Expression of placenta-specific 1 and its potential for eliciting anti-tumor helper T-cell responses in head and neck squamous cell carcinoma

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Supplemental materials and methods

Cell lines

Two HPV-positive human HNSCC cell lines, SCC090 (tongue SCC) and SCC152 (hypopharynx SCC) were purchased from ATCC. UM-SCC-47 (HPV-positive tongue SCC cell line) was supplied by Merck Millipore. All cell lines were maintained in tissue culture as recommended by the supplier.

Analysis of GEO and TCGA data

To compare *PLAC1* and *HLA-DR* expression between the four molecular subtypes of HNSCC (basal, mesenchymal, atypical and classical), the gene expression data of GSE39366 (ref. 52; based on the platform of GPL9053 Agilent-UNC-custom-4X44K), containing 138 HNSCC samples, was downloaded from the GEO database at the National Cancer for Biotechnology Information (NCBI). Comparative analysis was performed with the use of GeneSpring ver. 14.9.1 (Agilent Technologies, Inc., Santa Clara, CA). The differences were analyzed using the one-way ANOVA test with the TukeyHSD Post Hoc test.

TCGA data were analyzed by cBioPortal (http://www.cbioportal.org). *PLAC1* mutation frequencies in HNSCC were estimated using a Head and Neck Squamous Cell Carcinoma (TCGA, PanCancer Atlas) dataset with 523 samples (of which 515 samples have available mutation data).

Intracellular cytokine analysis

PLAC1-specific CD4 helper T cells were labeled using a CellTrace^{TM} CFSE Cell

Proliferation Kit (Invitrogen; Thermo Fisher Scientific, Inc.) and were co-cultured with autologous PBMCs pulsed with or without $PLAC1_{31-50}$ peptide (10 μg/ml) for 6 h in the presence of monensin solution (BioLegend). Collected cells were stained with PE/Cyamine7-conjugated anti-CD4 (RPA-T4) and APC/Cyamine7-conjugated anti-CD8 (RPA-T8) mAbs, were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences), and were subsequently stained intracellularly with PerCP/Cyanine5.5-conjugated anti-GM-CSF (BVD2-21C11), PE-conjugated anti-IFN-γ (B27) and APC-conjugated anti-granzyme B (QA16A02) mAbs. All antibodies were obtained from BioLegend. The stained cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA).

Statistical analysis

Correlations between all categorical variables were evaluated using Fisher's exact test. The relationship between IHC scores of PLAC1 and HLA-DR was evaluated using the Spearman rank correlation. *P* values <0.05 were considered statistically significant. GraphPad Prism 7 (GraphPad Software) was used for analyses.

Supplemental figure legends

Supplemental Figure S1. Expression of PLAC1 and HLA-DR in OCSCC specimens. **(A)** Representative immunohistochemical (IHC) images of PLAC1 and HLA-DR. Expression levels of tumor cells were classified into no, weak, moderate and strong staining by IHC staining intensity. Scale bar = 50 μm. **(B)** Distribution of the IHC score of PLAC1. **(C)** Distribution of the IHC scores of HLA-DR. In B and C, the IHC score was calculated in the same way as described in the legend of Figure 1B and 1C, respectively, and could range from 0 to 6. An IHC score ≥4 was defined as high expression, and the others were considered as low expression. **(D)** Distribution of high and low expression of PLAC1 and HLA-DR based on the IHC scores.

Supplemental Figure S2. Distribution (left matrix) and correlation (right scatter plot) between the IHC scores of PLAC1 and HLA-DR in OPSCC **(A)** and OCSCC **(B)** specimens. In the scatter plots, statistical significance was determined using the Spearman rank correlation test.

