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Supplemental information

TINF2 is a major susceptibility gene in Danish patients with multiple primary melanoma

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE

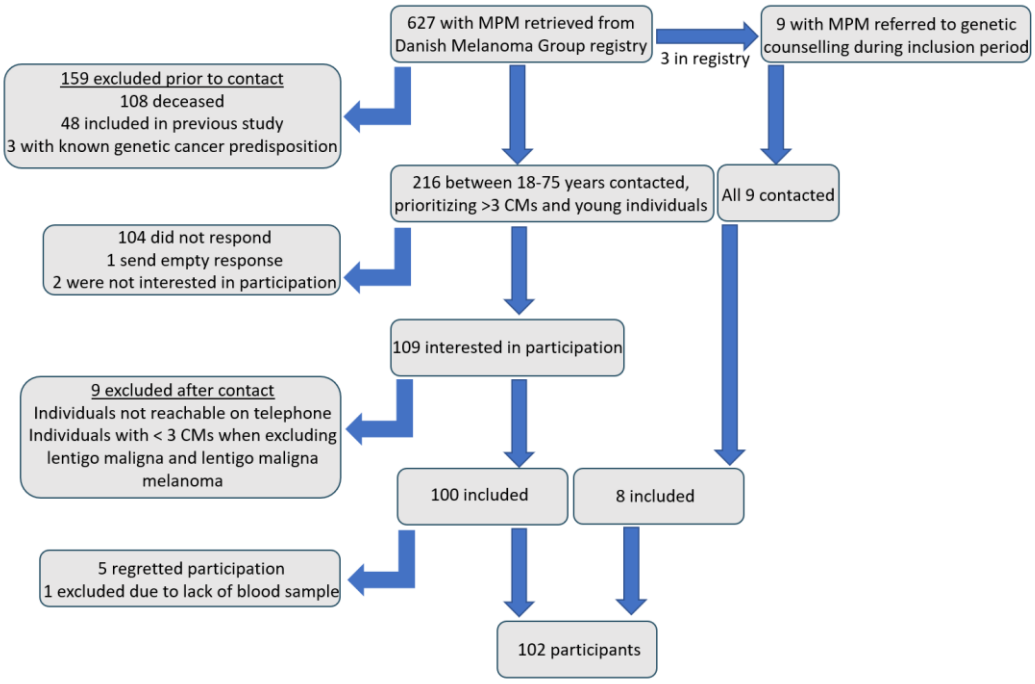

Figure S1. Inclusion flowchart. Flowchart showing the inclusion process, beginning with retrieval of MPM cases from the Danish Melanoma Group registry and patients referred to genetic counselling, ending with 102 included participants.

SUPPLEMENTAL TABLES

Gene	Variant identified[a]
ACD	NM_001082486.1:c.1332C>A , NP_001075955.1:p.(His444Gln)
ATM	NM_000051.4: c.9094G>C, NP_000042.3:p.(Val3032Leu)[b]
BAP1	NM_004656.3:c.2057-4G>T, NP_p.(?)[c]
	NM_004656.3:c.255+7T>C, NP_p.(?)
BRCA2	NM_000059.3:c.5021G>C, NP_000050.2:p.(Ser1674Thr)[d]
	NM_000059.3:c.3890_3892delATA, NP_000050.2:p.(Asn1297del)[b]
MC1R	NM_002386.3:c.836A>G, NP_002377.4:p.(Asn279Ser)
	NM_002386.4:c.637C>T, NP_002377.4:p.(Arg213Trp)[e]
	NM_002386.3:c.667C>T, NP_002377.4:p.(Arg223Trp)
	NM_002386.3:c.295C>A, NP_002377.4:p.(Leu99Ile)[f]
	NM_002386.3:c.292A>G, NP_002377.4:p.(Ile98Val)[f]
MET	NM_001127500.2:c.751C>G, NP_001120972.1:p.(His251Asp)[g]
MITF	NM_000248.3:c.895C>T, NP_000239.1:p.(Leu299Phe)
	NM_000248.3:c.719G>A, NP_000239.1:p.(Arg240His)
MLH1	NM_000249.3:c.2051A>G, NP_000240.1:p.(Tyr684Cys)
MSH2	NM_000251.3:c.698C>G, NP_000242.1:p.(Ser233Cys)[e]
MSH6	NM_000179.2:c.3647-5_3647-2dupAACA, NP_p.(?)
PMS2	NM_000535.6:c.608C>G, NP_000526.2:p.(Thr203Ser)[g]
TP53	NM_000546.5:c.572C>G, NP_000537.3:p.(Pro191Arg)[d]

[a]All variants were identified heterozygous. No VUSs were identified in more than one individual.

