

# Wild-Type p53 Epitope Naturally Processed and Presented by an HLA-B Haplotype on Human Breast Carcinoma Cells<sup>1</sup>

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## ABSTRACT

To broaden the clinical applicability of peptide-based immunotherapy in breast cancer, there is a need to identify further tumor-associated peptide epitopes that are specific for HLA alleles, in addition to HLA-A2. The HLA-B44 haplotype is one of the most common HLA-B haplotypes, occurring in 10–20% of the population. We performed the structural characterization of HLA class I-bound self-peptides presented by a human breast cancer cell line with a HLA-A68, A32, B40, B44 haplotype, to identify potential tumor-specific antigens. Of 13 sequenced peptides, 1 peptide had the HLA-A68 peptide binding motif and 12 peptides had the HLA-B40, B44 peptide binding motif. One of the latter peptides, FEVRVCACPG, shared 100% homology to residues 270–279 of wild-type P53 protein. Our study, thus, provides direct evidence for the natural processing and presentation of p53 epitope 270–279 by HLA-B40, B44-bearing human breast tumor cells. Epitopes spanning this region of P53 may have potential use for immunotherapy in patients expressing HLA-A2 and -B44 supertypes.

## INTRODUCTION

MHC class I and II molecules present self- and foreign peptides to CD4<sup>+</sup> and CD8<sup>+</sup> T cells for immune surveillance. Breast tumor cells characteristically accumulate a number of wild type or mutant proteins, including p53, HER2, and cyclin D1 (1, 2). Altered trafficking and degradation of such proteins by the proteolytic machinery associated with the endogenous antigen-processing pathway may result in an altered repertoire of MHC-bound peptides relative to nonmalignant cells. Presentation of normally cryptic epitopes to CD8<sup>+</sup> T cells by the MHC

class I molecules on the surface of tumor cells may provide unique targets that potentially can be harnessed for immunotherapy (3, 4). To date, only a limited number of tumor-specific or tissue-specific self-peptides with potential use in MHC-restricted immunotherapy have been identified in breast tumors (5–8), most in the context of the common MHC class I allele HLA-A2.

For wider applicability of peptide-based immunotherapy, definition of additional tumor-associated peptide epitopes that bind other common MHC alleles is required. It has been proposed that the major HLA-A and -B alleles can be grouped into four major supertypes, as defined by their peptide binding specificities (9). We have established a novel human breast cancer cell line, NiBrCa (10), with the HLA-B44-like supertype, expressing the common HLA-B44 and -B40 alleles that occur in 20–30% of the population. In the present study, the structural characterization of the MHC class I-bound self-peptides presented by the NiBrCa cell line was performed to identify tissue-specific or tumor-associated precursor proteins that could serve as immunogens in the context of CD8<sup>+</sup> T-cell antigen receptor/peptide/HLA class I interactions.

## MATERIALS AND METHODS

**Reagents.** The mAb W6/32 was obtained from murine hybridoma culture supernatants and purified by protein A affinity chromatography with reagents supplied by Bio-Rad (Richmond, CA). Cyanogen bromide-activated Sepharose 4B, purified normal mouse IgG, aprotinin, tosylleucylchloroacetone, tosylprolylchloroacetone, and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO). Pepstatin A, leupeptin, and phenylmethylsulfonyl fluoride were obtained from Boehringer Mannheim (San Diego, CA). All HPLC<sup>3</sup> solvents, TFA, Nonidet-40, and the Bicinchonnic Acid protein assay kit were obtained from Pierce Chemical Co. (Rockford, IL). All other reagents were of the highest quality commercially available.

**Cell Lines and Culture Conditions.** The NiBrCa cell line was established in 1997 from the malignant ascitic fluid of a woman with ductal cell adenocarcinoma of the breast. The primary tumor was aneuploid, estrogen receptor/progesterone receptor negative, and HER2 receptor negative. Ascitic fluid was placed in culture and gradually replaced with culture medium composed of DMEM supplemented with 10% FCS, 5 µg/ml hydrocortisone, 10 µg/ml insulin, 1% glutamine, and 0.5% gentamycin (all from Life Technologies, Inc., Grand Island, NY). After 12 weeks of stable growth, the cultured cells were phenotyped. The cells were weakly HLA class I positive. Cells were submitted to the National Cell Culture Center (Minneapolis, MN) and expanded to a total weight of ~42 g in

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<sup>3</sup> The abbreviations used are: HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

rollerbottle culture. The murine hybridomas producing the mAb W6/32 and anti-HLA-A, -B, and -C (obtained from American Type Culture Collection) was cultured in IgG-free media.

