
NEO4	A*02:06, A*11:01	96	33	31	1	0.03	3	3	0	0.00	1	1	0
NEO5	A*02:01, A*02:01	100	12	12	4	0.33	5	5	2	0.40	2	2	1
NEO6	A*02:01, A*24:02	107	24	22	6	0.27	9	7	2	0.29	4	3	1
AV10	A*32:01, A*33:03	143	11	3	0	0.00	5	3	0	0.00	0	0	0
AV13	A*02:01, A*30:04 [#]	109	21	17	6	0.35	15	13	5	0.38	4	2	2
AV38	A*01:01 [#] , A*31:01	76	14	10	0	0.00	10	9	1	0.11	0	0	0
Total		898	159	137	28	0.20	84	75	15	0.20	26	21	7
[#] Note: A*30:04 and A*01:01 matched peptides were not tested due to lack of donor blood													

Table 4
List of ELISpot-positive cancer-specific neoepitopes.

case	HLA Allele	Prediction algorithm	Candidate neoepitope (9 or 10mer)	Wild type sequence	Gene Symbol	NetMHCpan binding affinity mutant	NetMHCpan binding affinity Wild type	BindLevel
Neo1	A*24:02	N	SYGRLMFFC	SHGRLMFFC	CPA6	3276.94	24435.71	
Neo1	A*24:02	N	RFIPGSSLL	RFIRGSSLL	ZNF517	126.35	173.14	SB
Neo1	A*33:03	NP	IYFLIGTSR	IYFLMGTSR	SLC26A1	40.73	55.21	SB
Neo2	A*24:02	N	CYKMIGLTI	CYIMIGLTI	FAM162B	162.07	142.00	WB
Neo2	A*24:02	N	RYLQLQLHL	RYLQLQLYL	IKZF4	31.80	29.17	SB
Neo3	A*11:01	N	IAYNLYLIY	IAYNLSLIY	GTF3C3	195.64	115.19	WB
Neo3	A*11:01	N	ASVRKKLGK	ASVRKKLGE	SPTBN4	114.48	25706.04	SB
Neo3	A*02:06	N	FLGSHLLHI	FLGSRLHLI	RTL1	11.48	33.03	sB
Neo3	A*11:01	N	PTTMPYPLK	PTTMTYPLK	VSIG4	495.86	589.41	WB
Neo3	A*02:06	NP	IILRALCAL	IILRAVCAL	SLFN5	56.88	134.81	
Neo3	A*11:01	NP	(A)TACWSGLFK	(A)TACWSGLCK	PRPF4	22.26	166.48	WB
Neo3	A*02:06	P	VLIKGSINSV	VLIEGSINSV	ARPC4	43.69	14.37	SB
Neo3	A*11:01	P	HSNRLAVAYK	HTNRLAVAYK	DMXL1	18.12	12.66	WB
Neo4	A*02:06	N	YQDNVTIFA	YQDNVTVFA	ABCA2	30.64	31.39	SB
Neo5	A*02:01	N	LLAYSEYNL	LLAYSEYNLP	USP6	33.2	1872.93	WB
Neo5	A*02:01	N	AINSYRFLV	AINYYRFLV	GPR68	64.63	92.90	WB
Neo5	A*02:01	N	KLQPFEGM	KLKPFEGM	PACS1	98.06	747.38	SB
Neo5	A*02:01	NP	HLLQCAWLEI	HLLCAWLEI	AK9	99.66	18.76	
Neo5	A*02:01	P	YMNAIKDYEL	YINAIKDYEL	PLEC	8.21	106.30	WB
Neo6	A*24:02	N	YYQLFAATV	YYQLFAAAV	ANKRD53	45.53	285.69	SB
Neo6	A*24:02	N	IYETNVVGF	IYETNVLGF	TULP1	157.53	92.89	SB
Neo6	A*24:02	N	SFLKLAKLF	SFLKLAELF	SNX32	99.82	18.28	SB
Neo6	A*02:01	N	YIQTTLTPV	YIQTTLTV	GRM6	15.88	44.45	WB
Neo6	A*24:02	N	YHIFDQVF	YHIFDQVF	PGBD2	812.59	2762.52	WB
Neo6	A*24:02	NP	AYLPWSYFL	AYLPWSYFP	C4orf33	9.19	1014.65	SB
Neo6	A*02:01	P	KLFSRNSGL	KFFSRNSGL	ZNF304	55.09	9702.91	SB
AV13	A*02:01	N	ALRRFAFMV	ALMRFAFMV	LPGAT1	171.47	3.52	
AV13	A*02:01	N	LLLFCDVGL	LLLFCVDL	CHSY3	15.27	62.59	WB
AV13	A*02:01	N	AMTILILKV	AMTIWILKV	SGCZ	60.49	42.39	SB
AV13	A*02:01	N	YLLMISALM	YLIMISALM	PSEN1	13.49	20.89	WB
AV13	A*02:01	NP	LLTTCGEKV	LLTTCGEEV	STC2	54.01	24.09	WB
AV13	A*02:01	NP	GVANCLFPL	GVGNCLFPL	METTL6	21.62	91.66	WB
AV13	A*02:01	P	FVPEVFLKL	FVSPEVFLK	DNAAF5	64.82	10909.18	WB
AV13	A*02:01	P	FLLRGPPVPV	FLLRGPPGPV	C8orf82	4.60	6.90	SB
AV13	A*02:01	P	YLQRNAPTL	YLQRNALTL	KDM6A	21.75	35.01	SB