Supplemental Figure S3. Analysis of *PLAC1* and *HLA-DR* gene expression in HNSCC tissues from a GEO dataset (GSE39366). **(A)** Dot-plots show the expression of *PLAC1*, *HLA-DRA* and *HLA-DRB5* in four molecular subtypes of HNSCC (basal, mesenchymal, atypical and classical). Horizontal bars represent mean values. **(B)** The table shows the results of the one-way ANOVA test with the TukeyHSD Post Hoc test between the four molecular subtypes in the expression of *PLAC1*, *HLA-DRA* and *HLA-DRB5*. Significant differences between two subtypes were determined using a 2-fold change and $p<0.05$ as cut-offs.

Supplemental Figure S4. Expression of PLAC1 and HLA-DR in HPV-positive HNSCC cell lines. **(A)** Western blotting analysis of the expression of PLAC1 in tumor cell lines. β-Actin was used to confirm the amount of loaded protein. **(B)** Cell surface expression of HLA-DR on tumor cell lines as determined by flow cytometry. Cell lines were incubated with or without IFN-γ in the same way as described in the legend of Figure 2B. The black, red and blue lines show isotype control staining, HLA-DR staining of IFN-γ-untreated cells and that of IFN-γ-treated cells, respectively.

Supplemental Figure S5. Plot of *PLAC1* mutations in 515 samples from the Head and Neck Squamous Cell Carcinoma (TCGA, PanCancer Atlas) dataset. The figure was generated by the cBioPortal website, in which each lollipop denotes a unique mutation location for *PLAC1*. The table shows mutation types and protein changes. *PLAC1* mutation frequency was 0.6% (3 of 515 samples) in these samples, including one case of a nonsense mutation and two cases of missense mutations.

Supplemental Figure S6. IFN-γ production of induced PLAC1-specific CD4 helper T cells, T4, Y6 and H9. **(A)**, **(B)**, **(C)** and **(D)** show the amount of IFN-γ in culture supernatants measured by ELISA using the same experimental design as employed in Figure 3A, 3B, 3C and 4, respectively. In A, points represent the mean ± SD of duplicate measurements. In B, C and D, columns represent the mean ± SD of triplicate measurements. Statistical significance was assessed using an unpaired Student's *t*-test (∗ $p<0.05$).

Supplemental Figure S7. Intracellular staining of PLAC1-specific CD4 helper T cells, T4 **(A)** and Y6 **(B)**. CFSE-labeled T4 or Y6 cells were co-cultured with autologous PBMCs pulsed with or without $PLAC1_{31\text{-}50}$ peptide (10 μg/ml) for 6 h. Collected cells were stained for flow cytometric analysis using anti-CD4, anti-CD8, anti-GM-CSF, anti-IFN-γ and anti-granzyme B mAbs as described in Supplemental materials and methods. In A and B, the upper panels indicate the gating strategy and representative scatter plots of the flow cytometric analysis. Cells were initially gated by forward scatter area and side scatter area, followed by gating to CFSE-labeled cells. Almost all CFSE-labeled T4 or Y6 cells (more than 96%) were CD4 positive and CD8 negative. The production of GM-CSF, IFN-γ and granzyme B was analyzed in T cells gated on CFSE-labeled, CD4-positive and CD8-negative. Lower panels indicate the percentage of T4 or Y6 cells producing GM-CSF, IFN-γ and/or granzyme B in the presence of PLAC1 peptide. Columns represent the mean ± SD of triplicate measurements.

Supplemental Table S1 Clinical characteristics of oropharyngeal squamous cell carcinoma (OPSCC) patients

Characteristics	Cases (*n*=59)
Gender	
Female	7
Male	52
Age (years)	
Median (range)	67 (46-85)
Tobacco	
Current smoker	25
Former smoker	21
Never smoker	11
Not recorded	2
Alcohol	
Current drinker	36
Former drinker	6
Never drinker	16
Not recorded	1
HPV status	
Positve	29
Negative	30
Tumor classification	
T1	10
T2	24
T3	8
T4	17
Node classification	
N0	21
N1	8
N2	27
N3	3
Metastasis classification	
M0	58
M1	1
Stage	
Ⅰ	3
Ⅱ	11
Ⅲ	10
Ⅳ	35