**HLA Expression and Typing.** The cell surface expression of HLA class I molecules by NiBrCa was measured by direct immunofluorescence using a Becton-Dickinson FACscan instrument (San Jose, CA).

The cell line NiBrCa was typed for HLA class I and class II genotypes by genomic typing of *in vitro*-amplified DNA with sequence-specific oligonucleotide primers. The HLA genotype was HLA-A3201, -A6801, -B4406, -B4013, -DR0401, and -DR1101.

**Immunoaffinity Purification of Class I Molecules and Elution of Bound Peptides.** HLA class I molecules and bound peptides from  $\sim 10^{11}$  cells were purified by immunoprecipitation, as described previously (11). Briefly, the cells were lysed in detergent buffer [10 mM Tris HCl (pH 7.8), 150 mM NaCl, 2 mM EDTA, 1% v/v NP40] containing 2 mM phenylmethylsulfonylfluoride, 10  $\mu$ g/ml aprotinin, 1  $\mu$ M pepstatin A, 1  $\mu$ M leupeptin, 20  $\mu$ g/ml tosylprolylchloroketone, and 20  $\mu$ g/ml tosylleucylchloroketone. The lysate was cleared by centrifugation, followed by filtering (2  $\mu$ m; Nalge Co., Rochester, NY). The clarified lysate was then passed over a series of fast-flow Sepharose 4B columns conjugated to normal mouse immunoglobulin and anti-HLA-A, -B, and -C mAb (W6/32). The columns were washed with buffered detergent solutions, and the bound HLA molecules were eluted from the affinity columns with 0.1% TFA. Aliquots of the purified HLA class I protein obtained were analyzed by SDS-PAGE and silver staining and judged to be >90% pure. The final yield of HLA class I molecules was 112  $\mu$ g, as determined by the bicinchonnic acid protein assay. The eluted HLA class I molecules were incubated for 3 min at 100°C, then centrifuged through a 3000-Da MW cutoff ultrafiltration cartridge (Micron 3; Amicon, Danvers, MA). The low molecular weight fraction was stored at -70°C until use.

#### HPLC Purification and Sequencing of Eluted Peptides.

The low molecular weight, TFA eluates of HLA molecules were separated by reverse-phase HPLC using a C18 column (Vydac; 2.1  $\times$  250 mm) and Waters Associates (Waltham, MA) equipment. The gradient was created using a solution (A) of H<sub>2</sub>O/0.1% TFA in combination with a solution (B) of 80% acetonitrile/0.1% TFA at a flow rate of 0.200 ml/min. The low molecular weight fractions were reconstituted in solution A and applied to the column. One-minute fractions were collected from a linear 2-h gradient running from 2–80% solution B. From the absorbance trace at 215 nm of the eluate, the single fractions containing absorbance peaks were selected for microsequencing by NH<sub>2</sub> terminal Edman degradation using a Hewlett-Packard G1000A protein sequencer (Palo Alto, CA). For the assignment of major sequences from these data, we used previously published criteria. Although the material in the fractions was not homogeneous, we were able to identify the amino acid sequence of the major peptide species in 13 fractions. Sequences were assignable on the basis of the pm yield of each PTH amino acid derivative in the cycle (ranging from 5–20 pmol). The assignment was corroborated by the repetitive yield of each sequence. The repetitive yields for all reported sequences ranged from 80–90%, and the total amount of each

Table 1 Sequences from HLA class I-bound peptides of NiBrCa breast carcinoma cells

