Methods for identifying skin cancer, administering biopsies and treating skin cancer

Related application

[0001] This application claims the benefit of priority from Australian provisional application no. 2024900717 filed 18 March 2024, and Australian provisional application no. 2024901602 filed 29 May 2024, the entire disclosures of which are incorporated herein by reference.

Field of the disclosure

[0002] The present disclosure relates to the field of medical diagnostics, prognosis and identifying subjects in need of therapy. In particular, the disclosure relates to methods and kits for determining whether an individual has, or likely has, a melanoma or melanocytic naevus. The disclosure also relates to diagnostic methods for melanocytic lesions and methods for treating an individual whom has been determined to have melanoma or melanocytic naevus.

Background of the disclosure

[0003] Melanoma is a common skin cancer with a significant financial burden.

[0004] Early detection is closely associated with better survival, low morbidity and reduced healthcare costs. Histopathology, while regarded as the gold standard for diagnosis of pigmented skin lesions, is not infallible and can result in either over diagnosis – resulting in unnecessary treatment/associated stress - or under diagnosis, which can have fatal consequences.

[0005] Benign melanocytic naevi and melanomas are tumours that exhibit considerable morphological heterogeneity and for which many histological features are common. As a consequence, the pathological diagnosis of melanocytic tumours can be difficult. Currently, a high proportion of melanocytic tumours cannot be categorised as either melanoma or melanocytic naevus. These 'histologically ambiguous', 'borderline' tumours or 'melanocytic tumours of uncertain malignant potential' (MELTUMP) are treated as melanoma as a precaution. This means that currently treatment is more

conservative than ideal with more extensive excision/surgery than would be required if the number of histologically ambiguous tumours was reduced.

[0006] There is a need for new methods for identifying, diagnosing, and treating melanocytic lesions in patients. Preferably, these new methods have better sensitivity and specificity, for example resulting in reduced rates of over or under diagnosis. Preferably, the methods of treatment have reduced rates of over and/or under treatment. Optionally, the improvement results from use of a combination of the new methods in combination with the current histopathology methods.

[0007] Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

Summary of the disclosure

[0008] In one aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: noncoding mutations in *pTERT, RPL13A and SMUG1,*

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0009] Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SWI5, SYF2, UBXN8, YAE1D1 and ZNF778*. Optionally, the method further comprises detecting the presence

of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 31, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2, to 31, 3 to 5, 3 to 10, 3 to 15, 3 to 20, 3 to 31, 5 to 31, 10 to 31, 15 to 31 or 20 to 31 of these additional genes.

[0010] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *TP53, CDKN2A, RAC1, PTEN.* Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *BAP1, BRAF, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, RAF1, RB1, SF3B1, TERT,* and *TYRP1*, Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15, 3 to 25, 3 to 25, 10 to 25, or 20 to 25 of these additional genes.

[0011] Optionally, the method further comprises detecting the presence of copy number alterations of one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53.* Optionally, the method further comprises detecting the presence of copy number alterations for 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

[0012] In one aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *TP53, CDKN2A, RAC1, and PTEN*; and copy number alterations for *CDKN2A and CDK4.*

  wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0013] Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *BAP1, BRAF, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, RAF1, RB1, SF3B1, TERT,* and *TYRP1*. Optionally, the method further comprises detecting the presence of coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 21, 2 to 5, 2 to 10, 2 to 15, 2 to 21, 3 to 5, 3 to 10, 3 to 15 or 3 to 21, 10 to 21 of these additional genes.

[0014] Optionally, the method further comprises detecting the presence of copy number alterations for one or more of the following genes: *BAP1, BRAF, CCND1, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53*. Optionally, the method further comprises detecting the presence of copy number alterations for 1 to 5, 1 to 10, 1 to 15, 1 to 21, 2 to 5, 2 to 10, 2 to 15, 2 to 21, 3 to 5, 3 to 10, 3 to 15 or 3 to 21, 10 to 21 of these additional genes.

[0015] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: non-coding mutations in *pTERT, RPL13A, and SMUG1*. Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SWI5, SYF2, UBXN8, YAE1D1 and ZNF778*. Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 30, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 30 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 30, 10 to 30 or 20 to 30 of these additional genes.

[0016] In embodiments where there is a non-coding mutation in the pTERT gene, optionally the non-coding mutation is not in the promoter region of the *TERT* gene.

[0017] In some embodiments, the gene alterations are: coding mutations in *TP53, CDKN2A, RAC1* and *PTEN*; copy number alterations in *CDKN2A* and *CDK4; and* non-coding mutations in *pTERT, RPL13A, and SMUG1*.

[0018] In another aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: non-coding mutations in C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, ZNF778.

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0019] Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *ARHGEF18, BLCAP, CHCHD2, ERGIC3, NFKBIE, RALY/RP5-1125A11.1, RPL13A, RPS27, SMUG1, SWI5, SYF2, TERT and YAE1D1.* Optionally, the method further comprises detecting the presence of coding mutations in 1 to 3, 1 to 5, 1 to 10, 1 to 13, 2 to 3, 2 to 5, 2 to 10, 2 to 13, 3 to 5, 3 to 10, 3 to 13 or 5 to 13 of these additional genes.

[0020] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT and TYRP1.* Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *BRAF, CDKN2A, CDK4, NRAS, PLCB4, PTEN, RAC1 and TP53.* Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15, 3 to 25, 3 to 25, 10 to 25, or 20 to 25 of these additional genes.

[0021] Optionally, the method further comprises detecting the presence of copy number alterations in one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1,*

MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT and *TP53*. Optionally, the method further comprises detecting the presence of copy number alterations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

[0022] In another aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; and copy number alterations in *MDM2, MAP2K1 and RAC1*.

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0023] Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *BRAF, CDKN2A, CDK4, NRAS, PLCB4, PTEN, RAC1*, and *TP53*. Optionally, the method further comprises detecting the presence of coding mutations in 1 to 3, 1 to 5, 1 to 8, 2 to 3, 2 to 5, 2 to 8, 3 to 5, 3 to 8 of these additional genes.

[0024] Optionally, the method further comprises detecting the presence of copy number alterations for one or more of the following genes: *BAP1, BRAF, CCND1, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53*. Optionally, the method further comprises detecting the presence of copy number alterations for 1 to 5, 1 to 10, 1 to 15, 1 to 20, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 10 to 20 of these additional genes.

[0025] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: non-coding mutations in

C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, and ZNF778. Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *ARHGEF18, BLCAP, CHCHD2, DHX16, ERGIC3, NFKBIE, RALY/RP5-1125A11.1, RPL13A, RPS27 SMUG1, SWI5, SYF2, TERT, and YAE1D1*, Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 14, 2 to 5, 2 to 10, 2 to 14, 2 3 to 5, 3 to 10, 3 to 14, 5 to 14 or 10 to 20 of these additional genes.

[0026] In embodiments where there is a non-coding mutation in the pTERT gene, optionally the non-coding mutation is not in the promoter region of the *TERT* gene.

[0027] In some embodiments, the gene alterations are: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; copy number alterations in *MDM2, MAP2K1 and RAC1; and* non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, and ZNF778*.

[0028] In another aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: non-coding mutations in *ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE* and *SWI5*,

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0029] Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *AP3D1, C16ORF59, CDC20, DHX16, DPH3/OXNAD1, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, PES1,*

RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, SLC30A6, SMUG1, TERT, UBXN8, and *ZNF778*. Optionally, the method further comprises detecting the presence of coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15, 3 to 20, 3 to 23, 5 to 23, 10 to 23 or 15 to 23 of these additional genes.

[0030] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *PLCB4, BRAF, NRAS, CDK4*. Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *TP53, CDKN2A, RAC1, PTEN, BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT,* and *TYRP1*, Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 25, 5 to 25, 10 to 25, 15 to 25 or 20 to 25 of these additional genes.

[0031] Optionally, the method further comprises detecting the presence of copy number alterations for one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53*. Optionally, the method further comprises detecting the presence of copy number alterations for 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

[0032] In another aspect, the present disclosure provides a method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;
- detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *PLCB4, BRAF, NRAS* and *CDK4*; and copy number alterations in *BRAF, BAP1, CCND1, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K2, MYC, NRAS, PTEN, RAF1, RB1, TERT, and TP53*

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma). Optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0033] Optionally, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *TP53, CDKN2A, RAC1, PTEN, BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT,* and *TYRP1.* Optionally, the method further comprises detecting the presence of coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 22, 2 to 5, 2 to 10, 2 to 15, 2 to 22, 3 to 5, 3 to 10, 3 to 15 or 3 to 21, 10 to 22 of these additional genes.

[0034] Optionally, the method further comprises detecting the presence of copy number alterations in one or more of the following genes: *CDKN2A, CDK4, MDM2, MAP2K1, and RAC1.* Optionally, the method further comprises detecting the presence of copy number alterations in 1 to 3, 1 to 6, 2 to 4, 2 to 6, 3 to 6 of these additional genes.

[0035] In some embodiments, the method further comprises detecting in the sample the presence of gene alterations in each of the following genes: non-coding mutations in *ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE, SWI5.* Optionally, the method further comprises detecting the presence of gene non-coding alterations in one or more of the following genes: *pTERT, RPL13A, SMUG1, AP3D1, C16ORF59, CDC20, DHX16, DPH3/OXNAD1, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, PES1, RP5-1125A11.1, RNF185, RPL18A, RPL29, RPL34, RPS14, SLC30A6, UBXN8, and ZNF778*, Optionally, the method further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 24, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 24, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 24, 10 to 24 or 15 to 24 of these additional genes.

[0036] In embodiments where there is a non-coding mutation in the pTERT gene, optionally the non-coding mutation is not in the promoter region of the *TERT* gene.

[0037] In some embodiments, the gene alterations are: coding mutations in *PLCB4, BRAF, and NRAS*; copy number alterations in *BRAF, BAP1, CCND1, CTNNB1,*

CYSLTR2, GNA11, GNAQ, HRAS, KIT, MAP2K2, MYC, NRAS, PTEN, RAF1, RB1, TERT, and TP53; and non-coding mutations in *ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE, SWI5.*

[0038] In any embodiment or aspect, optionally the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

[0039] In any embodiment of the above aspect, the gene alterations are: non-coding mutations in *pTERT, RPL13A, SMUG1, C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8* and *ZNF778.*

[0040] In any embodiment of the above aspect, the gene alterations are: non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8* and *ZNF778.*

[0041] In any embodiment of the above aspect, the gene alterations are: non-coding mutations in *pTERT, RPL13A* and *SMUG1.*

[0042] In any embodiment of the above aspect, the gene alterations are non-coding mutations in *pTERT* and *RPL13A.*

[0043] In any embodiment of the above aspect, the gene alterations are: coding mutations in *TP53, CDKN2A, RAC1, PTEN, BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; non-coding mutations in *pTERT, RPL13A, SMUG1, C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8* and *ZNF778;* and copy number alterations for *CDKN2A, CDK4 and MDM2, MAP2K1 and RAC1.*

[0044] In any embodiment of the above aspect, the gene alterations are: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34,*

RPS14, *UBXN8* and *ZNF778* and copy number alterations for *MDM2, MAP2K1 and RAC1.*

[0045] In any embodiment of the above aspect, the gene alterations are: coding mutations in *TP53, CDKN2A, RAC1* and *PTEN*; non-coding mutations in *pTERT, RPL13A* and *SMUG1;* and copy number alterations for *CDKN2A* and *CDK4.*

[0046] In any embodiment of the above aspect, the gene alterations are: coding mutations in *TP53* and *CDKN2A*; non-coding mutations in *pTERT* and *RPL13A* and copy number alterations in *CDKN2A.*

[0047] In any embodiment or aspect, the method is for determining whether a lesion in a subject is melanoma or a melanocytic nevus. Optionally, clinical indicators of a melanocytic lesion in the absence of gene alterations in the one or more genes indicates that the individual has a melanocytic naevus.

[0048] In any embodiment or aspect, the method further comprises collecting a genetic sample from the lesion.

[0049] In another aspect, the present disclosure provides a method of determining that a lesion is (or is likely to be) a melanoma or a melanocytic naevus, the method comprising:

- receiving a genetic sample from the lesion;
- determining the presence of gene alterations in genes selected from: coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1*, non-coding mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778*; and copy number alterations for *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53,*

wherein the presence of gene alterations in one or more genes indicates that the individual has melanoma (or the lesion is a melanoma),

or

wherein clinical indicators of a melanocytic lesion in the absence of gene alterations in one or more genes indicates that the individual likely has (or the lesion is) a melanocytic naevus.

