Supplementary Online Content

Wong K, Robles-Espinoza CD, Rodriguez D, et al. Association of the *POT1* Germline Missense Variant p.I78T With Familial Melanoma. *JAMA Dermatol*. Published online December 26, 2018. doi:10.1001/jamadermatol.2018.3662

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

Supplementary Information

Supplementary Tables, Figures and Methods

eTable 1a: Coding mutations in established melanoma genes found in the melanoma from the *POT1* p.I78T mutation carrier (father) in pedigree 1 (Figure 1).

Chrom.	Position	ClinVar/dSNP ID	Ref.	Mut.	Gene	Amino Acid Change	Mutation Type
3	141586704	rs763528237	C	T	*RASA2*	R629*	Nonsense
4	54727495	rs121913513 COSM1290	T	C	*KIT*	L576P	Missense
17	7675190	rs587781288 COSM44911	C	A	*TP53*	C141F	Missense
17	31261809	rs137854555*	GG	AA	*NF1*	W1559*	Nonsense
18	32068313	rs748776580*	G	A	*RNF125*	E628K	Missense

Shown are mutations in known melanoma genes, identified in a melanoma from the father of the proband in pedigree 1. An asterisk (*) in the "ClinVar/dbSNP ID" column indicates the non-reference base in the ClinVar and/or dbSNP database is different than the base observed here. Nucleotide positions are from GRCh38.

Supplementary Table 1b: A tally of hotspot driver mutations in nevi from the son in pedigree 1.

Gene	Mutation	Samples
NRAS	Q61K	7
	Q61L	1
	Q61R	4
BRAF	V600D	1
	V600E	4
	F583L	1

Mutations were identified in nevi from the proband in pedigree 1. The *BRAF* V600D and F583L mutations were found in the same sample.

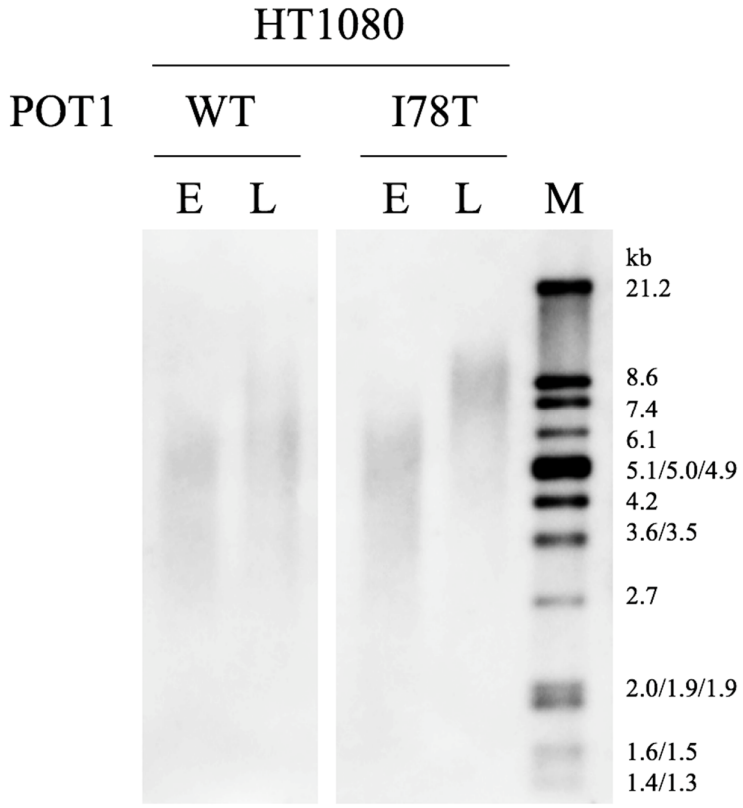
eTable 2: Mutation spectra of tumors from germline p.I78T carriers.