HPV: Human papilloma virus

Supplemental Table S2 Clinical characteristics of oral cavity squamous cell carcinoma (OCSCC) patients

Characteristics	Cases (*n*=52)
Gender	
Female	16
Male	36
Age (years)	
Median (range)	66 (41-100)
Tumor site	
Tongue	28
Floor of mouth	12
Gingiva	6
Buccal mucosa	6
Tobacco	
Current smoker	23
Former smoker	12
Never smoker	14
Not recorded	3
Alcohol	
Current drinker	24
Former drinker	6
Never drinker	19
Not recorded	3
Tumor classification	
T1	11
T2	18
T3	9
T4	14
Node classification	
N0	30
N1	3
N2	13
N3	6
Metastasis classification	
M0	52
M1	0
Stage	
Ⅰ	11
Ⅱ	9
Ⅲ	7
Ⅳ	25

Supplemental Table S3 Relationship between the clinical features of OPSCC patients and PLAC1 expression

Features	PLAC1 IHC score		*P*
	<4	≥4	
Gender (*n*=59)			0.6657
Female	1	6	
Male	14	38	
Age (years) (*n*=59)			0.5471
<70	11	28	
≥70	4	16	
Tobacco (*n*=57)			>0.9999
Current or former smoker	12	34	
Never smoker	3	8	
Alcohol (*n*=58)			0.194
Current or former drinker	13	29	
Never drinker	2	14	
HPV status (*n*=59)			0.3817
Positive	6	24	
Negative	9	20	
T classification (*n*=59)			0.5484
T1, 2 (early)	10	24	
T3, 4 (advanced)	5	20	
N classification (*n*=59)			0.5372
N0 (negative)	4	17	
N1, 2, 3 (positive)	11	27	
M classification (*n*=59)			>0.9999
M0	15	43	
M1	0	1	
Stage (*n*=59)			0.7659
I, II	7	24	
III, IV	8	20	

HPV: Human papilloma virus

IHC score: Immunohistochemical score

Supplemental Table S4 Relationship between the clinical features of OCSCC patients and PLAC1 expression

Features	PLAC1 IHC score		*P*
	<4	≥4	
Gender (*n*=52)			0.7683
Female	7	9	
Male	18	18	
Age (years) (*n*=52)			0.7743
<70	15	18	
≥70	10	9	
Tobacco (*n*=49)			0.928
Current or former smoker	17	18	
Never smoker	7	7	
Alcohol (*n*=49)			0.3205
Current or former drinker	13	17	
Never drinker	11	8	
T classification (*n*=52)			0.5883
T1, 2 (early)	15	14	
T3, 4 (advanced)	10	13	
N classification (*n*=52)			0.7852
N0 (negative)	15	15	
N1, 2, 3 (positive)	10	12	
M classification (*n*=52)			>0.9999
M0	25	27	
M1	0	0	
Stage (*n*=52)			0.5697
I, II	11	9	
III, IV	14	18	