Fraction	Amino acid sequence <sup>a</sup>										Homology (residues)	
	2	-	-	-	-	8	9	10	-			
28	F	E	V	R	V	C	A	C	P	G	P53 (270–279) 100%	
35	K	E	V	R	V	A	A	P	P	G	(-) <sup>b</sup>	
38	G	E	A	R	N	V	P	A	R	G	Homeobox H6 (78–87) 80%	
41	A	E	A	F	P	X	A	R	X	X	(-)	
42	G	E	D	H	R	E	F	E	X	X	(-)	
53	P	E	G	E	G	X	R	E	X	H	(-)	
54	D	V	H	P	H	H	N	X	R		(-)	
59	S	E	F	R	N	X	A	A	X	H	(-)	
62	F	E	V	A	V	A	V	A	C	X	P	(-)
63	D	E	V	K	R	N	G	X	R		(-)	
70	S	E	D	H	R	H	H	X	E	H	(-)	
71	A	E	H	D	K	E	N	H	X	N	(-)	
80	S	E	X	D	E	A	H	Y	P	S	(-)	

<sup>a</sup> Standard IUPAC amino acid abbreviations are used. X denotes an unidentifiable PTH amino acid derivative.

<sup>b</sup> (-), no homology to known proteins.

peptide reported in Table 1 ranged from 5–15 pmol. In some fractions, it was not possible to assign major or minor sequences. In certain instances, PTH amino acid derivatives did not correspond to the PTH standards. Under these circumstances, the residue is indicated by X. The sequences of the MHC-bound peptides were compared with existing protein sequences contained within the EMBL and Swissprot databases.

## RESULTS

### Expression of HLA Class I Molecules by NiBrCa Cells.

The breast cancer cells used in this study were continuously monitored for their cell surface expression of HLA class I molecules. Fig. 1 shows the cell surface expression of HLA-A, -B, and -C molecules on cells after large-scale culture, as determined by immunofluorescence. The low level and distribution of expression of HLA-A, -B, and -C molecules was not substantially different than that observed on cells from the primary effusion (data not shown). For purposes of comparison, the HLA-A, -B, and -C expression on the human myeloid leukemic cell line U937 is shown in Fig. 1.

**Structure of HLA Class I-bound Peptides Eluted from Cells.** Low molecular weight material, eluted from HLA class I heterodimers, was released by acid-heat denaturation, purified by ultrafiltration, and fractionated by reverse-phase HPLC. The absorbance trace for the separation, at 215 nm, is shown in Fig. 2.

The HLA class I-bound peptides of NiBrCa cells analyzed by sequential Edman degradation are shown in Table 1. The length of peptides in the pool isolated from the HLA class I molecules of NiBrCa ranged from 9–11 residues. The peptide sequences obtained generally conformed to the extended allele-specific motifs published for HLA-B44 or -B40. One peptide, DVHPHHNXR (fraction 54), had a HLA-A68 peptide binding motif.

### Precursor Proteins of MHC Class I-bound Peptide.

The sequences of the 13 MHC class I-bound peptides obtained from NiBrCa cells were compared with known sequences con-

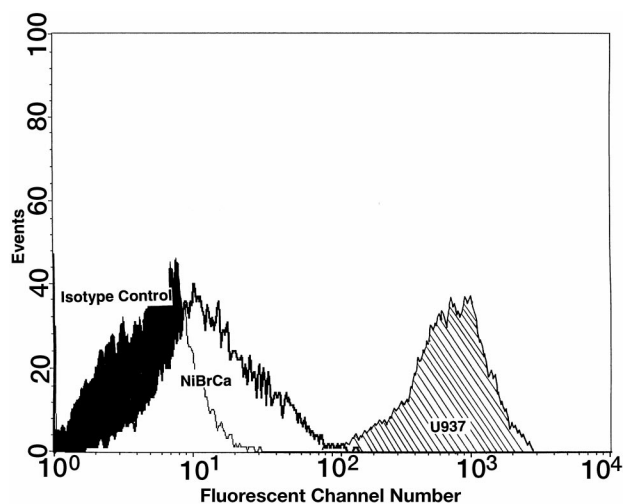


Fig. 1 Immunofluorescent cytometry analysis of HLA class I expression on NiBrCa and U937 cells. *Black area*, isotype control; *white area*, NiBrCa stained with W6/32 mAb (anti-HLA-A, -B, and -C); *hatched area*, U937 stained with W6/32 mAb.

tained within the GenomeNet databases.<sup>4</sup> The database searches for peptide sequence homology to known protein precursors revealed that the peptide FEVRVCACPG, from fraction 28, matched to residues 270–279 of the p53 protein. Synthetic monomeric FEVRVCACPG had the same retention time when chromatographed by HPLC under the same conditions as detailed in “Materials and Methods” (data not shown).