	Mutation					
	C>A	C>G	C>T	T>A	T>G	T>C
CLL	0	2	8	0	0	2
Melanoma	31	31	1597	54	40	86

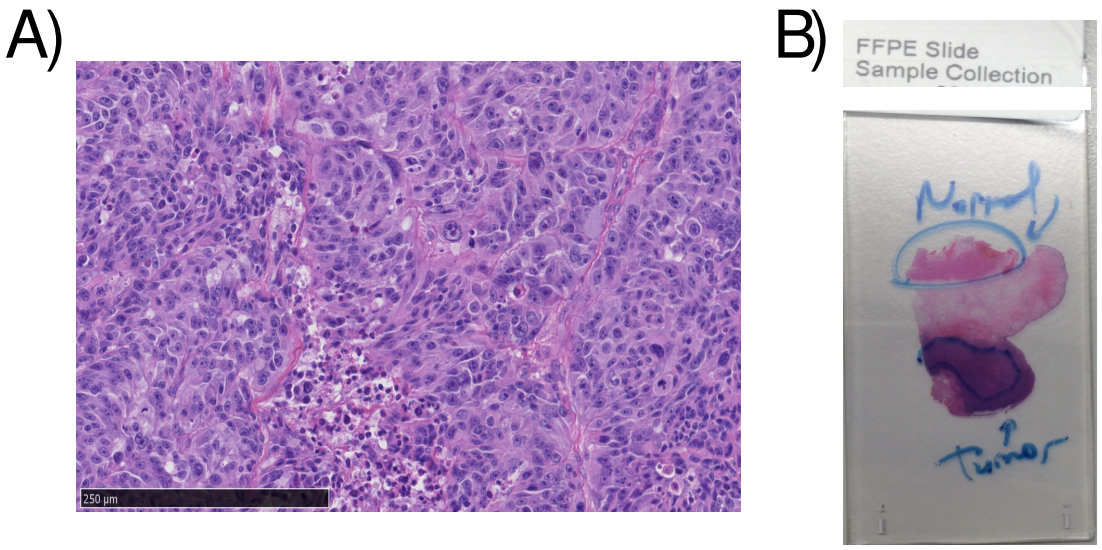
Mutations were identified from whole-exome sequencing of a chronic lymphocytic leukemia (CLL) sample from the proband and a melanoma from the proband's father (pedigree 1). Included are missense, synonymous, nonsense, and translational start mutations.

***Telo*TAGGG Telomere Length Assay** (Roche, Cat. No. 12 209 136 001)

Early passage (E) = 15.1 population doublings
Late passage (L) = 45.4 population doublings
(with an average doubling time of 25.4 h for each cell line)



eFigure 1: Telomere lengthening in HT1080 cells stably expressing mutant POT1. HT1080 cells were transduced to stably express POT1-WT or POT1-I78T, and telomere lengths were assessed at early (E, 15.1 population doublings) and late (L, 45.4 population doublings) time points after transduction. M, molecular weight marker.



eFigure 2: Histology of a melanoma tumor from a POT1 p.I78T carrier does not show defining features. A) Histology image showing a primary cutaneous polyploid nodular malignant melanoma, corresponding to the neck lesion in Figure 1A, invading deeply into the dermis with ulceration. B) Formalin-fixed paraffin-embedded slide of this specimen.

eMethods

Previous screening for mutations in established high-penetrance melanoma genes

All patients had been screened and found negative for mutations in *CDKN2A, BAP1* and *CDK4*. The patients in pedigrees 1 and 2 were also negative for the *MITF* E318K variant.

Whole exome sequencing

Whole exome sequencing was performed on DNA from 30 nevi, 3 normal skin samples, normal blood and a CLL sample from the proband, and a melanoma and normal blood sample from the proband's father (Figure 1, pedigree 1). Several nevi samples were divided into 2 samples and sequenced separately. Exome enrichment was performed using the Agilent SureSelectXT Human All Exon v5 platform following the manufacturer's protocol, and captured DNA fragments were indexed and paired-end sequenced using the Illumina HiSeq 2000 platform at the Wellcome Sanger Institute. The raw 75bp sequencing reads were aligned to the reference genome GRCh38 (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/GRCh38_reference_genome/), which includes the GRCh38 primary assembly, the mitochondrial genome, additional unplaced human contigs, alternate loci, HLA sequences, decoy sequences, and the sequence of the Epstein-Barr virus. Alignments were performed using the Burrow-Wheeler Aligner software package (version 0.7.13-r1039, [1]) to generate binary Sequence Alignment/Map (BAM) files. Two sequencing runs were performed for each sample. Although there are two different library identifiers in each raw BAM file, the same library preparations were used in the two sequencing runs. Because PCR duplicates are identified only within the same library using the library identifiers, the BAM files were modified to contain only a single library identifier prior to re-running Biobambam (v2.0.18, [2]) 'bammarkduplicates'. The mean PCR duplicate rate was 28% (range 13-50%). After removing PCR duplicates, secondary alignments, supplementary alignments, and reads failing Illumina chastity filtering, the mean sequencing depth was 126x (range 73-171x). These 'clean' BAMs were used to identify somatic mutations using MuTect (version 1.1.7,[3]) with default parameters. The nevi and CLL were compared to the proband's normal blood, and the father's blood sample was used as the matched normal for the father's melanoma (pedigree 1, Figure 1). In the variant calls from the FFPE nevus samples, we observed a high proportion of C>T/G>A mutations with low variant allele fractions (VAF). Mutation signature analysis using SomaticSignatures[4] (version