IHC score: Immunohistochemical score

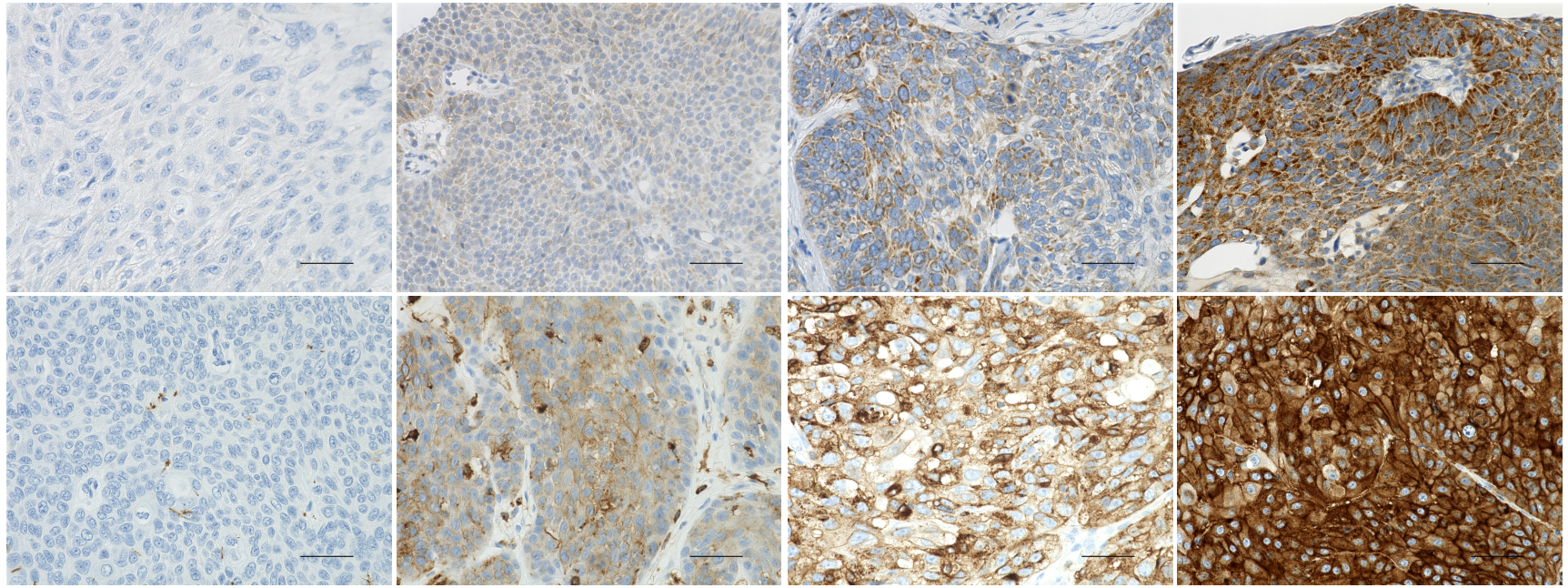
Supplemental Table S5 Relationship between the clinicopathological features of HNSCC patients and T-cell response to PLAC1 peptide

Features	T-cell response to $PLAC1_{31-50}$ peptide		*P*
	Negative	Positive	
Gender (*n*=12)			0.2045
Female	0	3	
Male	5	4	
Age (years) (*n*=12)			>0.9999
<70	2	4	
≥70	3	3	
Tobacco (*n*=12)			0.2424
Current or former smoker	4	2	
Never smoker	1	5	
Alcohol (*n*=12)			0.4697
Current or former drinker	5	5	
Never drinker	0	2	
HPV status (*n*=4)			>0.9999
Positive	1	1	
Negative	1	1	
T classification (*n*=12)			>0.9999
T1, 2 (early)	3	4	
T3, 4 (advanced)	2	3	
N classification (*n*=12)			0.0455
N0 (negative)	3	0	
N1, 2, 3 (positive)	2	7	
M classification (*n*=12)			>0.9999
M0	5	6	
M1	0	1	
Stage (*n*=12)			0.2222
I, II	3	1	
III, IV	2	6	
PLAC1 IHC score (*n*=11)			0.0606
<4	3	0	
≥4	2	6	
HLA-DR IHC score (*n*=11)			0.2424
<4	4	2	
≥4	1	4	

HPV: Human papilloma virus
IHC score: Immunohistochemical score

Supplemental Figure S1

A



B

PLAC1	Staining intensity score 0 No staining	1 Weak	2 Moderate	3 Strong
Quantity score 0 <5%	10	10	0	0
1 5-25%		5	0	0
2 26-50%		0	13	1
3 >50%		6	6	1

IHC score <4 | IHC score ≥4

C

HLA-DR	Staining intensity score 0 No staining	1 Weak	2 Moderate	3 Strong
Quantity score 0 <10%	17	7	6	0
1 10-25%		2	2	1
2 26-50%		0	2	5
3 >50%		3	3	4