[0050] In any embodiment, the gene alterations are:

coding mutations in BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53 and TYRP1; and non-coding mutations in AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1 and ZNF778,

wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

[0051] In any aspect or embodiment of the present disclosure, the method further comprises:

- determining a tumour mutational burden score (TMB) from the sample, wherein the TMB score is the number of gene alterations within the tested genes,
- comparing the TMB score with a reference TMB score,

  wherein a TMB score from the sample that is at or above the reference TMB score indicates that the individual likely has, or has, a melanoma or that the melanocytic lesion is melanoma (or is likely a melanoma),

  or

wherein a TMB score from the sample that is below the reference TMB score indicates that the individual likely has, or has, a melanocytic naevus or that the melanocytic lesion is a melanocytic naevus (or is likely a melanocytic naevus).

[0052] Optionally, the number of tested genes is at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20.

[0053] Optionally, the number of tested gene is 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 2 to 30, 2 to 35, 2 to 40, 2 to 45, 2 to 50, 2 to 55, 2 to 57, 3 to 15, 3 to 20, 3 to 25, 3 to 30, 3 to 35, 3 to 40, 3 to 45, 3 to 50, 3 to 55, 3 to 57, 4 to 15, 4 to 20, 4 to 25, 4 to 30, 4 to 35, 4 to 40, 4 to 45, 4 to 50, 4 to 55, 4 to 57, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 5 to 40, 5 to 45, 5 to 50, 5 to 55, 5 to 57, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 10 to 40, 10 to 45, 10 to 50, 10 to 55, 10 to 57, 20 to 25, 20 to 30, 20 to 35, 20 to 40, 20 to 45, 20 to 50, 20 to 55, 20 to 57, 30 to 35, 30 to 40, 30 to 45, 30 to 50, 30 to 55, 30 to 57, 40 to 50, 40 to 55, 40 to 57, or 50 to 57.

[0054] Optionally, the number of tested genes is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 ,14, 15,16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 or 57.

[0055] In any embodiment, the TMB score may be a coding mutation TMB score, ie a TMB score determined from the number of mutations and/or copy number alterations in the coding genes, optionally wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

[0056] In any embodiment, the TMB score may be a non-coding mutation TMB, ie a TMB score determined from the number of mutations in the non-coding genes, optionally wherein the non-coding mutation is not in the promoter region of the *TERT* gene.

[0057] In any embodiment, the TMB score may be a copy number alteration TMB, ie a TMB score determined from the number of copy number alterations in the coding genes.

[0058] In any embodiment, the TMB score may be a promoter region TMB, ie a TMB score determined by the presence of a non-coding mutation in the promoter region of the *TERT* gene.

[0059] In any embodiment, the reference TMB score may be a preassigned score. In some embodiments, the reference TMB score is between 1 and 50, between 1 and 40, between 1 and 30, between 1 and 20, between 1 and 10, between 1 and 9, between 1 and 8, between 1 and 7, between 1 and 6, between 1 and 5, between 1 and 4 or between 1 and 3. In some embodiments, the reference TMB score is at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9.5 or 10, preferably at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 or 7.5.

[0060] In any embodiment, wherein gene alterations are coding mutations and/or copy number alterations, the reference TMB score is the number of coding mutations and/or copy number alterations within the tested genes. Preferably, the reference TMB score is at least 3.

[0061] In any embodiment, wherein gene alterations are non-coding mutations, the reference TMB score is the number of coding mutations within the tested genes. Preferably, the reference TMB score is at least 6.

[0062] In any embodiment, wherein gene alterations are non-coding mutations, and the non-coding mutation is not in the promoter region of the *TERT* gene, the reference TMB score is the number of non-coding mutations within the tested genes. Preferably, the reference TMB score is at least 5.

[0063] In any embodiment, wherein gene alterations are coding mutations, and the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation, the reference TMB score is the number of non-coding mutations within the tested genes. Preferably, the reference TMB score is at least 3.

[0064] In any embodiment including a TMB score, the method comprising determining two or more tumour mutational burden scores (TMB) from the sample selected from a coding mutation TMB, a non-coding mutation TMB, a copy number alteration TMB, a promoter region TMB, and TMBs based on combinations of these.

[0065] In another aspect, the present disclosure provides a method of determining likelihood that an individual has melanoma or a melanocytic naevus, the method comprising:

- receiving a sample from the lesion;

- determining two or more tumour mutational burden scores (TMB) from the sample selected from a coding mutation TMB, a non-coding mutation TMB, a copy number alteration TMB, a promoter regions TMB, and TMBs based on combinations of these,

- wherein each TMB score is the number of gene alterations within the tested genes, wherein the gene alterations are selected from one of the following groups consisting of:

  (a) for a coding mutation TMB: coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1*, optionally wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation;

  (b) for a non-coding TMB: non-coding hotspot mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778*, optionally wherein the non-coding mutation is not within the promoter region of *TERT*;

  (c) for a copy-number TMB: copy number alterations for *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN RAC1, RAF1, RB1, TERT* and *TP53;* and

  (d) for a promoter region TMB: promoter regions mutations in *TERT*,

- comparing the two or more TMB scores from the sample with a reference data set using a multivariate statistical model to generate a multivariate score,

- comparing the multivariate score to a reference multivariate score,

  wherein a multivariate score from the sample that is at or above the reference multivariate score indicates that the individual has melanoma (or the lesion is a melanoma),

  or

  wherein a multivariate score from the sample that is below the reference multivariate score indicates that the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

[0066] In any embodiment, a TMB score may be determined for each of (a), (b) and (d) or (a), (b) and (c).

[0067] In any embodiment, two TMB scores are determined from the sample. Preferably, a first TMB score is determined from (b) and (c) and the second TMB score is determined from (a), preferably wherein the coding mutations of (a) does not include a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation

[0068] In any embodiment of the above aspect, the method comprises:

- determining two tumour mutational burden scores (TMB) from a sample from the individual,

  wherein the first TMB score is the total number of coding mutations in *TP53* and *CDKN2A*; and copy number alterations in *CDKN2A,*

  wherein the second TMB is the total number of non-coding mutations in *pTERT, RPL13A* and *SMUG1.*

[0069] Preferably, the gene alterations for the first TMB further include: the number of coding mutations in *RAC1* and *PTEN;* and the number of copy number alterations in *CDK4.*

[0070] Optionally, the gene alterations for the first TMB further include: the number of coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; and the number of copy number alterations for *MDM2, MAP2K1 and RAC1;* and the second

TMB further includes: the number of non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8* and *ZNF778*.

[0071] In any embodiment, the method comprises:

- determining two tumour mutational burden scores (TMB) from a sample from the individual,

  wherein the first TMB score is the number of gene alterations within the tested genes, wherein the gene alterations are: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; and copy number alterations for *MDM2, MAP2K1 and RAC1,*

  wherein the second TMB is the number of gene alterations within the tested genes, wherein the gene alterations are: non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8* and *ZNF778*.

[0072] In any embodiment of the above aspect, the reference data set is constructed using a multivariate statistical model based on two or more TMB scores determined from a plurality of reference melanoma and melanocytic samples.

[0073] In any embodiment, the TMB scores for the sample and reference data set are determined from the same groups of gene alterations, ie the two or more TMB score determined for the sample is equivalent to the TMB determined for the reference data set.

[0074] In any embodiment, the reference multivariate score may be a pre-assigned score. In some embodiments, the reference multivariate score is between about 0.4 to 1, between about 0.5 to 1, between about 0.55 to 1, between about 0.6 to 1, between about 0.65 to 1, between about 0.7 to 1, between about 0.75 to 1, between about 0.8 to 1, between about 0.85 to 1, between about 0.9 to 1 or between about 0.95 to 1. In some embodiments, the reference multivariate score is between 0.4 to 1, between 0.5 to 1, between 0.55 to 1, between 0.6 to 1, between 0.65 to 1, between 0.7 to 1, between 0.75

to 1, between 0.8 to 1, between 0.85 to 1, between 0.9 to 1 or between 0.95 to 1. In some embodiments, the reference multivariate score is greater than or equal to about 0.4, greater than or equal to about 0.45, greater than or equal to about 0.5, greater than or equal to about 0.55, greater than or equal to about 0.6, greater than or equal to about 0.65, greater than or equal to about 0.7, greater than or equal to about 0.75, greater than or equal to about 0.8, greater than or equal to about 0.85, greater than or equal to about 0.9 or greater than or equal to about 0.95. In some embodiments, the reference multivariate score is greater than or equal to 0.4, greater than or equal to 0.45, greater than or equal to 0.5, greater than or equal to 0.55, greater than or equal to 0.6, greater than or equal to 0.65, greater than or equal to 0.7, greater than or equal to 0.75, greater than or equal to 0.8, greater than or equal to 0.85, greater than or equal to 0.9 or greater than or equal to 0.95. In some embodiments, the reference multivariate score is 0.5, 0.51, 0.52, 0.53, 0.54, 0.55, 0.56, 0.57, 0.58, 0.59, 0.60, 0.61, 0.62, 0.63, 0.64, 0.65, 0.66, 0.67, 0.68, 0.69, 0.70, 0.71, 0.72, 0.73, 0.74, 0.75, 0.76, 0.77, 0.78, 0.79 or 0.80.

[0075] In any embodiment, the multivariate statistical model comprises multivariate linear regression (MLR) or multivariate generalised linear regression (GLM), preferably multivariate GLM. In any aspect or embodiment of the present disclosure, the method may further comprise using one or more additional diagnostic methods.

[0076] In any embodiment, the GLM equation is:

$$P = \left(1 + e^{(2.6042 - 1.8416 * \text{Non-BRAF Non-NRAS coding mutational load} - 1.1655 * \text{Non-coding mutational load})}\right)^{-1}$$

with $P \in [0,1]$ the probability of melanoma.

Non-BRAF/Non-NRAS coding mutational load ≥ 0 the number of coding gene alterations (including copy number alterations) within the tested genes, excluding those in *BRAF* V600 and *NRAS* Q61.

Non-coding mutational load ≥ 0 the number of non-coding gene alterations within the tested genes.

[0077] In another aspect, the present disclosure provides a method of treating an individual that has, or is likely to have, melanoma, the method comprising:

- selecting an individual having a lesion that has been genetically tested in accordance with this disclosure and the gene alterations indicate melanoma,
- providing wide local excision or resection of the melanoma.

[0078] In another aspect, the present disclosure provides a method of treating an individual that has, or is likely to have, melanocytic naevus, the method comprising:

- selecting an individual having a lesion that has been genetically tested in accordance with this disclosure and the gene alterations indicate melanocytic naevus,
- providing intralesional or marginal excision of the naevus.

[0079] In another aspect, the present disclosure provides a method of treating melanocytic lesions, the method comprising:

- selecting at least a first melanocytic lesion that has been genetically tested in accordance with this disclosure and the gene alterations indicate melanoma, and
- selecting at least a second melanocytic lesion that has been genetically tested in accordance with this disclosure and the gene alterations indicate melanocytic naevus;
- treating the first lesion with wide local excision or resection of the melanoma, and
- either not treating the second lesion or treating the second lesion with intralesional or marginal excision of the naevus.

[0080] In another aspect, the present disclosure provides a method of treating an individual that has, or is likely to have, melanocytic naevus, the method comprising:

- selecting an individual having a lesion that has been genetically tested in accordance with this disclosure and the gene alterations indicate melanocytic naevus,
- administering to the lesion a treatment for melanocytic naevus, optionally the treatment is surgical resection of the melanocytic naevus. Optionally, the treatment is therapeutic, pre-emptive or cosmetic.

[0081] In another aspect, the present disclosure provides a method comprising detecting in a sample the presence of gene alterations in one or more genes selected from:

(a) coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1,* optionally wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation;

(b) non-coding hotspot mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778,* optionally wherein the non-coding mutation is not within the promoter region of *TERT*;

(c) copy number alterations in *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN RAC1, RAF1, RB1, TERT* and *TP53;* and

(d) promoter regions mutations in *TERT*,

or any combination thereof.

[0082] Optionally, the method detects at least 3 genes with coding mutations from list (a), optionally at least 5 genes with non-coding mutations from list (b) and at least 3 genes with copy alteration mutations from list (c).

[0083] In any aspect or embodiment, the melanocytic lesion or melanoma is a superficial spreading melanoma, a nodular melanoma, a lentigo maligna melanoma, or a desmoplastic melanoma.

[0084] In any aspect or embodiment, the melanocytic lesion or melanoma is a naevoid melanoma.

[0085] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is a superficial spreading melanoma, a nodular melanoma, a lentigo maligna melanoma, a desmoplastic melanoma, a mitotically active naevus in pregnancy (MANP), or a naevoid melanoma.

[0086] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is not a BAP1-deficient tumour, a uveal melanoma, a blue naevus, or a melanoma arising from blue naevus.

[0087] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is not a Spitz naevus, a Spitz melanoma, or a pigmented epithelioid melanocytoma.

[0088] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is not a BAP1-deficient tumour, a uveal melanoma, a blue naevus, a melanoma arising from blue naevus, a Spitz naevus, a Spitz melanoma or a pigmented epithelioid melanocytoma.