[b]Identified in the same individual. [c]Identified in the same individual as one of the pathogenic *MITF* variants (p.Glu318Lys). [d]Identified in the same individual. [e]Identified in the same individual. [f]Identified in the same individual. [g]Identified in the same individual.

Table S1. VUSs in cancer-related genes.

Genes
ACD, APC, AXIN2, BAP1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CDKN2B, DICER1, EPCAM, FH, FLCN, GREM1, MET, MLH1, MLH3, MSH2, MSH3, MSH6, MUTYH, NTHL1, PALB2, PMS2, POLD1, POLE, POT1, PTEN, RAD51C, RAD51D, RNF43, SDHB, SDHC, SMAD4, STK11, TERF2IP, TERT, TP53 and *VHL.*

Table S3. Clinical cancer gene panel initially performed on MZ twins.

Variant	Variant carriers	Heterozygous carriers
NM_002386.3:c.252C>A, NP_002377.4:p.(Asp84Glu)	9	9
NM_002386.3:c.451C>T, NP_002377.4:p.(Arg151Cys)	39	34
NM_002386.3:c.478C>T, NP_002377.4:p.(Arg160Trp)	39	34
NM_002386.3:c.880G>C, NP_002377.4:p.(Asp294His)	3	3
NM_002386.3:c.425G>A, NP_002377.4:p.(Arg142His)	2	2
NM_002386.3:c.487C>T, NP_002377.4:p.(Arg163Ter)	1	1
NM_002386.3:c.86dupA, NP_002377.4:p.(Asn29LysfsTer14)	2	2
NM_002386.3:c.537dupC, NP_002377.4:p.(Ile180HisfsTer59)	1	1

Table S4. *MC1R* R-alleles identified in MPM study participants.

Variants
c.81C>A, p.(C27Ter)[a]
c.805C>T, p.(Q269Ter)[4]
c.811C>T, p.(Q271Ter)[4]
c.815G>A, p.(W272Ter)[5]
c.826delA, p.(R276GfsTer41)[6]
c.838A>T, p.(K280Ter)[7]
c.839delA, p.(K280RfsTer36)[4]
c.849delC, p.(T284QfsTer33)[6]
c.849_850insC, p.(T284HfsTer8)[7]
c.851_852delCA, p.(T284SfsTer7)[8]
c.851_855delCAGTC, p.(T284NfsTer6)[9]
c.857delTinsGC, p.(M296SfsTer6)[6]
c.866_867delCC, p.(P289LfsTer2)[10]
c.867_868insC, p.(F290LfsTer2)[7]
c.872_875delGGAA, p.(R291IfsTer25)[11]
c.889delA, p.(T297PfsTer20)[12]
c.892delC, p.(Q298RfsTer19)[7]
c.1061+1G>T[13]
c.1090dup, p.(L364PfsTer9)[a]
c.1221+1G>C[14]
[a]*To our knowledge, these variants have not been published. However, they have been registered in ClinVar.*

Table S5. Pathogenic and likely pathogenic nonsense variants, frameshift variants and splice variants identified in *TINF2* associated with DC.

SUPPLEMENTAL METHODS

Approvals

This study was approved by the Capitol Region Committee on Health Research Ethics (H-3-2011-050) and the Danish Data Protection Agency (P-2021-154). The study has been conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was collected from all study participants. All patients described in this article have provided written consent to publication.