IHC score <4 | IHC score ≥4

Immunohistochemical (IHC) score: 0 1 2 3 4 5 6

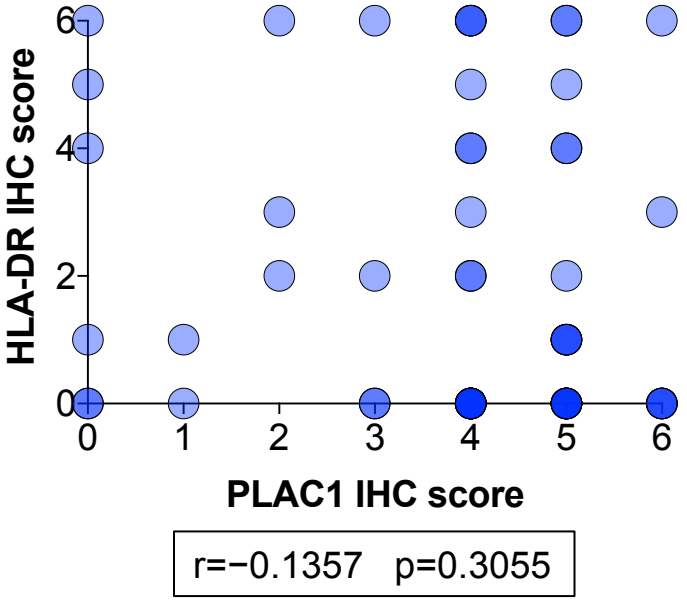
D

HLA-DR IHC score	PLAC1 IHC score <4	PLAC1 IHC score ≥4	
<4	16 30.8%	18 34.6%	34 65.4%
≥4	9 17.3%	9 17.3%	18 34.6%
	25 48.1%	27 51.9%	52

Supplemental Figure S2

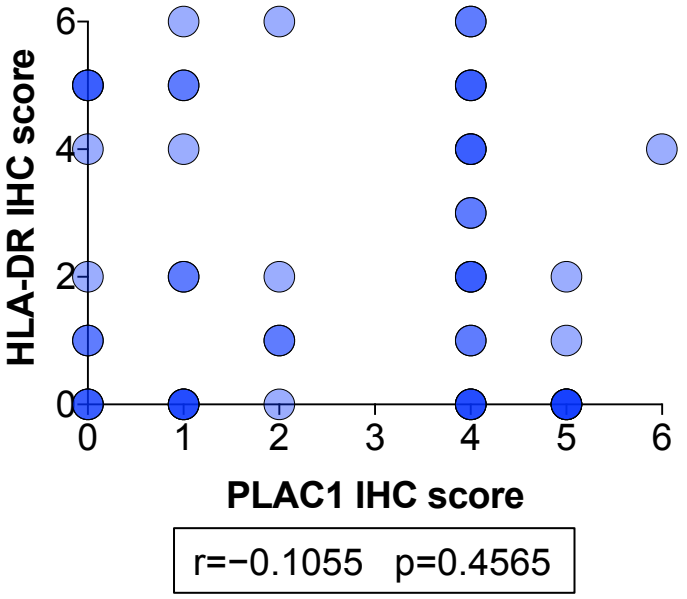
A

HLA-DR IHC score \ PLAC1 IHC score	0	1	2	3	4	5	6
0	2	1	0	2	11	8	4
1	1	1	0	0	0	4	0
2	0	0	1	1	2	1	0
3	0	0	1	0	1	0	1
4	1	0	0	0	2	2	0
5	1	0	0	0	1	1	0
6	1	0	1	1	3	2	1