[0089] In any aspect or embodiment, the subject is pregnant. Alternatively, the subject is not pregnant. Optionally, the melanocytic lesion or melanocytic naevus is a mitotically active naevus in pregnancy (MANP). Optionally, the melanocytic lesion or melanoma is a naevoid melanoma in pregnancy. Optionally, the subject is pregnant and the method is used to distinguish mitotically active naevus from naevoid melanoma.

[0090] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is positive for PRAME.

[0091] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is positive for one or more of 6p25 (RREB1), 6q23 (MYB), Cep6 (centromere 6), 11q13 (CCND1), 9p21 (CDKN2A) and 8q24 (c-MYC).

[0092] In any aspect of embodiment, the melanocytic lesion, melanoma or melanocytic naevus is positive for p16 (meaning p16 is lost).

[0093] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is negative for PRAME.

[0094] In any aspect or embodiment, the melanocytic lesion, melanoma or melanocytic naevus is negative for one or more of 6p25, 6q23, Cep6, 11q13, 9p21.

[0095] In any aspect of embodiment, the melanocytic lesion, melanoma or melanocytic naevus is negative for p16 (meaning p16 is retained).

[0096] Optionally, PRAME and/or p16 status is determined by immunostaining.

[0097] Optionally, 6p25 (RREB1), 6q23 (MYB), Cep6 (centromere 6), 11q13 (CCND1) 9p21 (CDKN2A) and/or 8q24 (c-MYC) status is determined by fluorescence in situ hybridisation (FISH).

[0098] In any aspect or embodiment, the sample is a skin sample, preferably a skin lesion sample, more preferably a melanocytic lesion.

[0099] In any aspect or embodiment, the individual is human.

[0100] In any aspect or embodiment, the individual may have been previously diagnosed as having a melanoma or a melanocytic naevus, preferably wherein the diagnosis was performed on a sample from the same skin sample or location, or lesion, for which the determination or diagnosis of the methods as described herein is to be performed. Preferably, the previous diagnosis was performed by histopathology. The methods of the disclosure may be used as genetic testing following, concurrently with, or be followed by traditional diagnosis such as histopathology. Optionally, a final diagnosis follows both forms of testing. Alternatively, the methods of the disclosure may follow treatment of a melanoma or a melanocytic naevus to detect recurrence.

[0101] In any aspect or embodiment, the individual may been previously diagnosed as having a borderline or indeterminate melanocytic lesion, preferably wherein the diagnosis was performed on a sample from the same skin sample or location, or lesion, for which the determination or diagnosis of the methods as described herein is to be performed. Preferably, the previous diagnosis was performed by histopathology. The methods of the disclosure may be used as genetic testing following, concurrently with, or be followed by traditional diagnosis such as histopathology. Optionally, a final diagnosis follows both forms of testing. Alternatively, the methods of the disclosure may follow treatment of a borderline or indeterminate melanocytic lesion to detect recurrence (and ideally provide a specific rather than a borderline/indeterminate diagnosis).

[0102] In another aspect, the present disclosure provides a kit, panel or microarray comprising at least one diagnostic reagent for detecting a gene alterations as described herein. In one embodiment, the kit comprises diagnostic reagents that detect gene alterations according to any method described herein.

[0103] In another aspect, the present disclosure provides a kit, panel or microarray comprising at least one diagnostic reagent for detecting the presence of gene alterations in one or more genes as set out in any of the aspects or embodiments of the disclosure.

[0104] In any aspect or embodiment, the gene alterations are determined by detecting a protein, nucleic acid, for example DNA or RNA, or amplification product. Where the gene alteration is detected by a nucleic acid or amplification product, the method includes detecting mutations and/or gene alterations within one or more genes described herein.

[0105] In any aspect or embodiment described herein, where there is reference to a gene, the disclosure may include determining or measuring the presence of, level of or amount of, as the case may be, the corresponding protein (translated from the gene) as a surrogate for detecting the gene alteration.

[0106] In any aspect of the present disclosure, the presence of gene alterations may be determined by detecting the gene alterations in DNA (eg genomic DNA). The skilled person would be well aware of suitable methods for detecting gene alterations, including mutations and copy number amplification as described herein.

[0107] In any aspect or embodiment, the sample is a lesion sample. The sample includes genetic material. Optionally, the sample is *ex vivo*. In any aspect or embodiment, the sample is optionally an *in vitro* sample. Optionally, the method is conducted using *in vitro* testing. Optionally the sample is a sample of cells from the lesion. Optionally, the sample is collected by biopsy.

[0108] In any aspect or embodiment, the genetic test is optionally performed with histopathology before diagnosis and/or treatment (or the deferral of treatment in the case of a melanocytic lesion that has no treatment). In any embodiment, the genetic test is performed prior to, simultaneous with or subsequently to the histopathology test.

[0109] In any embodiment, both the genetic test results and histopathology results are used for considering a diagnosis.

[0110] As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps.

[0111] Further aspects of the present disclosure and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

Brief description of the drawings

[0112] Figure 1. Scatter plot of mean target coverage and mean number of unique start sites across targets for melanoma, naevus and normal skin samples. Samples with a coverage below 300X were deemed outliers and removed from further analysis.

[0113] Figure 2. Coding and non-coding mutational landscape of melanoma and naevus. Mutations in *BRAF* V600 and *NRAS* Q61 hotspots are indicated with a dot. CN: Copy Number.

[0114] Figure 3. Distribution of total, coding and non-coding mutational load against pathological and clinical factors. (A) Distribution in melanoma. (B) Distribution in naevus. LOESS regression is shown for thickness, TMR and age at biopsy. NS: Not significant. (*): p-value<0.05. (**): p-value<0.01. (***): p-value<0.001.

[0115] Figure 4. Performance of coding genes, promoter regions and mutational loads for discriminating melanoma and naevus. Area under the ROC curve (AUC) against Specificity (A) and AUC against Sensitivity (B) on the discovery cohort. Only features with an AUC greater 60% on the discovery set are included.

[0116] Figure 5. Performance of the genomic multivariate model on the classification of melanoma and naevus. ROC curves (A) and boxplot of scores in discovery and validation (B) are shown. The optimal cut-off that maximises the difference between true positive rate (TPR) and false positive rate (FPR) corresponding

to the Youden index is identified at 0.65. Horizontal line in panel (B) indicates the optimal cut-off.

[0117] Figure 6. Scatter plot of mean target coverage and mean number of unique start sites across targets for borderline melanocytic lesions. Samples with a coverage below 300X (+ shape) were deemed outliers and removed from further analysis.

[0118] Figure 7. Coding and non-coding mutational landscape of borderline melanocytic lesions. Mutations in *BRAF* V600 and *NRAS* Q61 hotspots are indicated with a dot. CN: Copy Number. T1: Tier 1. T2: Tier 2. T3: Tier 3. DP: Deep Penetrating. Conv: Conventional.

[0119] Figure 8. Distribution of total, coding and non-coding mutational load against age at time of biopsy and anatomical location in borderline lesions. LOESS regression is shown for age at biopsy. NS: Not significant. (*): p-value<0.05. (**): p-value<0.01. (***): p-value<0.001.

[0120] Figure 9. Performance of the genomic multivariate model on the classification of borderline melanocytic lesions. Boxplot of score values are shown. The optimal cut-off that maximises the difference between true positive rate (TPR) and false positive rate (FPR) corresponding to the Youden index identified at 0.65 in the discovery cohort is shown as a horizontal line.

[0121] Figure 10. Mutational load in melanoma and naevus. (A) Mutational load in melanoma and naevus by mutational load type. (B) Mutational load in melanoma stratified by driver mutation and mutational load type; BRAF-V600 left box, NRAS-Q61 middle box, non-BRAF-non-NRAS right box. NS: Not Significant. (*): p-value<0.05 (***): p-value<0.001.

[0122] Figure 11. Distribution of borderline melanocytic lesions across pathology-defined tiers and likely pathways of origin.

[0123] Figure 12. Mutational load distribution in melanoma and naevus.

[0124] Figure 13. Performance of the bivariate genomic model on the classification of melanoma and nevus. ROC curves indicating the performance of the

model in the discovery and validation cohort are shown for patients stratified by clinically relevant groups (A-G).

[0125] Figure 14. Coding and non-coding mutational landscape of borderline melanocytic tumors. (A) Distribution of mutations across tiers and likely pathways of malignancy. Mutations in BRAF V600 and NRAS Q61 hotspots are indicated with a dot. CN: Copy Number. T1: Tier 1. T2: Tier 2. T3: Tier 3. Conv: Conventional. PEM: Pigmented epithelioid melanocytoma. WNT: WNT-activated. (B) Total, coding and non-coding mutational load distribution for conventional and non-conventional tumors. (C) A Tier 2 borderline melanocytic tumor (BL103), with features of a WNT-activated tumor in a 71-year-old female. Low power view (C-1) shows the tumor with a dermal based nodular architecture, with a dominant clone centrally, and second clonal population superficially and on the left edge. PNL2 immunohistochemistry (C-2) highlights the clonal populations, with a gradient staining pattern on the left, and diffuse staining in the dominant clone. High power view (C-3) shows atypical cytomorphology in the dominant clone, with irregular nuclear membranes, conspicuous nucleoli, lacking intranuclear pseudoinclusions, along with scattered melanophages. Beta-catenin immunohistochemistry (C-4) shows aberrant nuclear and cytoplasmic staining in the dominant clonal population. PRAME immunohistochemistry (C-5) was negative in the entire tumor.

[0126] Figure 15. Bivariate genomic model on conventional borderline melanocytic tumors. (A) Boxplot of scores across pathology-based tiers of malignancy. (B) A borderline conventional melanocytic tumor (BL80) showing a predominantly junctional melanocytic tumor (B-1) in a 45-year-old male with a thin tumor (0.5mm from the granular layer) from the mid thoracic region and a high genomic score (0.96, with 5 non-coding mutations and no coding mutations. Atypical histomorphological features, including irregular large nests and epidermal consumption, together with dermal inflammation were focally present (B-2, B-3), in addition to less atypical regions (B-4). Immunostains PRAME, BRAF V600E, RAS Q61R, ALK, ROS1 and panTRK were all negative and p16 was retained. Diagnosis at sign-out favored at least melanoma in-situ arising from a dysplastic nevus.

Detailed description of the embodiments

[0127] It will be understood that the disclosure disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the disclosure.

[0128] Further aspects of the present disclosure and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

[0129] Reference will now be made in detail to certain embodiments of the disclosure. While the disclosure will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the disclosure to those embodiments. On the contrary, the disclosure is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present disclosure as defined by the claims.

[0130] Melanoma refers to a condition involving a malignant tumor of melanocytes. Melanocytes are cells that produce the dark pigment, melanin, which is responsible for the color of skin. They predominantly occur in skin, but are also found in other parts of the body, including the bowel and the eye. Thus primary melanomas can occur in areas of the body other than the skin (e.g., uveal melanoma). A primary melanoma is neoplasia at the site of origin; even if the primary tumor has metastasized the original site remains primary and the distant site is the metastasis.

[0131] Naevus (plural naevi, the terms which are herein used interchangeable) refer to a condition involving a benign tumour of melanocytes. Naevus typically present as a sharply circumscribed pigmented spot on the skin, or other part of the body, such as the bowel or eye. Naevus may be commonly referred to as birthmarks or moles. Naevus comprise melanocytes, which contribute to the nevi's pigmented appearance. Typically, naevus are considered benign. However, a dysplastic naevus (also sometimes referred to as an atypical mole) is a type of naevus with abnormal features. A dysplastic naevus may be bigger than and its color, surface, and border may be different from a non-dysplastic naevus. On the skin surface, a dysplastic naevus can appear as having a mixture of several colors (e.g., from pink to dark brown), a smooth or slightly scaly or

pebbly surface, and irregular edges that may fade into the surrounding skin. Dysplastic naevus are more likely than "ordinary" naevus to develop into melanoma, and about half of melanomas arise from dysplastic naevi. However, most dysplastic naevus never become malignant; thus, it is important to be able to determine which naevus (whether dysplastic or non-dysplastic) may, in fact, mistakenly be or be biologically transforming (e.g., at the molecular level) to primary melanoma.

[0132] Advantages of aspects or embodiments of the disclosure is that the present method focuses on detecting the presence of gene alterations in specific genes to provides improved sensitivity and specificity for determining or diagnosing melanocytic lesions. The disclosure therefore reduces the likelihood that patients receive unnecessary treatment, or receive delayed treatment, or no treatment at all due to a misdiagnosis using the existing less sensitive methods known in the art.

General

[0133] Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter. Thus, as used herein, the singular forms “a”, “an” and “the” include plural aspects, and vice versa, unless the context clearly dictates otherwise. For example, reference to “a” includes a single as well as two or more; reference to “an” includes a single as well as two or more; reference to “the” includes a single as well as two or more and so forth.