## DISCUSSION

Breast cancer cells may down-regulate MHC class I molecules by various mechanisms, including HLA-B core promoter repression by wild-type P53, to escape immune surveillance (12, 13). Low levels of MHC class I molecule expression makes isolation and sequencing of naturally processed MHC peptides technically challenging. On the basis of previous results, we estimated the expression of HLA class I molecules by NiBrCa cells to be approximately two orders of magnitude less than that measured on U937 cells (and lymphoblastoid cell lines), and probably corresponding to 1000–3000 MHC molecules per cell (11). The total amount of MHC-bound peptides isolated, as determined by the absorbance signature of the peptides separated by reverse-phase HPLC, was similar to previous experiments, in which the total amount of MHC-bound peptides isolated was in the hundred pmol range.

The HLA-B44 haplotype is one of the most common HLA-B haplotypes [25% of Caucasians (14), 10% of African-Americans], whereas the frequencies for HLA-B40 are 20% in Asians and 8% and 4.5% in Caucasians and African-Americans, respectively (15). The consensus peptide binding motif for HLA-B44 and -B40 are similar, sharing predominance of glutamic acid at position 2, a hydrophobic residue in position 3, and

a polar residue at position 9/10. The majority of naturally processed and presented peptides sequenced in this study conform, in general, to this HLA-B44 supertype binding motif. One peptide with a HLA-A68 peptide binding motif, which features V or L at position 2 and R at position 9, was also identified.

In this study, we isolated a naturally processed MHC-bound peptide with the sequence FEVRVCACPG. This sequence has 100% homology to residues 270–279 of wild-type P53 protein. P53 is a nuclear phosphoprotein constitutively expressed at low levels in normal cells. The p53 gene is mutated in 50–60% of human cancers, altering p53 protein half-life, intracellular location, quantity, and processing by the ubiquitination system. Both wild-type and mutated p53 have been the focus of strategies to induce CTL lysis of tumors (8, 16). Proteasomal degradation of p53 is important in determining the peptide repertoire and may be affected by mutations (17), but alternative pathways of peptide degradation likely also play a role in generating epitopes for HLA molecules (9). Synthetic peptides based on the primary sequence of p53 have been shown *in vitro* to bind HLA-A1, -A2, -A11, -B7 and -B8 (18–22). Furthermore, CTL responses can be induced against both the wild-type and mutant p53 peptides 25–35, 65–73, 187–197, and 264–272 bound to HLA-A\*0201 *in vitro* (19, 23–25). In animal models, Vierboom *et al.* (26) provide data consistent with the hypothesis that CTLs, specific for p53-derived peptides bound to MHC class I molecules, are able to discriminate between normal and p53 overexpressing tumor cells *in vivo* (26).

Our study provides direct evidence for the natural processing and presentation of p53 epitope 270–279 by HLA-B40 and -44-bearing human breast tumor cells. In HLA-A2 transgenic mice, CTL generated against p53 epitopes 264–272 recognize and lyse human tumor cell lines (16, 27). Human CTL specific for p53 peptide (264–272) recognize and lyse HLA-A2 squamous cell carcinoma cells (28) and breast and melanoma tumor cell lines that overexpress p53 (29), providing further evidence for natural processing and presentation of the 264–272 p53 epitope in the context of this allele. Together, these data suggest that this DNA binding region of wild-type p53 protein has features favoring its presentation by both *HLA-A* and *HLA-B* alleles. Self-proteins with DNA-binding activity may have favored processing and presentation by MHC class I molecules. For example, IRE-BP, E2Fa, 5′ nucleotidase, and *c-myc* DNA-binding proteins are sources of high copy number peptides to HLA class I molecules (30).

The transgenic mice experiments show evidence for elimination of CTL with high avidity for the HLA-A2-wild-type p53 peptide complex (31), suggesting that tumor lysis may be restricted to low avidity CTL (8). Other data suggest that the proteasomal processing and presentation of the 264–272 epitope may be impaired by mutations at the “hotspot” position 273 (17). Of note is the finding that the 264–272 epitope has the highest predicted binding affinity for HLA-A2 of p53 protein-derived nanomers; the predicted disassociation half-life of 270–279 for HLA-B40 and -44 is lower (32). The lower binding affinity of 270–279 may potentially provide a target for T cells, which have escaped negative selection directed by other p53-derived MHC-bound self-peptides (33, 34). We are currently

<sup>4</sup> [http://www.fasta.genome.ad.jp/ideas/fasta/fasta\\_genes.html](http://www.fasta.genome.ad.jp/ideas/fasta/fasta_genes.html).