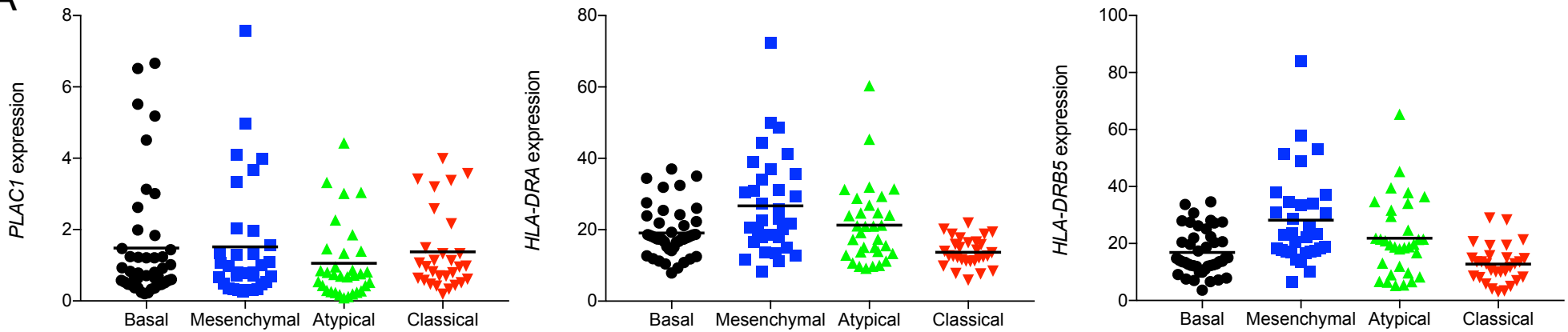
B

HLA-DR IHC score \ PLAC1 IHC score	0	1	2	3	4	5	6
0	3	4	1	0	4	5	0
1	2	0	2	0	2	1	0
2	1	2	1	0	3	1	0
3	0	0	0	0	2	0	0
4	1	1	0	0	3	0	1
5	3	2	0	0	3	0	0
6	0	1	1	0	2	0	0