Inclusion

The study was performed from February 2021 to December 2022. 627 individuals with ≥ 3 primary cutaneous melanomas were identified using the Danish Melanoma Group registry (a nationwide registry established in 1985). 216 individuals aged 29-75 years were contacted by e-Boks (a Danish personal digital mailbox), prioritizing individuals with a history of > 3 CMs and secondly the youngest individuals at the time of data extraction. Participants ≥ 18 years of age with ≥ 3 primary CMs were offered inclusion. Cases of lentigo maligna or lentigo maligna melanoma were not included in the 3 CM cases due to their strong association to sun exposure. In situ melanomas, such as superficial spreading malignant melanoma in situ (SSMM in situ), were included in the 3 CM cases. Exclusion criteria were individuals with known genetic cancer predisposition syndromes involving melanoma susceptibility (3 individuals), deceased individuals (108 individuals), and individuals participating in a previous study of familial melanoma published by our group (44 individuals)[1].

112 of the 216 responded by e-Boks; two were not interested in participation and one send an empty response. 104 did not respond. The remaining (109) respondents agreed to participate (i.e., 50.5 % of contacted individuals). All 109 were subsequently contacted by telephone, of which 100 were included. The remaining 9 were excluded due to one of several reasons (patients not reachable by telephone and patients with < 3 CMs when excluding lentigo maligna and lentigo maligna melanoma).

Of the 100 left, 5 regretted their participation, several due to health-related issues or lack of time. Further, one participant was excluded due to lack of blood sample. Thus, 94 participants included by e-Boks fulfilled participation. During the study, patients with ≥ 3 primary CMs referred to genetic counseling for suspicion of familial melanoma in the Department of Clinical Genetics in Copenhagen and Roskilde were offered inclusion in the study, of which 8 agreed to participate. 3 of the patients were in the registry and thus not contacted by e-Boks. Thus, a total of 102 participants. One of the participants had one of the three melanomas as "uncertain if primary tumor or metastasis", all other participants had ≥ 3 confirmed primary cases. None of the participants were near relatives, to our knowledge. A flow chart of the inclusion process can be viewed in Figure S1.

Participation

All participants received project information by telephone or in person, as well as written information. All participants performed a questionnaire by telephone or in-person in the Department of Clinical Genetics, Rigshospitalet. The questionnaire included a questionnaire developed by GenoMEL (https://genomel.org/) with questions regarding sun-seeking behavior, sunburns, use of sunscreen and other precautions to sun exposure, as well as sunbed use, and questions regarding tobacco use. Our questionnaire further included questions regarding skin type (skin-, hair- and eye color, skin reaction to sun exposure, freckling) and number of nevi.

In the interview, a pedigree covering 1st to 3rd generation family members was constructed for all participants regarding their history of cancer. Parents and siblings were defined as 1st degree relatives, grandparents, parents' siblings and patient's half-siblings as 2nd degree relatives, and great grandparents, grandparents' siblings, cousins and half-siblings of parents, 3rd degree relatives. After obtainment of consent, the participants' melanoma cases as well as other cancers were verified by pathological descriptions and/or medical records. Cancer cases in living family members were seized verified by pathological description (in the Danish Pathology Data Bank)

and/or medical records after obtainment of written consent. Cancer cases in deceased relatives were seized verified as well. For participants that had received genetic counseling prior to participation, the pedigree from previous counseling was retrieved if consent from the patient.

Whole exome sequencing (WES)

DNA library preparation and exome sequencing

DNA library preparation was performed using the Twist human core exome kit with custom region enrichment (Twist Bioscience). Exome sequencing was performed on a NovaSeq 6000 sequencer (Illumina) with a coverage of at least 20X in 98 % of the exome in all samples.

Data analysis pipeline

A clinical pipeline was used to generate BAM/CRAM and VCF files. Annotation and variant filtering was performed using VarSeq (https://www.goldenhelix.com). Initially, we identified families with pathogenic alterations in the known high penetrance melanoma genes (*CDKN2A, CDK4, BAP1, POT1, TERT, ACD, TERF2IP*), and selected known or putative cancer predisposition genes (in-house panel of 390 genes).

Further, structural rearrangements were investigated by searching for copy number variations (CNVs) in exome data.

