Supplementary Online Content

Gezgin G, Luk SJ, Cao J, et al. PRAME as a potential target for immunotherapy in metastatic uveal melanoma. *JAMA Ophthalmol.* Published online April 27, 2017. doi:10.1001/jamaophthalmol.2017.0729

eAppendix. Methods

eReference

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Methods

PRAME Expression by Real-Time Quantitative PCR on UM Cell Lines

Total RNA was isolated using the micro RNAqueous kit (Ambion, Thermo Fisher Scientific Inc., Waltham, MA, USA) before DNA was fragmented with DNAse and removed by RNeasy mini kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using oligo dT primers with M-MLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). *PRAME* expression was measured on the Roche Lightcycler 480 (Roche, Basel, Switzerland) using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotium, Hayward, CA) with forward primer 5' CGTTTGTGGGGTTCCATTC 3' and 5' GCTCCCTGGGCAGCAAC 3'. Each sample was run in triplicate using cDNA from 20ng of total RNA. Relative mRNA expression of *PRAME* in samples was calculated using the $2^{-\Delta\Delta CT}$ method[1] with the expression of *PBGD* serving as endogenous reference gene and the average expression of *PRAME* in Mel1.14 serving as calibrator.

PRAME Expression by mRNA Fluorescence in situ Hybridization

An oligonucleotide probe for *PRAME* mRNA was designed using the Stellaris ®FISH Probe Designer (Biosearch technologies Inc., Petaluma, CA, USA). All probes were individually analyzed and probes that contained less than two mismatched bases with any non-*PRAME* mRNA molecule were removed. A total of 46 probes was selected. Oligonucleotide probes recognizing *GAPDH* were commercially available from BioSearch Technologies (Petaluma, CA, USA). For both genes, two probe sets were ordered: one labelled with the Quasar 570 fluorescent dye and one with the Quasar 670

fluorescent dye. In all experiments, *GAPDH* served as an internal control for mRNA quality of the FFPE slides. There was no spectral overlap between Quasar 570 and 670 probe sets.

To validate our *PRAME* probe sets, mRNA FISH was performed on FFPE slides from 14 of 64 primary UM of which we had RNA expression data available on the Illumina HT-12v4 platform. Then, *PRAME* expression in UM metastases from 16 unrelated patients was assessed by mRNA FISH. Hybridizations were performed according to the manufacturer's protocol with the following modifications. FFPE tissue sections were deparaffinized in xylene and hydrated in decreasing concentrations of ethanol (EtOH) to 70% EtOH, in which they were permeabilized for 30 min before autofluorescence was reduced by incubating slides in 70% EtOH with 0.25% ammonia for 1 h. Tissue sections were further digested by incubation with 10µg/ml proteinase K (Promega, Madison, WI, USA) at 37°C and dehydrated in ascending series of ethanol. Hybridisation was performed under a cover slip in a moist chamber at 37°C for 24h. The hybridization mix consisted of 2X Saline-Sodium Citrate buffer (SSC), 10% deionised formamide, 10% dextran sulphate, 0.1 mg/ml yeast tRNA and probe sets at a final concentration of 125nM each. Post-hybridization washes were done in 2X SSC with 10% formamide at 37°C, subsequent washing in 2X SSC to remove formamide and dehydration. Tissue sections were then embedded using ProLong® Gold (Thermo Fisher Scientific, Waltham, MA, USA) with DAPI (4',6-Diamidino-2-Phenylindole Dilactate). Pictures were taken using a Leica DM5500 microscope equipped with a 63X oil immersion lens with a numerical aperture of 1.4, using appropriate filter sets and in-house capturing software.

Targeted mRNA molecules could be seen as refraction limited dots. For each tumor, two slides were hybridized. One slide was hybridized with the *GAPDH* probe set with the Quasar 670 label and the *PRAME* probe set with the Quasar 570 label and the other slide was hybridized with the same probe sets but with reversed labels. Multiple images were taken throughout each slide and scored by two independent observers (M.H.M.H. and S.J.L.). If initial scoring differed between observers, a consensus was reached after re-evaluation of the images. The Cohen's kappa statistic was used to test interrater agreement. A tumor was scored as *PRAME* positive when *PRAME* mRNA molecules could be visualized in pictures from both reversed-dye experiments. When *GAPDH* but not *PRAME* was present in both colour combinations, the tumor was scored as *PRAME* negative. If there was no clear *GAPDH* signal in both colour combinations in any of the pictures, the tumor was excluded from the analysis.

HLA Class I Staining and Scoring

Monoclonal mouse antibodies against HCA2 (anti-HLA-A) and HC10 (anti-HLA-B/C) (Nordic-MUbio, Susteren, The Netherlands) were used at concentrations of 1:50 and 1:100, respectively. A poly-rabbit anti-β2M antibody (1:2000) was used to stain β2M (A-072, DAKO Denmark A/S, Glostrup, Denmark). The following secondary antibodies with Alexa fluorochromes were used: goat-anti-rabbit IgG Alexa 647 to detect β2M, goat-anti-mouse IgG1 Alexa 488 to detect HLA-A and goat-anti-mouse IgG2 Alexa 546 to detect HLA-B/C (Molecular Probes, Bleiswijk, The Netherlands; all 1:200). Blood vessels, infiltrating leukocytes and stromal cells served as internal positive controls. Sections were analyzed and scored by two independent researchers (G.G. and E.S.J.) with

a confocal microscope (LSM 700, Zeiss, Germany).

For the HLA staining, intensity was scored as 0, 1, 2 and 3, indicating an absent, weak, positive or strong expression, respectively. The percentage of positive tumor cells was scored as: 0 for 0% (absent), 1 for 1-5% (sporadic), 2 for 6-25% (local), 3 for 26-50% (occasional), 4 for 51-75% (majority), 5 for 76-100% (large majority). The scores for intensity and percentage of positive tumor cells were added and were used to identify three categories of HLA class I expression: positive (total score 7 to 8), weak (3 to 6) or negative (0 to 2).

eReference

1. Livak KJ, Schmittgen TD. Analysis of relative 15 gene expression data using real-time quantitative PCR 16 and the 2(-Delta Delta C(T)) method. *Methods*. 17 2001;25(4):402-408.