Supplemental Figure S3

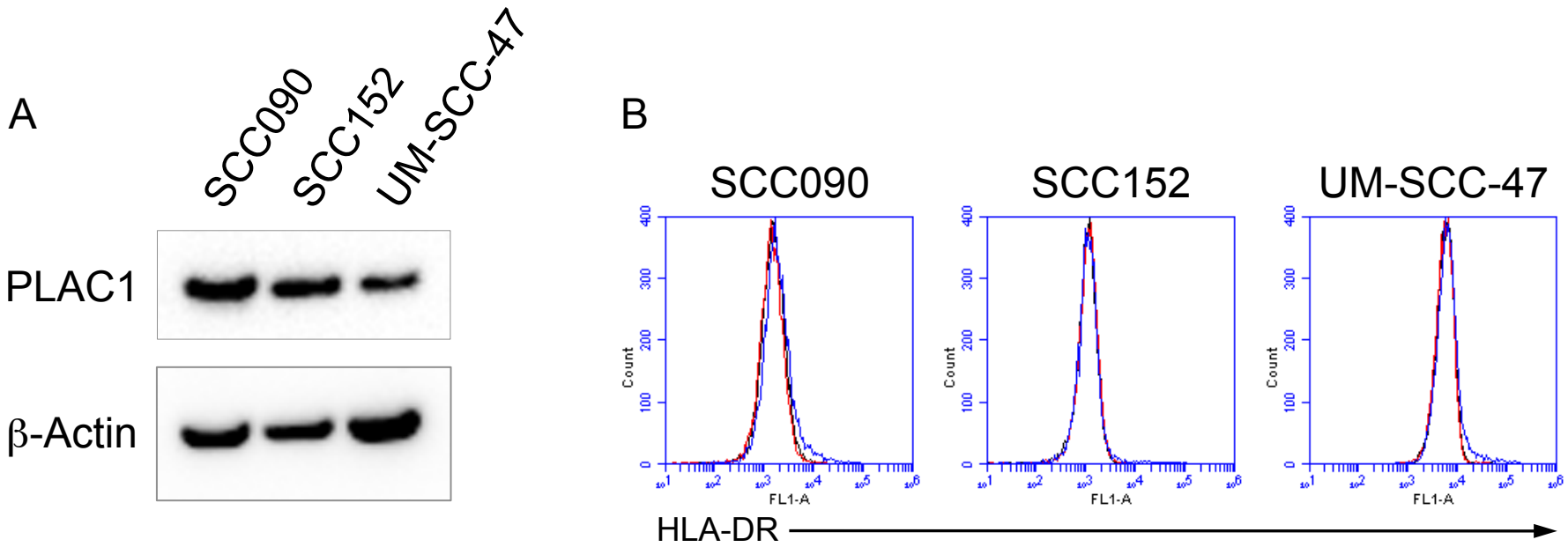
A



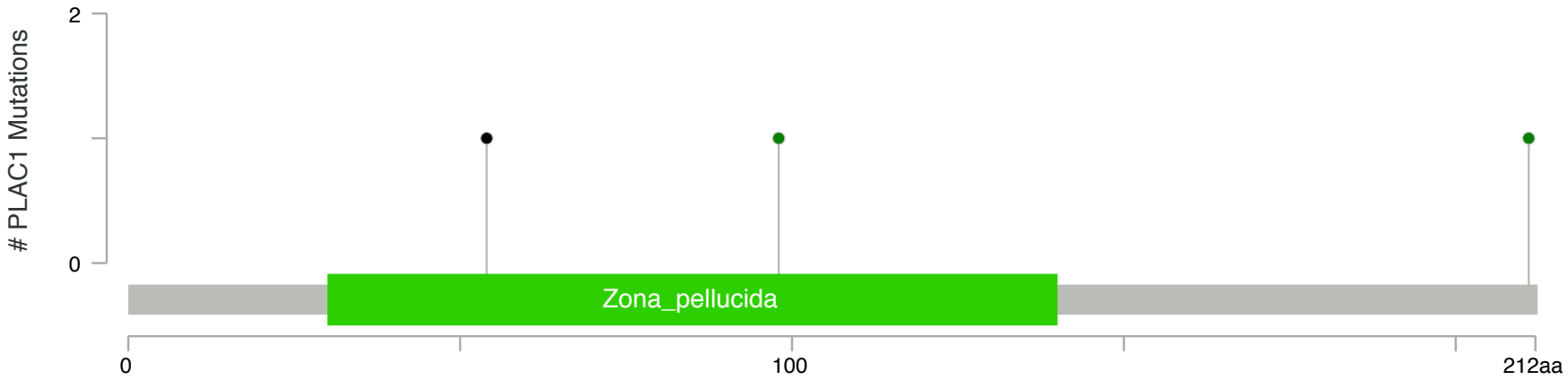
B

	Oneway ANOVA	Atypical vs Basal		Atypical vs Classical		Atypical vs Mesenchymal		Basal vs Classical		Basal vs Mesenchymal		Classical vs Mesenchymal	
	p-Value	p-Value	Fold change	p-Value	Fold change	p-Value	Fold change	p-Value	Fold change	p-Value	Fold change	p-Value	Fold change
PLAC1	2.501E-01	3.889E-01	1.396	2.868E-01	1.510	3.492E-01	1.450	9.837E-01	1.082	9.978E-01	1.039	9.981E-01	-1.041
HLA-DRA	2.419E-06	9.171E-01	-1.064	2.160E-03	-1.457	1.581E-01	1.238	7.966E-03	-1.369	1.970E-02	1.317	7.806E-06	1.804
HLA-DRB5	1.503E-06	5.241E-01	-1.195	3.945E-03	-1.632	1.142E-01	1.367	9.590E-02	-1.366	9.229E-04	1.634	7.820E-06	2.232