[0134] Those skilled in the art will appreciate that the present disclosure is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

[0135] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present

disclosure. The present disclosure is in no way limited to the methods and materials described.

[0136] All of the patents and publications referred to herein are incorporated by reference in their entirety.

[0137] The present disclosure is not to be limited in scope by the specific examples described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the present disclosure.

[0138] Any example or embodiment of the present disclosure herein shall be taken to apply *mutatis mutandis* to any other example or embodiment of the disclosure unless specifically stated otherwise.

[0139] Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (for example, in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

[0140] Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel *et al.* (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

[0141] The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

[0142] As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Selected definitions

[0143] As used herein, the terms "tumor mutational burden score" and "TMB score," which may be used interchangeably, refers to the number of genes with gene alterations (e.g., one or more gene alterations) in a pre-determined set of genes (e.g., in the coding regions, non-coding regions or promoter regions of the pre-determined set of genes) detected in a suspected tumor tissue sample (e.g., a formalin-fixed and paraffin-embedded (FFPE) tumor sample, an archival tumor sample, a fresh tumor sample, or a frozen tumor sample).

[0144] As used herein, the term "reference TMB score" refers to a TMB score against which another TMB score is compared, e.g., to make a determination or diagnosis using a method as described herein. For example, the reference TMB score may be a TMB score in a reference sample, a reference population, and/or a pre-determined value. In some instances, the reference TMB score is a cut-off value that significantly separates a first subset of individuals (e.g., patients) who have, or are likely to have, or have been diagnosed with a melanoma and a second subset of individuals who have, or are likely to have, or have been diagnosed with a melanocytic naevus.

[0145] The term "equivalent TMB" refers to a numerical value that corresponds to a TMB score that has been generated by scoring the same genes and gene alterations that were used to generate the TMB score.

[0146] A TMB score can be determined using any approach described in International Patent Application Publication No. WO 2017/151524 or in International Patent Application No. PCT/US2017/055669, both of which are incorporated herein by reference in their entirety.

[0147] As used herein, “gene alterations” include, but are not limited to, point mutations (e.g., the exchange of a single nucleotide for another (e.g., silent mutations, missense mutations, and nonsense mutations)), insertions and deletions (e.g., the addition and/or removal of one or more nucleotides (e.g., indels)), amplifications, gene duplications, copy number alterations (CNAs), rearrangements, and splice variants, or any combination thereof. In some embodiments, an indel may be a frameshift mutation or in-frame mutations of one or more nucleotides (e.g., about 1-40 nucleotides).

[0148] The term "diagnosis" is used herein to refer to the identification or classification of a molecular or pathological state, disease or condition (e.g., cancer). For example, "diagnosis" may refer to identification of a particular type of cancer. "Diagnosis" may also refer to the classification of a particular subtype of cancer, for instance, by histopathological criteria, or by molecular features (e.g., a subtype characterized by expression of one or a combination of biomarkers (e.g., particular genes or proteins encoded by said genes)).

[0149] The term "aiding diagnosis" is used herein to refer to methods that assist in making a clinical determination regarding the presence, or nature, of a particular type of symptom or condition of a disease or disorder (e.g., cancer). For example, a method of aiding diagnosis of a disease or condition (e.g., cancer) can comprise measuring certain somatic mutations in a biological sample from an individual.

[0150] In any aspect or embodiment, the present disclosure also provides a method of aiding diagnosis of a melanocytic lesion using a method described herein.

[0151] The term "detection" includes any means of detecting, including direct and indirect detection. The term "biomarker" as used herein refers to gene alterations in a sample that are a determinative or diagnostic indicator of melanoma or a melanocytic naevus. The term “biomarkers” and “gene alterations” are used interchangeable herein.

[0152] “Reference data set” as used herein refers to the source of the reference biomarker levels. The reference data set may be reference values, or reference ranges, or a reference standard. The “reference data set” is preferably provided by using the same assay technique and methods as is used for determining the test sample’s TMB in the reference samples or population, to avoid any error in standardization. The reference data set may be, alternatively, a numerical value, a predetermined cutpoint, a

mean, an average, a numerical mean or range of numerical means, a numerical pattern, a ratio, a graphical pattern derived from the same biomarker or biomarkers in a reference subject or reference population.

[0153] A "reference sample," as used herein, refers to a sample, cell, tissue, standard, or level that is used for comparison purposes.

[0154] The word "label" when used herein refers to a compound or composition that is conjugated or fused directly or indirectly to a reagent such as a polynucleotide probe and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. The term is intended to encompass direct labeling of a probe by coupling (i.e., physically linking) a detectable substance to the probe, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin.

Subject

[0155] As used herein, the terms "individual," "patient," or "subject" are used interchangeably and refer to any single animal, more preferably a mammal (including such non-human animals as, for example, dogs, cats, horses, rabbits, zoo animals, cows, pigs, sheep, and non-human primates). In particular embodiments, the individual herein is a human.

Sample

[0156] The term "sample," as used herein, refers to a composition that is obtained or derived from a subject and/or individual of interest that contains a cellular and/or other molecular entity that is to be characterized and/or identified, for example, based on physical, biochemical, chemical, and/or physiological characteristics.

[0157] Samples include, but are not limited to, tissue samples, primary or cultured cells or cell lines, cell supernatants, cell lysates, tumor lysates, and tissue culture medium, tissue extracts such as homogenized tissue, tumor tissue, cellular extracts, and combinations thereof. A "tumor cell" as used herein, refers to any tumor cell present in a

tumor or a sample thereof. Tumor cells may be distinguished from other cells that may be present in a tumor sample, for example, stromal cells and tumor-infiltrating immune cells, using methods known in the art and/or described herein.

[0158] In preferred embodiments the sample is obtained or derived from a skin sample suspected of being malignant. In other preferred embodiment is obtained or derived from a skin sample suspected that is borderline malignant.

Detection of gene alterations

[0159] Any aspect of the present disclosure involves detecting the presence of gene alterations as described herein.

[0160] In certain embodiments, any gene described herein may comprise a silent mutation (e.g., a synonymous alteration). In other embodiments, any gene described herein may comprise a non-synonymous single nucleotide variant (SNV). In other embodiments, any gene described herein may comprise a passenger mutation (e.g., an alteration that has no detectable effect on the fitness of a clone). In certain embodiments, any gene described herein may comprise a variant of unknown significance (VUS), for example, an alteration, the pathogenicity of which can neither be confirmed nor ruled out. In certain embodiments, any gene described herein may comprise a gene alteration has not been identified as being associated with a cancer phenotype.

[0161] In certain embodiments, any gene described herein may comprise a gene alteration that is not associated with, or is not known to be associated with, an effect on cell division, growth, or survival. In other embodiments, any gene described herein may comprise a gene alteration that is associated with an effect on cell division, growth, or survival.

[0162] The "copy number of a gene" refers to the number of DNA sequences in a cell encoding a particular gene product. Generally, for a given gene, a mammal has two copies of each gene. The copy number can be increased, e.g., by gene amplification or duplication, or reduced by deletion. Unless otherwise specified, the mutational load (or TMB score as described herein) for copy number alterations is calculated as the sum of copy number alterations across all tested genes. Preferably, copy number alterations in this disclosure are copy number amplifications.

[0163] In some embodiments, the listed genes have at least one extra copy number.

[0164] In some embodiments, any gene described herein may comprise a functional alteration that compared with a reference sequence (e.g., a wild-type or unmutated sequence) has an effect on cell division, growth, or survival (e.g., promotes cell division, growth, or survival). In certain embodiments, the functional alteration is identified as such by inclusion in a database of functional alterations, e.g., the COSMIC database (see Forbes et al. Nucl. Acids Res. 43 (D1): D805-D81 1 , 2015, which is herein incorporated by reference in its entirety). In other embodiments, the functional alteration is an alteration with known functional status (e.g., occurring as a known somatic alteration in the COSMIC database). In certain embodiments, the functional alteration is an alteration with a likely functional status (e.g., a truncation in a tumor suppressor gene). In certain embodiments, the functional alteration is a driver mutation (e.g., an alteration that gives a selective advantage to a clone in its microenvironment, e.g., by increasing cell survival or reproduction).

[0165] In certain embodiments, the functional alteration is not a passenger mutation (e.g., is not an alteration that has no detectable effect on the fitness of a clone of cells). In certain embodiments, the functional alteration is not a variant of unknown significance (VUS) (e.g., is not an alteration, the pathogenicity of which can neither be confirmed nor ruled out).

[0166] The presence of an increased number of pre-determined genes with gene alterations is indicative of a melanoma determination or diagnosis using the methods described herein or can be used in further methods described herein to determine or diagnose melanoma. For example, the biomarkers may be used to generate a TMB score or a multivariate score, wherein a relatively high TMB score or a relatively high multivariate score is indicative of a melanoma determination or diagnosis. Accordingly, a decreased number of pre-determined genes with gene alterations is indicative of a melanocytic naevus determination or diagnosis using the methods described herein or can be used in further methods described herein to determine or diagnose melanoma. For example, the biomarkers may be used to generate a TMB score or a multivariate score, wherein a relatively low TMB score or a relatively low multivariate score is indicative of a melanocytic naevus determination or diagnosis.

[0167] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single- stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs.

[0168] A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps," substitution of one or more of the naturally-occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, and the like) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, and the like), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, and the like), those with intercalators (e.g., acridine, psoralen, and the like), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, and the like), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal

OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-0-methyl-, 2'-0-allyl-, 2'-fluoro-, or 2'-azido-ribose, carbocyclic sugar analogs, a-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs, and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(0)S ("thioate"), P(S)S ("dithioate"), "(0)NR_2 ("amidate"), P(0)R, P(0)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1 -20 C) optionally containing an ether (-0-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. A polynucleotide can contain one or more different types of modifications as described herein and/or multiple modifications of the same type. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0169] Oligonucleotide," as used herein, generally refers to short, single stranded, polynucleotides that are, but not necessarily, less than about 250 nucleotides in length. Oligonucleotides may be synthetic. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

[0170] The term "primer" refers to a single-stranded polynucleotide that is capable of hybridizing to a nucleic acid and allowing polymerization of a complementary nucleic acid, generally by providing a free 3'-OH group.

[0171] "Amplification," as used herein generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies" mean at least two copies. A "copy" does not necessarily mean perfect sequence complementarity or identity to the template sequence. For example, copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template), and/or sequence errors that occur during amplification.

[0172] The technique of "polymerase chain reaction" or "PCR" as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described, for example, in U.S. Pat. No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage, or plasmid sequences, etc. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51 :263 (1987) and Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

[0173] Gene alterations data may be analyzed by a variety of methods to identify the genes with gene alterations and determine the statistical significance of differences in genes with gene alterations between test and reference expression profiles. In certain embodiments, patient data is analyzed by one or more methods including, but not limited to, multivariate simple linear regression model (SLM), multivariate generalised linear regression model (GLM), multivariate linear discriminant analysis (LDA), receiver operating characteristic (ROC) analysis, principal component analysis (PCA), ensemble data mining methods, bayesian generalized linear model, gaussian process, naive bayes, elastic net, *k*-nearest neighbors, lasso, penalized logistic regression, partial least squares, prediction analysis for microarrays (PAM), poisson linear discriminant analysis, negative-Binomial linear discriminant analysis, neural networks, support vector machines, significance analysis of microarrays (SAM), cell specific significance analysis of microarrays (csSAM), spanning-tree progression analysis of density-normalized events (SPADE), Uniform Manifold Approximation and Projection (UMAP) and multi-dimensional protein identification technology (MUDPIT) analysis. (See, e.g., Hilbe (2009) Logistic Regression Models, Chapman & Hall/CRC Press; McLachlan (2004) Discriminant Analysis and Statistical Pattern Recognition. Wiley Interscience; Zweig et

al. (1993) Clin. Chem. 39:561-577; Pepe (2003) The statistical evaluation of medical tests for classification and prediction, New York, NY: Oxford; Sing et al. (2005) Bioinformatics 21:3940-3941; Tusher et al. (2001) Proc. Natl. Acad. Sci. U.S.A. 98:5116-5121; Oza (2006) Ensemble data mining, NASA Ames Research Center, Moffett Field, CA, USA; English et al. (2009) J. Biomed. Inform. 42(2):287-295; Zhang (2007) Bioinformatics 8: 230; Shen-Orr et al. (2010) Journal of Immunology 184: 144-130; Qiu et al. (2011) Nat. Biotechnol. 29(10):886-891; Ru et al. (2006) J. Chromatogr. A. 1111(2): 166- 174, Jolliffe Principal Component Analysis (Springer Series in Statistics, 2nd edition, Springer, NY, 2002); McCarthy et al., (2017) R Bioinformatics, 33: 1179-1186; Koren et al. (2004) IEEE Trans Vis Comput Graph 10:459-470; herein incorporated by reference in their entireties).