2.6.0) revealed that the majority of these were due artefacts generated from 5-methylcytosine (5mC) deamination. In the variant calls from the FFPE melanoma sample, a high number of C>T/G>A mutations were present at both high and low VAF, and the dominant mutation signature present was associated with UV-damaged DNA (Alexandrov signature 7, [5]). In contrast, the CLL sample from blood had very few mutations and no C>T/G>A bias. Thus, to reduce artefacts, somatic mutations were removed from the FFPE sample mutation catalogue if one of the following was true: the coverage at the site in the lesion/cancer was less than 10, the VAF was less than 0.05, the VAF was less than 0.15 with 5 or less supporting reads, or the VAF was less than 0.20 and the total depth was less than 20. Analysis of the remaining mutation calls revealed the presence of signature 7 in several nevus samples, while other samples had too few mutations for a signature analysis. However, with this additional filtering, some mutations in known hotspot positions (in *NRAS*) were lost due to low cellularity in the DNA samples. Thus, the number of hotspot mutations we report might be an underestimate of the true mutation frequency for the reasons outlined above.

Capillary sequencing

All p.I78T mutation carriers were confirmed using capillary sequencing. PCR products for sequencing were amplified with the primers I78T_For: CATTTCTGGGGAATGAAAGC and I78T_Rev: AACTTGCCTGCTCTTTAGTGG.

Genotyping arrays

Illumina CoreExome arrays were used to genotype individuals from pedigrees 1-3 using the method described by the manufacturer.

Telomere gel shift assay

POT1-telomere gel shift assays were performed as described previously [6]. POT1 WT, Y89C, I78T and POT1Δ cDNA constructs were synthesized by GeneArt and fully sequence-validated.

Cell lines and culture conditions

The cell lines HT1080 and Phoenix-GP were purchased from ATCC and cultured under standard conditions in DMEM (Sigma Aldrich) supplemented with 10% FBS, 100 Units/ml penicillin, 100 µg/ml streptomycin and 0.29 mg/ml L-glutamine (Gibco). Cells were subcultured every 2-3 days and tested for mycoplasma contamination. The population doubling time of HT1080 cells was determined at three time points and used to calculate an average doubling time of 25.4 h according to the least square fitting method.

***POT1* cDNA overexpression in HT1080 cells for telomere length studies**

Wildtype (WT) and mutant POT1 (I78T) cDNAs were generated by GeneArt and cloned into the pLPCX-IRES-GFP vector (Addgene, # 65436) replacing the sequence encoding IRES-GFP. Vectors were validated using Sanger sequencing (Eurofins). VSV-G pseudotyped retroviral particles were produced by transfecting Phoenix-GP cells with pCMV-VSV-G and either pLPCX-POT1-WT or pLPCX-POT1-I78T using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). Supernatants containing retroviral particles were harvested 48 h and 72 h after transfection, pooled and filtered using Millex-HP Filter Units (0.2 µm, Millipore). Cell lines stably expressing POT1-WT or POT1-I78T were generated by incubating HT1080 cells with retroviral supernatant and 0.8 µg/ml polybrene (Thermo Fisher Scientific) for one day on two consecutive days followed by puromycin selection (1 µg/ml, InvivoGen) for seven days. Expression of cDNAs was confirmed by qPCR.

Telomere length assay

Genomic DNA (gDNA) was isolated from HT1080 cells transduced with pLPCX-POT1-WT or pLPCX-POT1-I78T at different time points after transduction (as indicated in the legend of **Supplementary Figure 1**) using the DNeasy Blood and Tissue Kit (Qiagen). Telomere length was assessed by performing the *Telo*TAGGG Telomere Length Assay (Roche) with 1.5 µg of gDNA according to the manufacturer`s protocol.

eReferences:

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