Supplemental Figure S4

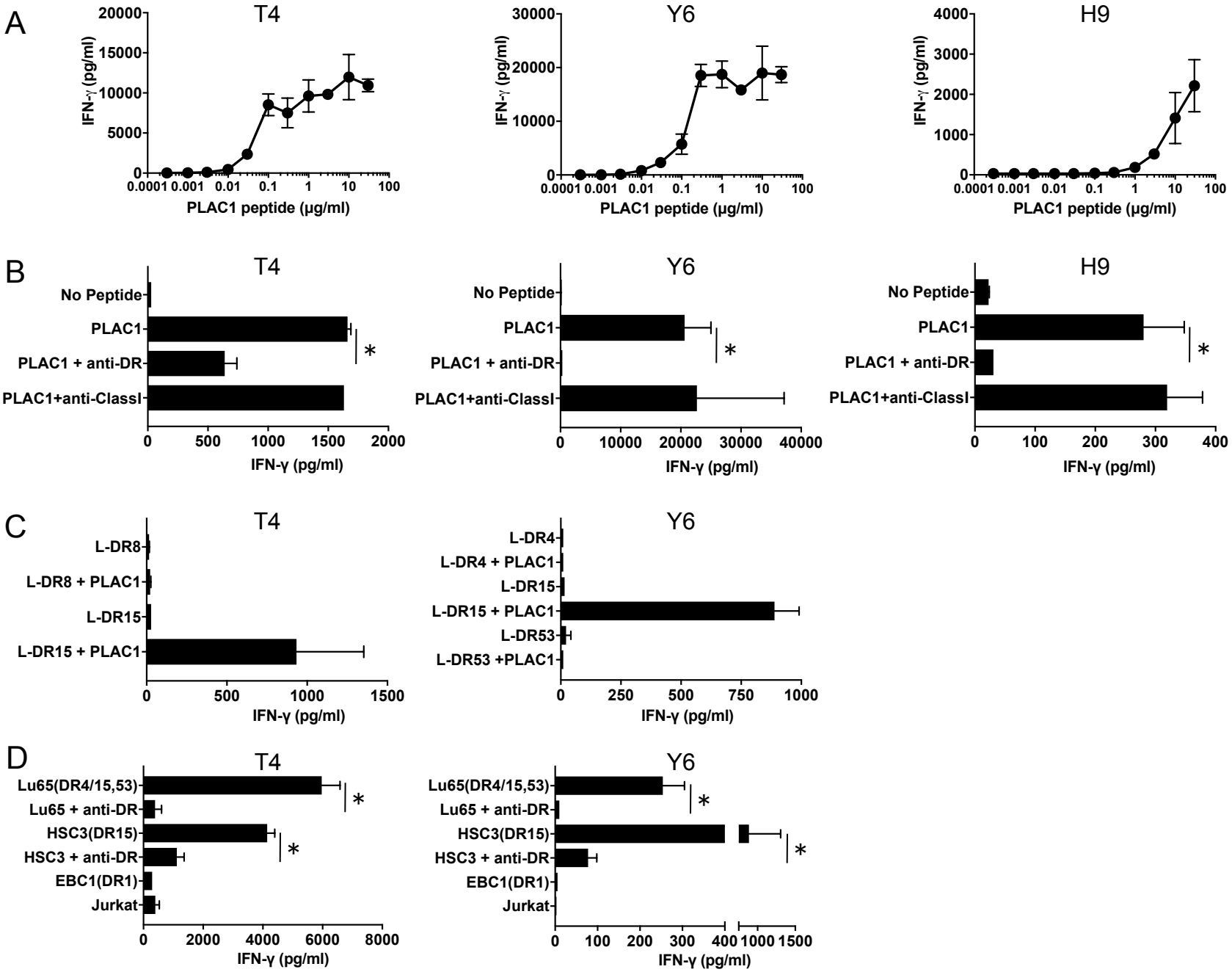


Supplemental Figure S5



Sample ID	Mutation type	Protein change
TCGA-QK-A8Z8-01	Nonsense	E54*
TCGA-CV-7415-01	Missense	I98M
TCGA-UF-A7J9-01	Missense	S211T

Supplemental Figure S6



Supplemental Figure S7