Methods of treatment

[0174] In any method described herein, the determination may that an individual has, or likely has, a melanoma. As used herein, reference that an individual has, or likely has, a melanoma may be taken as a reference to determination that an individual requires an intervention in the form of pre-emptive therapy. Therefore, in any method or use of the disclosure, where a determination is made that an individual requires an intervention in the form of pre-emptive therapy, the method or use may further comprise the step of administering an intervention in the form of pre-emptive therapy (for example, any pre-emptive therapy described herein).

[0175] In any method as described herein, melanocytic lesion may be diagnosed as melanoma. As used herein, reference to a melanocytic lesion being diagnosed as melanoma may be taken as a reference to determination that an individual requires an intervention in the form of a therapy. Therefore, in any method or use of the disclosure, where a determination is made that an individual requires an intervention in the form of a therapy, the method or use may further comprise the step of administering an intervention in the form of a therapy (for example, any therapy described herein).

[0176] In any method described herein, the determination that an individual has, or likely has, a melanocytic naevus. As used herein, reference that an individual has, or likely has, a melanocytic naevus may be taken as a reference to determination that an individual requires an intervention in the form of pre-emptive therapy. Therefore, in any method or use of the disclosure, where a determination is made that an individual

requires an intervention in the form of pre-emptive therapy, the method or use may further comprise the step of administering an intervention in the form of pre-emptive therapy (for example, any pre-emptive therapy described herein).

[0177] As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

[0178] As used herein, "administering" is meant a method of giving a dosage of a compound (e.g., an antagonist) or a pharmaceutical composition (e.g., a pharmaceutical composition including an antagonist) to a subject (e.g., a patient). Administering can be by any suitable means, including parenteral, intrapulmonary, topical and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include, for example, intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g., by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic or by topical administration with a dose per volume of topical for administration to a specific area. Various dosing schedules including but not limited to single or multiple administrations over various time-points.

[0179] Treatment regimens for melanoma include wide local excision, systemic drug therapies(such as chemotherapies) and radiation therapy.

[0180] Typically, wide local excision is performed with the aim of achieving complete histological clearance. In any embodiment, the wide local excision margin may be 2mm to 20mm. The skilled person will appreciate that the excision margin may depend on the thickness of primary melanoma and clinical practice guidelines. Preferably the wide local excision margins are from 5mm to 10mm, more preferably 5mm or 10mm.

[0181] In any method as described herein, melanocytic lesion may be diagnosed as melanocytic naevus. As used herein, reference to a melanocytic lesion being diagnosed

as melanocytic naevus may be taken as a reference to determination that an individual requires an intervention in the form of a therapy. This can be pre-emptive therapy such as to prevent problems associated with growth of the lesion or standard therapy to achieve resolution of problems associated with the current lesion, eg to allow restoration of a typical skin barrier. Therefore, in any method or use of the disclosure, where a determination is made that an individual requires an intervention in the form of a therapy, the method or use may further comprise the step of administering an intervention in the form of a therapy (for example, any therapy described herein).

[0182] Preferably, the therapy is intralesional (excision not extending beyond the visible boundaries of the lesion) or marginal excision of the melanocytic naevus. Intralesional or marginal excision are typically performed to reduce the visible bulk of the naevus. Marginal excision generally does not extend significantly beyond the visible boundaries of the naevus and/or results in residual tumour margins that are visible by sight or by histological analysis.

[0183] Some melanocytic naevus do not require treatment. Optionally, the lesion can remain untreated. Alternatively, the lesion may be treated for cosmetic reasons. The lesion may also be treated to restore consistent skin barrier (eg a normal dermis/skin). Presently treatment also occurs when there is diagnostic ambiguity. Treatment can be excision or topical therapy.

Examples

Example 1 – Material and Methods

Sample collection

[0184] This study was conducted with Human Research Ethics Committee approval (The Sydney Local Health District Human Research Ethics Committee, Protocol No. X15-0454 and HREC/11/RPAH/444). The archives of the Department of Tissue Pathology and Diagnostic Oncology at Royal Prince Alfred Hospital were searched for cases of primary melanoma, naevus and borderline melanocytic lesions. A total of 371 formalin-fixed, paraffin-embedded (FFPE) melanocytic neoplasms - 118 melanomas (from 117 patients), 132 naevi (from 123 patients) and 121 borderline melanocytic lesions – with sufficient amounts of melanocytic cells for macrodissection were retrieved. Nevi and melanoma were reviewed by a second pathologist with melanoma

pathology expertise (NM/AP/EP). Cases were included when a concordant diagnosis (either benign or malignant) was reached. Spitz tumors, known BAP1-inactivated tumors and pigmented epithelioid melanocytomas (PEM) were excluded. Additionally, 15 FFPE normal skin samples were retrieved. All cases were reviewed by an expert pathologist to confirm the diagnosis and identify the areas of interest for molecular analysis.

[0185] For further evaluation of the bivariate genomic model on borderline melanocytic tumors, the inventors selected 110 cases (from 110 patients) with pathological uncertainty over diagnosis and/or behavior, without discrimination by subtype or age. PRAME, p16 and fluorescent in-situ hybridization (FISH) ancillary investigation results for assessing malignant risk were recorded for the borderline tumors.

[0186] For all cases, a representative tissue block was selected, and slides were marked for macrodissection when required.

DNA extraction

[0187] Tumour enriched DNA was extracted from archival formalin-fixed biopsies using the High Pure FFPET DNA Isolation Kit following the manufacturer's protocols (Roche, Australia). DNA quality was assessed using a quantitative PCR, pre-sequencing quality control assays were performed to generate a PreSeq DNA QC score (PreSeq DNA QC assay; ArcherDX, USA).

Next generation custom amplicon panel

[0188] Recurrently mutated coding genes and hotspot non-coding genes (promoter regions) were designed into a custom next generation sequencing panel in the Archer Assay Designer (ArcherDX). The panel covered coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53 and TYRP1*, non-coding hotspot mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1 and ZNF778*, as well as copy number alterations in *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ,*

HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN RAC1, RAF1, RB1, TERT and TP53. Primers designed to target genomic regions of these genes are known and can be identified by the skilled person.

Library preparation and sequencing

[0189] Next generation sequencing (NGS) libraries were generated for this custom amplicon DNAseq panel (Archer® VariantPlex® Melanoma panel, dSA15666081/dSA15666082) using the ArcherDX chemistry. Libraries were prepared following the FFPE DNA Archer® VariantPlex® Somatic Protocol for Illumina. Input DNA was determined by the PreSeq DNA QC assay, with 100 ng of DNA used for samples with high quality scores, 200 ng for low quality scores and 300 ng for samples with bad quality scores as defined by the PreSeq DNA QC assay. NGS libraries were quantified using the KAPA Library Quantification Kit for Illumina (KK4824), and pooled to a concentration of 1.2pM for the NextSeq500 and 250pM for NextSeq2000, both with 20% PhiX. Sequencing was performed on the Nextseq 500/ 2000 without custom primers using paired-end sequencing (151bp for Read 1 and Read 2) with index reads (8bp for Index Read 1 and Index Read 2). FASTQ were generated onboard the instrument and export for secondary analysis within the ArcherDx Analysis Software.

Alignment, sequencing quality control and initial variant calling

[0190] FASTQ files were processed using the Archer Analysis Pipeline v6.2.7 with default settings, except for down-sampling default option which was disabled (changed to 0) to ensure use of all read pairs. The pipeline aligns the reads against the human reference genome version hg19. The quality of the sequencing data for each sample is then assessed based on the average number of unique starts sites found across all target regions (GSP2s) in the panel. While all samples passed QC based on this number (a value of 50 or greater is the recommended threshold by the manufacturer) inspection of coverage distribution suggested 8 melanomas, 3 naevi and 5 skins with coverage lower than 300X to be outliers and were excluded from further analysis. Somatic variants are called using Freebayes (Garrison & Marth, *arXiv preprint*, 2012; arXiv: 1207.3907) and LoFreq (Wilm et al., *Nucleic Acids Res.* 2012; 40(22):11189-201). Copy number alterations are detected using an *ad-hoc* implementation provided within the pipeline. The set of skin samples (n=10) were used as reference for variant calling.

Variant calling optimisation

[0191] To assess the accuracy of the variant calling generated by the ArcherDx Analysis Pipeline, the inventors compared variant calls on matched samples derived from the ArcherDx panel as well as from whole genome sequencing of snap frozen blood and tumour tissue that was taken adjacent to the FFPE tissue. In total, 22 melanomas and 12 naevi from the Australian Melanoma Genome Project were analysed (Colebatch et al., *J Invest Dermatol,* 2019; 139: 1762-8; Hayward et al., *Nature,* 2017; 545: 175-80; Newell et al., *Cancer Discov,* 2022; 12: 2856-79; Newell et al., *Nat Commun,* 2022; 11: 5259; Newell et al., *Nat Commun,* 2019; 10: 3163). These tumours have available point mutation and copy-number alteration calls from whole-genome sequencing (WGS). Library preparation, sequencing and variant calling for these samples were processed as described above. The set of patient skin samples (n=10) were used as reference for copy number calling. Based on this assessment, the following filters were identified to optimize the point-mutation calling: (1) Exclusion of calls on promoter regions of genes *CHCHD2, BLCAP, RPS27* (prone to false positives) as well as *ERGIC3, RALY, YAE1D1, SYF2* (prone to false negatives), (2) UDP >10, UAO >6 (except *TERT* promoter), DAO >1 (except *TERT* promoter), (3) HomopolymerCount<6, (4) gnomAD AD<0.01 and Global AF<0.01, (5) No positive call for sequence direction bias or Sample strand bias, (6) Sequence Direction Strand Bias Probability >0.1 (except *TERT* promoter), (7) If the call was made with FreeBayes, then Sample Strand Bias Probability>0.05 or Sample Strand Bias Ratio >0.4, as well as 95MDAF<0.2, (8) If the call was made with LoFreq, AF>0.1 and AF<=0.2 as well as 95MDAF<0.1, and (9) For protein-coding mutations, only those with a High or Moderate impact based on Ensembl predicted consequences were kept. For non-coding mutations, only those with a sequence ontology term of 5_prime_UTR_variant or upstream_gene_variant consequence were kept.

[0192] A total of 20 mutations were called across naevi, 14 of which were deemed true positives, 6 false positives and 0 false negatives. One hundred and forty four mutations were called in melanomas, 96 of which were deemed true positives, 8 false positives and 40 false negatives. Inspection of the false negatives revealed that the majority were called by LoFreq – a variant caller designed to be highly sensitive in samples of low purity – but eventually filtered out, or not detected at all in the raw calls. This suggests that most of these false negatives are due to low purity. Additionally, some of these calls

may represent false positives in the AMGP WGS-based calls. For *BRAF* and *NRAS* hotspots a sensitivity and specificity of 100% was achieved across naevi and melanomas. For p*TERT* promoter mutations, a sensitivity of 91% and a specificity of 82% was achieved in melanomas. In naevi, only one sample generated a call for *TERT* promoter, in agreement with the absence of p*TERT* hotspot mutations in naevi.

[0193] For copy number alteration calling, correlation assessment of the copy number calls against those on the matched AMGP samples revealed best performance for *BRAF, CDK4, KIT, MAP2K1, MDM2 and RAC1* (Oncogenes) and *CDKN2A* (Tumour Suppressor). For oncogenes, a CN Ratio >=1.5 and a p-value <=0.01 maximised the product of sensitivity and specificity, except for *CDK4* were CN Ratio was set to >= 2. For CDKN2A, a CN Ratio <=0.7 and a p-value <=0.05 maximised the product of sensitivity and specificity. Based on a definition of gains (defined as a log2 ratio of gene copy number to genome-level ploidy >= log2(3/2) in the WGS calls) and high-level amplifications (defined as a log2 ratio >=log2(4/2) in the WGS calls), a sensitivity and specificity of 59% and 96.7% was achieved for oncogenes respectively. Within high-level amplification calls, sensitivity increased to 85.7%, with a specificity of 96.2%. For *CDKN2A,* a sensitivity and specificity of 80% and 79% was achieved, respectively.

[0194] These optimal settings identified for variant calling were applied to the unfiltered set of mutations and copy number alterations generated by the ArcherDx Pipeline on each melanoma, naevi and borderline melanocytic lesion.

Definition of summary statistics

Based on the filtered somatic point mutation and copy number calling, the following summary statistics were defined and calculated for each sample:

(i) ***total mutational load***: the sum of coding and non-coding point mutations and copy number alterations,

(ii) ***coding mutational load***: the sum of coding point mutations and copy number alterations,

(iii) ***non-coding mutational load***: the sum of non-coding point mutations,

(iv) ***non-BRAF/non-NRAS coding mutational load***: the sum of coding point mutations and copy number alterations, excluding those point mutations present in codon V600 of *BRAF* and Q61 of *NRAS*, and,

(v) ***non-pTERT non-coding mutational load***: the sum of non-coding point mutations, excluding those in the promoter region of the *TERT* gene.