Note: mutations and matching wild-type sequences are in bold character, N= predicted by NeoPepsee, P= predicted by pVACseq, NP=predicted by both NeoPepsee and pVACseq

For our first secondary aim of comparing the positive predictive value of NeoPepsee versus pVACseq, 28 of 137 tested NeoPepsee candidates were ELISpot positive (PPV 0.20) compared to 15 of 75 tested pVACseq candidates (PPV 0.20). These PPVs were not significantly different ($p=0.95$). Among 21 tested candidates predicted by both algorithms, seven were positive by ELISpot (PPV 0.33). Therefore, we conclude that NeoPepsee and pVACseq identify different pools of candidate neoepitopes from sequencing data and are complementary to each other, which in combination provide a reasonable number of candidates to screen for vaccine design, reducing the number of candidates that need to be screened experimentally from 8082 (898 times 9 possible positions for a mutation within a 9-mer nucleotide) to 217 with a median of 24 candidates per case (range 10 to 43) with a PPV of 18.8%.

case	HLA Allele	Prediction algorithm	Candidate neoepitope (9 or 10mer)	Wild type sequence	Gene Symbol	NetMHCpan binding affinity mutant	NetMHCpan binding affinity Wild type	BindLevel
AV38	A*31:01	P	RQDIDFGVSR	RQDIDLGVSR	NFE2L2	49.25	115.92	SB
Note: mutations and matching wild-type sequences are in bold character, N= predicted by NeoPepsee, P= predicted by pVACseq, NP=predicted by both NeoPepsee and pVACseq								
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Evaluation of TESLA recommended criteria for neoepitopes

Previously, the Tumor Neoantigen Selection Alliance (TESLA) suggested that potential immunogenic peptides are characteristic of MHC binding affinity stronger than 34 nM¹⁴. According to these criteria, we compared the validated neoepitopes based on an MHC binding affinity of 34 nM (Table 5).

Table 5
Summary of *in vitro* validated neoepitopes by ELISpot assay according to MHC binding affinity of 34 nM.

	IC ₅₀ <34 nM	IC ₅₀ ≥34 nM	Marginal row total
ELISpot positive	14 (38.9%)	22 (61.1%)	36
ELISpot negative	47 (30.3%)	108 (69.7%)	155
Marginal column total	61 (31.9%)	130 (68.1%)	191

Fourteen of 36 (38.9%) ELISpot-positive peptides had NetMHCpan IC₅₀ below 34 nM, whereas 47 of 155 (30.3%) ELISpot-negative peptides had IC₅₀ below 34 nm (Fisher's exact test, *p*=0.32, NS). If we were to predict based on the IC₅₀ value, 61/191 (31.9%) peptides were predicted to be high-affinity binders, and among them, 14 (22.9%) were true positives. Therefore, we could not corroborate the findings from the TESLA consortium.

Discussion

Clinical trials have demonstrated that therapeutic vaccines targeting neoepitopes derived from somatic mutations are promising therapeutic modalities for solid tumors with a high mutation burden.^{8–11} However, with a small sample size, we demonstrated that luminal B breast cancer does express at least one or more immunogenic neoepitopes. Since there is little therapeutic option for luminal B BC patients after distant recurrence, it is worth considering them for inclusion in therapeutic vaccine trials. Epitope spreading after therapeutic neoepitope vaccination suggests that immune ignorance may be one of the immune evasion mechanisms.³⁵ A study by Westcott et al suggested that for patients with immune ignorance of clonal neoepitopes due to low level expression, therapeutic vaccination of neoepitopes may be able to trigger robust immune responses.³⁶