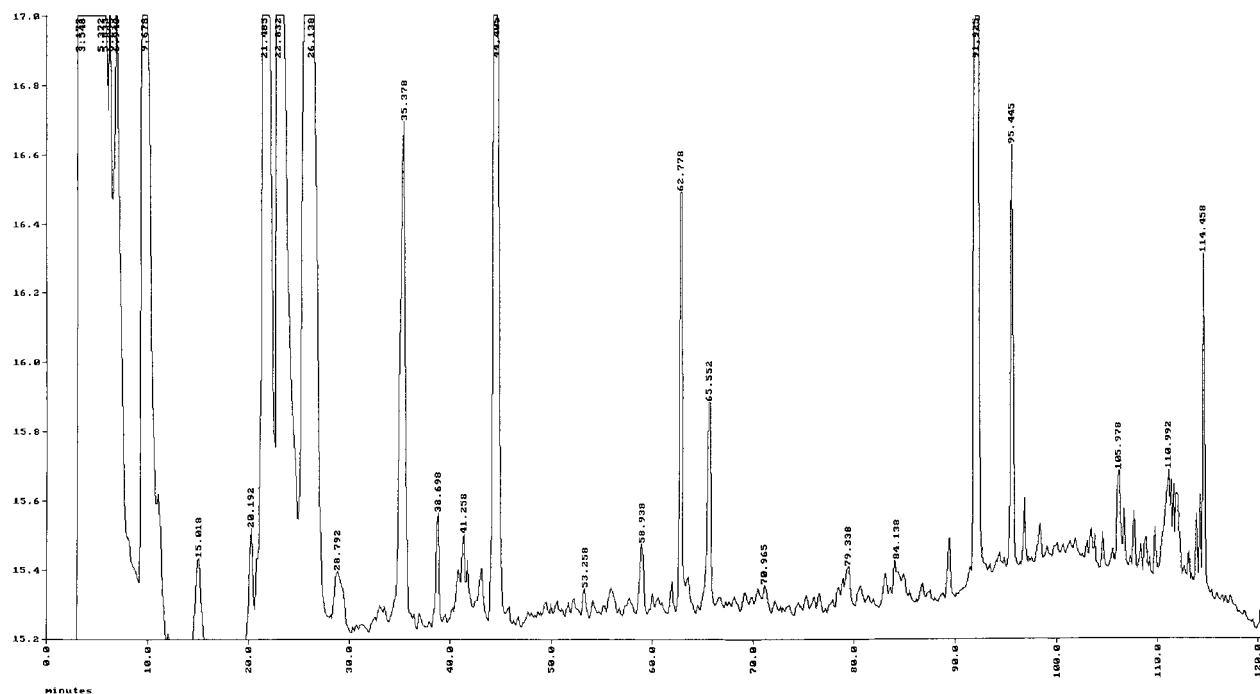


Fig. 2 HPLC signature of low molecular weight peptides eluted from HLA class I molecules purified from the breast cell line NiBrCa. The reverse-phase gradient was created using a solution of  $\text{H}_2\text{O}/0.1\%$  TFA (A) in combination with a solution of  $80\%$  acetonitrile/ $0.1\%$  TFA (B) at a flow rate of  $0.200$  ml/min. The low molecular weight fractions were reconstituted in solution A and applied to the column. One-minute fractions were collected from a linear 2-h gradient running from  $2$ – $80\%$  solution B. Vertical axis represents the absorbance at  $215$  nm ( $1.8$  mAu fullscale) and the horizontal axis is elution time (120 min full scale).

studying the immunogenicity of p53 270–279 and its feasibility for immunotherapy. For example, minigene vectors incorporating the 262–279 region of p53, together with residues from regions of the core domain shown to bind MHC class II molecules (35), may prove useful agents in adaptive immunotherapy in patients expressing the HLA-A2 or -B44 supertypes.

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