Discovery and validation of multivariate classifier

[0195] The 110 melanomas and 129 naevi after QC were randomly split into two cohorts: a 75% discovery cohort (179 lesions – 82 melanomas and 97 naevi) and a 25% validation cohort (60 lesions – 28 melanomas and 32 naevi). A generalized linear regression multivariate model (logistic regression bivariate model) combining *non-BRAF/non-NRAS coding mutational load*, *non-pTERT, non-coding mutational load and pTERT promoter mutation status (present/absent)* against the sample label (melanoma or naevi) as response variable was trained on the discovery cohort using the *glm* function of the *stats* package in R v3.6.3 with the *family* parameter set to binomial. This multivariate model generated a score from 0 to 1 – with increasing values indicative of increased likelihood of melanoma. The performance of this model was measured on the discovery and the independent validation cohort using area under the ROC curve (AUC), sensitivity and specificity. Based on the identification of a Youden index in the discovery set – the score that maximizes the difference between true positive rate and false positive rate - sensitivity and specificity were calculated on the discovery and validation cohorts. These and other statistics including the Youden index were obtained using the *pROC* v1.18.0 and *ROCit* v2.1.1 packages in R v3.6.3.

Example 2 - Clinical features of melanomas and naevi

[0196] The cohort of 118 invasive melanomas included in this study had a median thickness of 2.8 mm. (range 0.2 mm to 28 mm), with 31% presenting ulceration and a median tumor mitotic rate (TMR) of 4.5 mitoses/mm^2 (range 0-45 mitoses/mm^2) (Table 1). In agreement with the higher prevalence of melanomas in older male patients, significant differences were observed between melanomas and the 132 naevi included in this study for sex (p-value= 0.002), age at time of biopsy (p-value<0.001) as well as anatomical location (p-value = 0.02) (Table 1). Naevi were thinner compared to melanomas (median 2.8 mm in melanomas versus 0.8 mm in nevi, p-value<0.001) from

younger age patients (median 53 years versus 75 years), represented a higher proportion of females (52% of naevi (69/132) versus 32% of melanomas (38/118 were from females), showed a higher prevalence of a trunk location (41% in naevi versus 22% in melanomas) and a lower prevalence of a head and neck location (28% of naevi versus 41% of melanomas).

[0197] A wide range of melanomas and nevi histological subtypes composed this cohort. Melanomas included superficial spreading melanoma (n=47, 40%), nodular melanoma (n=35, 30%), lentigo maligna melanoma (n=13, 11%), desmoplastic melanoma (n=12, 10%) and acral melanoma (n=4, 3%). Nevi included a variety of subtypes, including dysplastic (n=48, 37%), blue (n=8, 6%) and WNT-activated nevi (n=3, 2%).

Table 1. Clinical and Pathological Characteristics of Melanomas and Naevi

Variable	Category/Statistic	Melanomas (n=118)	Naevi (n=132)	P-value
Age at Biopsy (years)	median [min – max]	74.73 [18.29 – 95.05]	52.96 [11.35 – 88.17]	1.18707E-18
Thickness (mm.)	median [min – max]	2.8 [0.2 – 28]	0.8 [0.2 - 4]	7.31956E-21
TMR (mitoses/mm2)	median [min – max]	4.5 [0 – 45]	N/A	N/A
Clark Level	median [min – max]	4 [2 – 5]	N/A	N/A
Ulceration Status	Absent - count (%)	79 (67%)	N/A	N/A
	Present - count (%)	36 (31%)	N/A	
	Unknown - count (%)	3 (2%)	N/A	
Sex	Female - count (%)	38 (32%)	69 (52%)	0.002
	Male - count (%)	80 (68%)	63 (48%)	
Anatomical Location	Head & Neck - count (%)	48 (41%)	37 (28%)	0.003
	Trunk - count (%)	26 (22%)	54 (41%)	
	Upper Limb - count (%)	26 (22%)	18 (14%)	
	Lower Limb - count (%)	18 (15%)	22 (17%)	
	Unknown - count (%)	0 (0%)	1 (1%)	

Example 3 - The coding and non-coding mutational profiles of melanomas and naevi carry large differences in distinct genes and overall mutational load

[0198] The 118 melanomas and 132 naevi were collected and processed with the ArcherDx library preparation kit and sequenced. Additionally, 15 skin samples were sequenced as a pooled reference for variant calling. The genes in the genomic panel include protein-coding genes known to drive melanoma across different subtypes as well as non-coding hotspot promoter regions known to be mutated throughout the progression from naevi to melanoma. While all samples passed QC based on the mean number of unique start sites across targets (a value of 50 or greater is the recommended threshold by the manufacturer) inspection of coverage distribution (Figure 1) suggested 8 melanomas, 3 naevi and 5 skins with coverage lower than 300X

to be outliers and were excluded from further analysis. No significant differences in coverage were observed across melanomas, naevi and skin samples (median 842X, 775X and 688X, respectively, Kruskal-Wallis p-value=0.1). Using an optimized set of filters (see *Example 1 - Materials and Methods*), somatic point mutations and copy number alterations were called on the remaining 110 melanomas (from 109 patients) and 129 naevi (from 120 patients), using the remaining 10 skin samples that passed QC as reference.

[0199] The landscape of somatic point mutations and copy number alterations across naevi and melanomas revealed distinct mutational events as well as differences in the overall mutational load (Figure 2). In naevi, *BRAF* and *NRAS* were the most frequently mutated genes. Across naevi, *BRAF* and *NRAS* mutations were mutually exclusive, with 56.5% (73/129) of naevi being *BRAF* mutants and 10% (13/129) of naevi being *NRAS* mutants, located exclusively in hotspots V600 and Q61, respectively. In melanomas, 31.8% (35/110) were *BRAF* mutants (20/35 V600 hotspot) and 25.4% (28/110) were *NRAS* mutants (18/28 Q61 hotspot). Four melanomas had both *BRAF* and *NRAS* mutations involving codons other than *BRAF* V600 and *NRAS* Q61 (MEL18 *BRAF*:Lys483Glu and *NRAS*:Gly12Ser; MEL78 *BRAF*:Gly466Glu and *NRAS*:Gly12Ser; MEL87 *BRAF*:Asp594Asn and *NRAS*:Gly60Glu; MEL94 *BRAF*:Gly469Arg and *NRAS*:Gly13Val).

[0200] In contrast, the most frequent event in melanomas was the mutation of *TERT* promoter (*pTERT)* in 72.7% of samples (80/110), compared to only 0.8% of naevi (1/129). Importantly, several protein-coding genes and non-coding promoter regions were found mutated exclusively in melanomas, including protein-coding genes *RAC1* (n=9), *BAP1* (n=4), *RAF1* (n=4), *KRAS* (n=3) and *SF3B1* (n=3) and promoter regions of genes *RPL34* (n=19), *RPS14* (n=15), *NSUN6* (n=12), *KBTBD8* (n=6), and *ZNF778* (n=6). Deletion of *CDKN2A* tumor suppressor (n=10) as well as amplification of oncogenes *CDK4* (n=7), *MDM2* (n=3), *MAP2K1* (n=3), *BRAF* (n=1) and *RAC1* (n=1) were exclusively identified in melanomas. No genes were found to be mutated exclusively in naevi.

[0201] The median total mutational load (Figure 10A) in melanomas - including coding and non-coding mutations as well as copy number alterations - was 7 mutations (range 0-22 mutations), significantly higher than the median load of 1 mutation in naevi (range 0-6 mutations, Wilcoxon test p-value < 0.0001). This difference remained significant in

the subsets of protein-coding gene mutations/alterations (median 2 mutations for melanomas (range 0-6) and 1 mutation for naevi (range 0-2), Wilcoxon test p-value < 0.0001) and non-coding promoter region mutations (median 5 mutations for melanomas (range 0-18) and 0 mutations for naevi (range 0-5), Wilcoxon test p-value < 0.0001). For 0.9% (4/110) of melanomas and 28% (36/129) of naevi, no mutations or copy number alterations were detected. Non-coding mutational load was higher in *NRAS* Q61* melanomas (median 4.5 mutations, range 2-17) compared to *BRAF* V600* melanomas (median 3 mutations, range 0-11, Wilcoxon test p-value=0.03, Figure 10B). Melanomas wild type for both *BRAF* and *NRAS* (n=51) had a median non-coding mutational load of 7 mutations (range 0-18), borderline significantly higher than *BRAF* V600* melanomas (Wilcoxon p-value=0.05) but not than *NRAS* Q61* melanomas (Wilcoxon p-value=0.92). Coding and non-coding mutational loads showed distinct associations with clinicopathological features, indicative of these mutation types being driven by different mutational processes.

[0202] Assessment of total mutational load against clinical-pathological factors in melanomas (Figure 3A) showed association with age at time of biopsy (spearman coefficient=0.33, p-value=0.0005), anatomical location (median 11.5, 6.5, 5.5 and 4 mutations in head & neck, upper limb, trunk and lower limb, respectively, Kruskal Wallis p-value=0.001) and TMR (Spearman coefficient=0.28, p-value=0.003). No association of total mutational load with Breslow thickness or ulceration were observed. Still, coding and non-coding mutational loads contributed differently to the significance of these associations. Age at time of biopsy was strongly associated with non-coding mutational load (Spearman p-value=0.0003), but not with coding mutational load (Spearman p-value=0.12). In contrast, Breslow thickness and ulceration were strongly associated with coding mutational load (Spearman p-value=0.007 and Wilcoxon p-value=0.006, respectively), but not with non-coding mutational load (Spearman p-value=0.86 and Wilcoxon p-value=0.11, respectively). In the case of TMR, while both coding and non-coding mutational load were significant, the association was much stronger for coding load (Spearman p-value=0.000002) than non-coding load (Spearman p-value=0.04).

[0203] In naevi (Figure 3B), total mutational load was associated with thickness (Spearman p-value=0.004). This association was highly significant with coding mutational load (Spearman p-value=0.00001) with non-coding mutational load (Spearman p-value=0.94).

[0204] No associations between mutational loads and age or anatomical location were identified within naevi (data not shown). No significant associations between mutational loads and sex were identified in melanomas or naevi (data not shown). Overall, these results suggest that coding and non-coding mutational loads are driven by different mutational processes.

Example 4 - Mutations in non-coding promoter regions and non-coding mutational load have best performance for classification of melanomas and naevi

[0205] To understand the ability of mutated genes as well as total mutational load to discriminate between melanomas and naevi, the 110 melanomas and 129 naevi were randomly split into two cohorts: a 75% discovery cohort (179 lesions – 82 melanomas and 97 naevi) and a 25% validation cohort (60 lesions – 28 melanomas and 32 naevi). No significant differences between discovery and validation cohorts across clinical and pathological features were observed within melanomas and naevi (Table 2).

Table 2. Clinical and pathological characteristics of melanomas and naevi in discovery and validation cohorts.

subtype	variable	group	min	max	median	pvalue
Melanoma	Age at Biopsy	Discovery	32.39	95.05	76.11	0.07
		Validation	18.29	90.11	71.745	
Naevus		Discovery	11.35	83.08	56.05	0.58
		Validation	21.24	79.39	49.485	
Melanoma	Clark	Discovery	2	5	4	0.18
		Validation	2	5	4	
Melanoma	Thickness	Discovery	0.2	21	2.75	0.78
		Validation	0.6	28	2.85	
Naevus		Discovery	0.3	4	0.7	0.88
		Validation	0.2	2.2	0.8	
Melanoma	TMR	Discovery	0	45	4	0.78
		Validation	1	32	4.5	

subtype	variable	group	category	n	percentage	pvalue
Melanoma	Anatomical Location	Discovery	HN	32	39.02	0.93
			LL	14	17.07	
			TRUNK	19	23.17	
			UL	17	20.73	
		Validation	HN	10	35.71	
			LL	4	14.29	
			TRUNK	7	25	
			UL	7	25	
Naevus		Discovery	HN	25	25.77	0.59
			LL	15	15.46	
			TRUNK	44	45.36	
			UL	13	13.4	
		Validation	HN	9	28.12	
			LL	7	21.88	
			TRUNK	10	31.25	
			UL	5	15.62	
			NA	1	3.12	
Melanoma	Sex	Discovery	Female	24	29.27	0.28
			Male	58	70.73	
		Validation	Female	12	42.86	
			Male	16	57.14	
Naevus		Discovery	Female	46	47.42	0.20
			Male	51	52.58	
		Validation	Female	20	62.5	
			Male	12	37.5	
Melanoma	Ulceration Status	Discovery	No	56	68.29	1.00
			Yes	25	30.49	
			NA	1	1.22	
		Validation	No	19	67.86	
			Yes	8	28.57	
			NA	1	3.57	

[0206] For each protein-coding gene and non-coding promoter region, the presence of a mutation was evaluated as a criterion to discriminate melanomas from naevi in the discovery cohort (Figure 4). Among coding genes, *TP53* mutations had the best performance with an AUC of 62% , followed by *CDKN2A* mutations (AUC = 58%). When either a mutation or copy number loss of *CDKN2A* was considered as an event, the performance of *CDKN2A* increased to an AUC of 63%. In contrast, the top performing promoter region was p*TERT*, with an AUC of 87%. Several other promoter regions, including *RPL13A* (AUC = 70%), *SMUG1* (AUC = 69%), *C16orf59* (AUC =

68%) and *AP3D1* (AUC = 67%) significantly outperformed *TP53* and *CDKN2A,* highlighting the diagnostic potential of non-coding promoter regions.