Clinical trials for personalized therapeutic neoepitope vaccines have demonstrated that T-cell immune responses were induced for only a subset of candidate neoepitopes.^{8–11} Although almost all vaccine trials reported thus far selected candidates based on computational algorithms, one trial selected candidates based on *in vitro* T-cell response to candidate neoepitopes presented by bacteria.³⁷ Intriguingly, in the latter study, some mutated peptides suppressed the immune response, and investigators named such peptides ‘inhibigens’. Inhibigens suppressed immune activation by otherwise immune-stimulatory neoepitopes when injected together. Since *in vitro* validation of every candidate neoepitope is demanding and time-consuming, it is challenging to incorporate it into a routine process. Thus, designing personalized therapeutic vaccines requires a robust computational neoepitope prediction algorithm with a high positive predictive value, ideally followed by *in vitro* testing of immunogenic responses with patient or HLA-matched donor PBMCs.

In 9 cases prospectively screened using the ELISpot assay with HLA-matched donor PBMCs, the PPV was 20% for both NeoPepsee and pVACseq. There was relatively little overlap between the predicted epitopes between the two algorithms. The PPV increased to 33% for overlapping candidates between NeoPepsee and pVACseq. This finding is consistent with the TESLA report in that ensemble models outperform individual prediction models. The ensemble of NeoPepsee and pVACseq provided a reasonable number of candidates to screen for vaccine design, reducing the number of candidates that need to be screened experimentally from 8082 (898 times 9 possible positions for a mutation within a 9-mer nucleotide) to 217 with a median of 24 candidates per case (range 10 to 43) with a PPV of 18.8%. However, it would be ideal to validate the immunogenicity of the predicted neoepitope candidates with an *in vitro* screening method before designing a therapeutic vaccine.

TESLA identified a set of thresholds for several variables that in combination could filter out 93% of nonimmunogenic peptides while maintaining 55% of immunogenic peptides.²⁴ This threshold set is composed of binding affinity less than 34 nM, tumor abundance greater than 33 TPM, and binding stability greater than 1.4 h. We could not corroborate the findings from the TESLA consortium. Unlike TESLA, which used a multimer assay to validate the neoepitopes, we used an ELISpot assay. While a multimer assay quantifies T-cells with T-cell receptors that can bind MHC-presented neoepitopes, the ELISpot assay quantifies the interferon-γ secreted by the T-cells activated by the MHC-presented neoepitopes. The differences in sensitivity and specificity between the two assays may be partially responsible for the observed difference with the TESLA data.

Our study has several limitations: 1) We did not screen mutated peptides that were not predicted by Neopepsee or pVACseq. Since there is a possibility that some of them might be immunogenic, we do not know the true false-negative rate of each algorithm. 2) We used HLA-matched donor blood for the ELISpot assay. Strønen et al. reported that HLA-matched healthy donor blood showed a five times higher response rate than blood from patients.²⁵ Thus, our result of 20% PPV could be an overestimate. 3) For the ELISpot assay, we used strict criteria of requiring a memory response upon rechallenge with the candidate peptides after ten days of incubation with the peptides. Some of the peptides showed increased spot numbers over control wells that had never contacted the peptides but did not show increased spot numbers in comparison to the no peptide control at the time of rechallenge. Such results could be interpreted as positive responses by some investigators. 4) ELISpot is known to be influenced by many factors.³⁸ Compared to viral antigens, which show strong positive ELISpot results, most of the neoepitopes we found showed only a moderate increase in spot numbers. Since we did not validate our results with a multimer assay, some peptides could be false-positive findings.

In conclusion, our data demonstrate that luminal B BC can be immunogenic and replicate the findings from many studies that the current generation of neoepitope prediction algorithms suffers from low PPV, and more *in vitro* validation data are required to train the prediction algorithms to achieve clinically meaningful PPV. In the absence of prediction algorithms with high PPV, it may make sense to design vaccines based on *in vitro* validated peptides only. For the latter aim, developing robust and cost-efficient methods for real-time *in vitro* screening of large numbers of candidate neoepitopes is necessary. For example, although a tandem minigene screen allows screening of 10 mutated genes in a single construct and thus allows experimental screening of all mutations with ELISpot, individual peptides eventually need to be synthesized to pinpoint the neoepitope within the positive minigene. While several innovative high-throughput screening methods have been reported recently, they do not fulfill such needs due to the high cost of peptide synthesis required for the assay or due to low sensitivity.^{39–41}

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