Exome variant interpretation

All exonic non-synonymous variants were evaluated for their potential effect on protein function using a suite of publicly available tools, including CADD, Alamut (also including SIFT and Polyphen), and PMut. Moreover, selected variants were evaluated for their putative effect on splicing (MaxEntScan). Variants were evaluated and classified according to the ACMG guidelines[2] jointly by three researchers (e.g., M.R.J., T.v.O.H. and K.W.).

Identified pathogenic variants were reported back to the participants, and family members at risk of carrying an identified pathogenic variant were offered genetic counseling as well, either at the Department of Clinical Genetics at Rigshospitalet or their regional Department of Clinical Genetics.

Telomere length analysis

3 of the 4 clinical study participants carrying the identified *TINF2* p.(Arg265Ter) variant had subsequently performed telomere length analysis by RepeatDx Europe (Aachen, Germany), using Flow-FISH analysis on DNA from patient peripheral blood lymphocytes. A description of the technique can be viewed at RepeatDx's homepage (https://repeatdx.com/).

Polymerase chain reaction (PCR) and sequencing

Carrier screening of relatives of the *TINF2* variant carriers agreeing to be tested was performed on purified genomic DNA from whole blood sample. The *TINF2* c.793C>T, p.(Arg265Ter) was amplified using the following primers: 5'-CTGACTCAGTGAACCTGGCTGAGC-3' and 5'-GCTGCTCTTGTGCCCATGGCTAGG-3', followed by sequencing using an ABI3730 DNA analyzer (Applied Biosystems).

***BRAF* analysis**

BRAF analysis was performed in a clinical setting on two invasive melanomas of one individuals with the Idylla *BRAF* method that detects presence of the variants: 1. V600E/V600D and 2. V600K/ V600R/V600M. The analysis does not differentiate between variants within the two groups. The sensitivity of the analysis is 5% tumor cells.

Haplotype analysis

The age of the mutation was estimated using a statistical model[3]. A pedigree was built as a binary tree with the five carriers as leaf nodes, three ancestral nodes and seven edges. Each edge is associated with a number of meioses, *M*, describing the closeness between the two nodes the edge connects. The probability that a recombination has occurred between loci *m* and *n* and not

between locus *m* and the *TINF2* mutation is $\theta_{n,m}$ = exp(-$\lambda x_n M$) – exp(-$\lambda x_m M$), where x_i is the distance from the *TINF2* mutation to locus *i*, and lambda is 10^{-8} assuming 1cM equals 1 Mb. The joint likelihood of the genotypes (g) across the *N* loci is then given by

$$P(g|M) = \sum_{n_1}^{N} \theta_{n_1,n_1+1} \sum_{n_2}^{N} \theta_{n_2,n_2+1} \sum_{n_3}^{N} \theta_{n_3,n_3+1} \sum_{n_4}^{N} \theta_{n_4,n_4+1} \sum_{n_5}^{N} \theta_{n_5,n_5+1} \sum_{n_6}^{N} \theta_{n_6,n_6+1} \sum_{n_7}^{N} \theta_{n_7,n_7+1} P(g|n),$$

where we for simplicity set $x_{N+1}=\infty$ and $P(g|n)$ is given by

$P(g|n) = \prod_{i=1}^{N} P(g_i|n) = \prod_{i=1}^{N} \prod_{j=1}^{G} P(g_{ij}|n)$, where *i* runs over the loci and *j* runs over groups of individuals defined by the recombination pattern, *n*, such that individuals are identical by descent (i.e. sharing a haplotype) if and only if they are in the same group. For singletons $P(g_{ij}|n)$ is provided by the genotype caller as Phred-scaled genotype likelihoods and capped at 0.999 and for larger groups the likelihood is calculated similarly by conditioning on a shared allele within the group. This can viewed as a hidden Markov model in which the state of the model is defined by the grouping, i.e., which individuals are in linkage at that locus and it only depends on the grouping at the previous locus and the transition probabilities, i.e., the probabilities for recombination. The Viterbi method was used to determine the most likely path of the model, which corresponds where the recombinations occurred. The number of meioses, and thereby the age of the mutation, was estimated by maximizing the likelihood for the model and each permutation of the pedigree. The confidence interval was determined using Monte Carlo summation such that $\sum_{age \in CI} L \Big/ \sum L < 0.95$.

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