[0207] The inventors next sought out to evaluate total, coding and non-coding mutational load in their ability to classify tumors in the discovery cohort (Figure 4). The total mutational load had the best performance (AUC = 92%), followed closely by non-coding mutational load (AUC = 90%) and coding mutational load (AUC = 74%, Figure 4). This suggests that the discriminatory ability of total mutational load is contributed mainly by the non-coding mutational burden.

[0208] When an optimal threshold (Youden index), defined as the number of mutations that maximizes the difference between true positive rate and false positive rate in the discovery cohort) threshold of 3.5 mutations was used to discriminate melanoma from naevi with total mutational load, this variable had a sensitivity and specificity of 82% and 94%, respectively (Figure 4). Similarly, for non-coding mutational load, an optimal threshold of 1.5 mutations resulted in a sensitivity and specificity of 82% and 94%, respectively. To assess the impact of p*TERT* on the performance of non-coding mutational load, a measure of non-coding mutational load that excludes *pTERT* – named non-p*TERT* non-coding mutational load – see Methods of Example 1 – was evaluated. This resulted in a slight decrease in AUC from 90% to 89%, with same specificity (94%) and decreased sensitivity (77% when pTERT was excluded versus 82% when pTERT was included). These results further highlight the diagnostic potential of non-coding promoter regions other than *pTERT* in their ability to achieve sensitive and specific discrimination between melanomas and naevi.

[0209] In contrast, coding mutational load presented a much lower sensitivity of 55% at the optimal threshold (1.5 mutations) compared to non-coding mutational load, with a high specificity of 96%. A measure of coding mutational load that excludes counts of *BRAF* V600* and *NRAS* Q61* - named *non-BRAF/non-NRAS coding mutational load* – see Methods of Example 1 – achieves an AUC of 81%, outperforming coding mutational load that includes those driver mutations (AUC = 74%). At an optimal threshold of 0.5 mutations, this increase in AUC comes with an increase in sensitivity (66% versus 55%) and slight decrease in specificity (93% versus 96%).

Example 5 - A bivariate logistic regression model that combines non-BRAF/non-NRAS coding mutational load and non-coding mutational load outperforms single genes and individual measures of mutational load

[0210] The distinct biology and association with clinical-pathological features captured by coding and non-coding mutational load prompted the inventors to assess their joint significance in discriminating melanomas and naevi. To achieve this, a logistic regression model was developed using melanoma or naevus as binary outcome and including as explanatory variables (i) non-coding mutational load, and (ii) non-*BRAF*/non-*NRAS* coding mutational load. The resulting model trained on the discovery cohort showed both variables to be highly statistically significant (p-value<0.001, Table 3), with a stronger significance achieved for non-coding mutational load (p-value = 1.16×10^{-7}) compared to the non-*BRAF*/non-*NRAS* coding mutational load (p-value=0.0001). This indicates the independent diagnostic value for the discrimination of melanomas from naevi contributed by both mutational loads.

Table 3. Summary statistics of a multivariate logistic model that predicts melanoma

Term	Odds Ratio	Std. Error	Statistic	P-value	95% CI
Intercept	0.074	0.38	-6.77	1.29204E-11	[0.03-0.15]
Non-*BRAF*/non-*NRAS* coding mutational load	6.31	0.48	3.83	0.000127005	[2.67-17.9]
Non-coding mutational load	3.21	0.22	5.30	1.1585E-07	[2.18-5.20]

[0211] This bivariate genomic model generated a score from 0 to 1 – indicative of increased likelihood of melanoma – and showed an AUC of 95% in the discovery cohort. When applied to the validation cohort, the model had an AUC of 96% (Figure 5A). The equation for this bivariate genomic model is

$$P = \frac{1}{1 + e^{(2.6042 - 1.8416 * \text{Non-BRAF Non-NRAS coding mutational load} - 1.1655 * \text{Non-coding mutational load})}}$$

with $P \in [0,1]$ the probability of melanoma.

Non-BRAF/Non-NRAS coding mutational load ≥ 0 the number of coding gene alterations within the tested genes, excluding those in *BRAF* V600 and *NRAS* Q61.

[0212] Non-coding mutational load ≥ 0 the number of non-coding gene alterations within the tested genes.

[0213] The model score that maximizes the difference between true positive rate and false positive rate in the discovery cohort – the Youden Index – for this model was identified as 0.65. When this cut-off was applied to the discovery cohort, a sensitivity and specificity of 84.1% and 95.6%, respectively, was achieved (Figure 5B). When applied to the validation cohort, a sensitivity and specificity of 82.1% and 96.9%, respectively, was achieved. These results indicate that, at a 0.65 score cut-off, the bivariate genomic-based model is highly specific in discriminating melanomas from naevi. On review of nevi with scores >0.65 (n=5), one case with a score >0.9 and negative PRAME immunostain was considered to potentially represent a difficult to recognize/evolving melanoma.

[0214] The differences in age between melanomas and naevi in this cohort (Table 1) prompted the evaluation of performance of the bivariate genomic model across different age ranges (Table 4). At age thresholds of 55, 65, and 75, the genomic model maintains a high range of AUC for discovery and validation cohorts (AUC range = [89% - 100%]). Using the 0.65 score cut-off, the genomic model retained high specificity across age groups (Specificity range = [91%-100%]).

Table 4. Performance of the bivariate genomic model stratified by age. A youden threshold of 0.65 was used

Cohort (size)	AUC	Specificity	Sensitivity	True Negatives	True Positives	False Negatives	False Positives
Discovery - All Patients (n = 179)	0.95	0.96	0.84	93	69	13	4
Validation - All Patients (n = 60)	0.96	0.97	0.82	31	23	5	1
Discovery - Age <=55 y. (n = 52)	0.97	1.00	0.67	46	4	2	0
Discovery - Age >55 y. (n = 127)	0.94	0.92	0.86	47	65	11	4
Validation - Age <=55 y. (n = 26)	0.98	1.00	0.80	21	4	1	0
Validation - Age >55 y. (n = 34)	0.96	0.91	0.83	10	19	4	1
Discovery - Age <=65 y. (n = 87)	0.90	0.97	0.63	69	10	6	2
Discovery - Age >65 y. (n = 92)	0.95	0.92	0.89	24	59	7	2
Validation - Age <=65 y. (n = 34)	0.95	0.96	0.78	24	7	2	1
Validation - Age >65 y. (n = 26)	1.00	1.00	0.84	7	16	3	0
Discovery - Age <=75 y. (n = 126)	0.89	0.95	0.72	83	28	11	4
Discovery - Age >75 y. (n = 53)	1.00	1.00	0.95	10	41	2	0
Validation - Age <=75 y. (n = 45)	0.96	0.96	0.82	27	14	3	1
Validation - Age >75 y. (n = 15)	1.00	1.00	0.82	4	9	2	0

Example 6 - The likelihood of melanoma estimated by the bivariate genomic model is associated with a pathology-based tier system on retrospectively-collected borderline melanocytic lesions

[0215] To assess the utility of the genomic model in routine clinical testing, an additional set of 121 borderline melanocytic lesions were collected (Table 5a). This cohort is composed of difficult-to-diagnose cases from predominantly young patients (median age at biopsy 28.6 years) compared to the melanomas and naevi in the discovery and validation cohorts, females (62%) and with most lesions located in the trunk (31%) and lower limb (28%). A pathology-based tier system was systematically evaluated on each lesion, assigning one of three tiers: *Tier 1* – favours naevi (n = 18), *Tier 2* – unresolved malignancy (n = 82) – and *Tier 3* – favours melanoma (n = 21). Additionally, pathological assessment of these cases revealed a spectrum of conventional (*eg.* acral, mucosal, congenital as well as Spitzoid tumours from patients older than 10 years without immune histochemistry (IHC) evidence of kinase fusion) and non-conventional types (*eg. BAP1*-inactivated tumours, blue family, pigmented epithelioid melanocytoma (PEM), deep penetrating tumours, Spitzoid tumours with IHC evidence of kinase rearrangement (any age) and Spitzoid tumours from patients younger than 10 years).

[0216] Each borderline lesion was classified into its most likely pathway, including conventional (n = 62), acral (n = 2), *BAP1* (n = 4), blue (n = 9), deep penetrating (n = 9), Spitz/Spitzoid (n = 32) and other (n = 3) (Figure 11; Table 5b). Three samples did not meet QC criteria based on a coverage threshold of 300X and were excluded from further analysis (Figure 6). The remaining 118 samples have a median coverage of 1400X.

Table 5a. Clinical and Pathological Characteristic of Borderline Melanocytic Lesions

Variable	Category/Statistic	Borderline Lesions (n=121)
Age at Biopsy (years)	median [min – max]	28.6 [1.2 – 90.6]
Sex	Female - count (%)	75 (62%)
	Male - count (%)	46 (38%)
Anatomical Location	Head & Neck - count (%)	30 (25%)
	Trunk - count (%)	37 (31%)
	Upper Limb - count (%)	18 (15%)
	Lower Limb - count (%)	34 (28%)
	Unknown - count (%)	2 (2%)

Table 5b. Distribution of borderline melanocytic lesions across pathology-defined tiers and likely pathways of origin

	Acral	BAP1	Blue	Conventional	Deep penetrating	Other	Spitz / Spitzoid
Tier 1	2	0	3	6	3	0	4
Tier 2	0	3	6	37	6	2	28
Tier 3	0	1	0	19	0	1	0

[0217] The landscape of mutations and copy number alterations across borderline lesions reveals distinct mutational events in agreement with their likely pathway of origin and tier-based classification (Figure 7). *BRAF* (n = 27) and *NRAS* (n = 15) were the most frequently mutated coding genes, with these mutations being mutually exclusive across samples. The majority of *BRAF* mutations were in the V600* codon (24/27) and all *NRAS* mutations were in the Q61* codon. Hotspot mutations in *TERT* promoter (n = 14) and *RPL13A* promoter (n = 14) were the most frequent in non-coding regions, detected only in *Tier 2 (5/81 and 7/81 for TERT and RPL13A, respectively) and Tier 3 (9/20 and 7/20 for TERT and RPL13A, respectively) lesions. Among copy number alteration events, one CDKN2A deletion occurred in a Tier 2 lesion, whereas one BRAF amplification and two CDKN2A deletions were identified in Tier 3 lesions.*

[0218] Other oncogenic mutations detected for individual lesions agreed with the likely pathway of origin assigned based on morphology. For example, all 7 samples with mutations in *GNAQ* Q209* codon have morphologies that resemble blue family characteristics (BL10, BL23, BL62, BL63, BL71, BL86, and BL95). Two samples (BL69 and BL114) with morphologies resembling *BAP1*-inactivated tumours have a frameshift indel in *BAP1* that results in early truncation (and likely inactivation) of the protein. Two samples with deep-penetrating morphologies have oncogenic missense mutations in *CTNNB1* (Beta-Catenin) in the T41 (BL103) and S45 (BL106) codons. Two Spitzoid tumors of uncertain malignant potential (BL1 and BL51) carry an *HRAS* Q61 mutation.

[0219] Tier 1, Tier 2 and Tier 3 lesions have a median total mutational load of 1 (range 0 - 2), 1 (range 0 - 7) and 3.5 (range 0 - 9) mutations, respectively. The median coding mutational load is 1 (0 - 1), 0 (0 - 3) and 1 (0 - 3) mutations for Tier 1, Tier 2 and Tier 3, respectively. The median non-coding mutational load is 0 (range 0 - 2), 0 (range 0 - 5)

and 2.5 (range 0 - 6) mutations for Tier 1, Tier 2 and Tier 3, respectively. For 41% (7/17) of Tier 1, 49% (40/81) of Tier 2 and 20% (4/20) of Tier 3, no mutations or copy number alterations were detected. The high prevalence of lesions with no mutational load detected is expected given the young age of this cohort (7 and 21 patients in Tier 1 and Tier 2, respectively, are younger than 18 years). As observed in melanomas, non-coding mutational load was higher in NRAS Q61* borderline lesions (median 1 mutation, range 0-6) compared to BRAF V600* borderline lesions (median 0 mutations, range 0-4, Wilcoxon p-value=0.01).

[0220] Assessment of total mutational load against clinical factors (Figure 8) showed a strong association with age at time of biopsy (spearman coefficient=0.39, p-value=0.00001). This association was weak for the coding mutational load (spearman coefficient=0.21, p-value=0.02) and strongest for the non-coding mutational load (spearman coefficient=0.44, p-value=7.6E-07). Non-coding mutational load was also weakly associated with anatomical location (median 1 mutation in samples from the upper limb compared to median 0 mutations in other sites, Kruskal-Wallis p-value=0.02). No association between gender and mutational load were identified.

[0221] The probability of melanoma as calculated with the bivariate genomic model is in agreement with the pathology-based estimate of malignancy for each borderline lesion across tiers (Figure 9).

[0222] The distribution of genomic score values was significantly different across tiers (Kruskal-Wallis test p-value=0.001). Lesions in *Tier 1* had a median genomic score of 0.07, with a range of values between 0.07 and 0.6. Based on a threshold of 0.65 (the optimal threshold identified in the discovery cohort), none of these lesions would be classified as melanoma. Lesions in *Tier 2*, while having the same median genomic-based score as those in *Tier 1*, had a broader range of values – 0.07 to 1, with 7 lesions having a probability of melanoma greater than 0.65. Lesions in *Tier 3* had a median genomic score of 0.65, ranging from 0.07 to 1, and with 10 of 20 lesions having a genomic score greater than 0.65.

Example 7 – A logistic regression bivariate model built on a primary set of mutations remains diagnostically useful compared to the full logistic regression bivariate model

[0223] A set of primary, secondary and tertiary genes in the panel has been identified (Table 6).

[0224] **Genes in the primary set** have the best AUC and specificity performance in the Discovery set within each group of coding, non-coding and copy number alteration. When combined into a bivariate logistic regression model – aka a *reduced-primary* model - this set of genes is diagnostically useful, as defined by a statistically insignificant (p-value>0.05) AUC difference to the AUC obtained with the *full* model (that includes all genes in the panel) in both discovery and validation cohorts.

[0225] Specifically, the inventors have identified:

Primary Coding Set: TP53, CDKN2A, RAC1, PTEN

Primary Non-Coding Set: *pTERT, RPL13A, SMUG1*

Primary Copy Number Alteration Set: *CDKN2A, CDK4*

[0226] The AUC of the *reduced-primary* bivariate logistic regression model is 91% in discovery and 96% in validation cohorts. The AUC of the *full* bivariate logistic regression model is 95% in discovery and 96% in validation. In discovery cohort, the difference in AUC between the two models is 3.16%, which is not statistically significant (delong paired test for AUC difference p-value>0.05, as implemented by Vergara *et al. BMC Bioinformatics,* 2008; 9:265). In validation cohort, the difference in AUC between the two models is 0.84%, which is not statistically significant (delong paired test for AUC difference p-value>0.05, as implemented by Vergara *et al. (Id).*

[0227] **Genes in the secondary set** have intermediate AUC and specificity performance within each group of coding, non-coding and copy number alteration. When combined into a bivariate logistic regression model – aka a *reduced-secondary* model - this set of genes has a statistically significant AUC difference to the AUC obtained with the *full* model (that includes all genes in the panel) in both discovery and validation cohorts.

[0228] Specifically, the inventors have identified:

Secondary Coding Set: BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT, TYRP1

Secondary Non-Coding Set: C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, ZNF778

Secondary Copy Number Alteration Set: *MDM2, MAP2K1, RAC1*

[0229] The AUC of the *reduced-secondary* bivariate logistic regression model is 90% in discovery and 84% in validation cohorts. The AUC of the *full* bivariate logistic regression model is 95% in discovery and 96% in validation. In discovery cohort, the difference in AUC between the two models is 4.93%, which is highly statistically significant (delong paired test for AUC difference p-value<0.001, as implemented by Vergara *et al.* (*Id*). In validation cohort, the difference in AUC between the two models is 12%, which is highly statistically significant (delong paired test for AUC difference p-value<0.005, as implemented by Vergara *et al.* (*Id*).

[0230] Genes in the tertiary set have either (i) the lowest performance based on AUC and specificity within each group of coding, non-coding and copy number alteration, or (ii) were not identified in this specific cohort so its performance could not be assessed, or (iii) they were excluded from analysis due to low individual performance in a separate cohort.

Tertiary Coding Set: *PLCB4, BRAF, NRAS, CDK4*

Tertiary Non-Coding Set: ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE, SWI5

Tertiary Copy Number Alteration Set: BRAF, BAP1, CCND1, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, *KRAS,* MAP2K2, MYC, NRAS, PTEN, RAF1, RB1, TERT, TP53

Table 6. Performance of coding gene mutations, non-coding gene mutations and copy number alterations for discriminating melanomas from naevi on the discovery cohort.

variable	variable type	Set	AUC discovery	Specificity Discovery	Sensitivity Discovery
CDK4	Coding Gene - Copy Number Alteration	Primary	0.52	1.000	0.05
CDKN2A	Coding Gene - Copy Number Alteration	Primary	0.55	1.000	0.10
RAC1	Coding Gene - Copy Number Alteration	Secondary	0.51	1.000	0.01
MAP2K1	Coding Gene - Copy Number Alteration	Secondary	0.52	1.000	0.04
MDM2	Coding Gene - Copy Number Alteration	Secondary	0.52	1.000	0.04
BRAF	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
BAP1	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
CCND1	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
CTNNB1	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
CYSLTR2	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
GNA11	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
GNAQ	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
HRAS	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
KIT	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
MAP2K2	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
MYC	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
NRAS	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
PTEN	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
RAF1	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
RB1	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
TERT	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
TP53	Coding Gene - Copy Number Alteration	Tertiary	NA	NA	NA
TP53	Coding Gene - Mutation	Primary	0.62	0.969	0.27
PTEN	Coding Gene - Mutation	Primary	0.53	1.000	0.06
CDKN2A	Coding Gene - Mutation	Primary	0.58	1.000	0.16
MAP2K2	Coding Gene - Mutation	Secondary	0.51	0.990	0.02
TERT	Coding Gene - Mutation	Secondary	0.51	0.990	0.02
HRAS	Coding Gene - Mutation	Secondary	0.52	0.990	0.05
MAP2K1	Coding Gene - Mutation	Secondary	0.53	0.990	0.07
BAP1	Coding Gene - Mutation	Secondary	0.51	1.000	0.02
EIF1AX	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
GNA11	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
KIT	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
KRAS	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
MYC	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
RAF1	Coding Gene - Mutation	Secondary	0.51	1.000	0.02
RB1	Coding Gene - Mutation	Secondary	0.51	1.000	0.02
TYRP1	Coding Gene - Mutation	Secondary	0.51	1.000	0.01
CYSLTR2	Coding Gene - Mutation	Secondary	0.52	1.000	0.04
SF3B1	Coding Gene - Mutation	Secondary	0.52	1.000	0.04
RAC1	Coding Gene - Mutation	Secondary	0.54	1.000	0.07

variable	variable type	Set	AUC discovery	Specificity Discovery	Sensitivity Discovery
BRAF	Coding Gene - Mutation	Tertiary	0.38	0.557	0.68
NRAS	Coding Gene - Mutation	Tertiary	0.58	0.907	0.24
PLCB4	Coding Gene - Mutation	Tertiary	NA	NA	NA
RPL13A	Promoter Region - Mutation	Primary	0.7	0.959	0.45
SMUG1	Promoter Region - Mutation	Primary	0.69	1.000	0.38
pTERT	Promoter Region - Mutation	Primary	0.87	1.000	0.73
PES1	Promoter Region - Mutation	Secondary	0.66	0.959	0.37
UBXN8	Promoter Region - Mutation	Secondary	0.56	0.979	0.15
OXNAD1	Promoter Region - Mutation	Secondary	0.62	0.979	0.27
CDC20	Promoter Region - Mutation	Secondary	0.65	0.979	0.33
FTH1	Promoter Region - Mutation	Secondary	0.65	0.979	0.32
AP3D1	Promoter Region - Mutation	Secondary	0.67	0.979	0.35
C16orf59	Promoter Region - Mutation	Secondary	0.68	0.979	0.39
RPL29	Promoter Region - Mutation	Secondary	0.53	0.990	0.06
HSBP1	Promoter Region - Mutation	Secondary	0.54	0.990	0.09
DHX16	Promoter Region - Mutation	Secondary	0.56	0.990	0.13
MRPS31	Promoter Region - Mutation	Secondary	0.56	0.990	0.12
MRPS33	Promoter Region - Mutation	Secondary	0.56	0.990	0.12
RNF185	Promoter Region - Mutation	Secondary	0.56	0.990	0.12
RPL18A	Promoter Region - Mutation	Secondary	0.65	0.990	0.32
KBTBD8	Promoter Region - Mutation	Secondary	0.53	1.000	0.06
ZNF778	Promoter Region - Mutation	Secondary	0.54	1.000	0.07
NSUN6	Promoter Region - Mutation	Secondary	0.57	1.000	0.13
RPS14	Promoter Region - Mutation	Secondary	0.57	1.000	0.15
RPL34	Promoter Region - Mutation	Secondary	0.59	1.000	0.18
SLC30A6	Promoter Region - Mutation	Secondary	0.64	1.000	0.28
ARHGEF18	Promoter Region - Mutation	Tertiary	NA	NA	NA
RPS27	Promoter Region - Mutation	Tertiary	NA	NA	NA
CHCHD2	Promoter Region - Mutation	Tertiary	NA	NA	NA
BLCAP	Promoter Region - Mutation	Tertiary	NA	NA	NA
ERGIC3	Promoter Region - Mutation	Tertiary	NA	NA	NA
RALY	Promoter Region - Mutation	Tertiary	NA	NA	NA
YAE1D1	Promoter Region - Mutation	Tertiary	NA	NA	NA
SYF2	Promoter Region - Mutation	Tertiary	NA	NA	NA
NFKBIE	Promoter Region - Mutation	Tertiary	NA	NA	NA
SWI5	Promoter Region - Mutation	Tertiary	NA	NA	NA

Example 8 – Logistic regression bivariate models built on subsets of the primary set of mutations remain diagnostically useful compared to the full logistic regression bivariate model

[0231] Using the same definition of primary gene set above, the inventors have identified subsets of primary genes that lead to diagnostically useful logistic regression bivariate models, as defined by a statistically insignificant (p-value>0.05) AUC difference to the AUC obtained with the full model (that includes all genes in the panel) in both discovery (AUC=95%) and validation (AUC=96%) cohorts (Table 7). These models include a minimum number of 5 total primary genes, 2 coding primary genes, 2 non-coding primary genes and 1 copy number alteration primary gene.

Table 7. Performance of bivariate genomic models when trained on different subsets of genes in the primary set. Performance was measured on discovery and validation cohorts. P-value for the difference between the AUC of each reduced model and the AUC of the full model was calculated with the delong test for paired AUC differences (Vergara et al.(Id)).

Total primary genes	Coding primary	Non-coding primary	CNA primary	Discovery_AUC reduced model	Validation_AUC reduced model	Discovery Diff AUC pvalue	Validation Diff AUC pvalue
5	2	2	1	0.90	0.92	0.06	0.07
6	2	3	1	0.92	0.92	0.08	0.17
7	2	3	2	0.91	0.93	0.09	0.22
7	2	3	2	0.91	0.96	0.09	0.22
7	2	3	2	0.92	0.92	0.09	0.22
7	3	2	2	0.90	0.93	0.06	0.79
7	3	2	2	0.91	0.94	0.06	0.79
7	3	2	2	0.92	0.96	0.06	0.79
7	3	3	1	0.91	0.95	0.08	0.08
7	3	3	1	0.92	0.90	0.08	0.08
8	3	3	2	0.91	0.96	0.05	0.80
8	3	3	2	0.92	0.96	0.05	0.80
8	3	3	2	0.91	0.94	0.05	0.80
8	4	2	2	0.92	0.96	0.06	0.70
8	4	3	1	0.91	0.94	0.05	0.63
8	4	3	1	0.91	0.95	0.05	0.63
9	4	3	2	0.92	0.96	0.06	0.73

Example 9 – Tumour mutational burden (TMB) scores indicative of melanomas

[0232] Across the mutational load measurements proposed, the following thresholds (inclusive) were identified to occur only in melanomas across the discovery and validation cohorts (Figure 12).

Total Mutational Load = 7

Coding Mutational Load = 3

Noncoding Mutational Load = 6

Non-pTERT Non-coding Mutational Load = 5

Non-BRAF/non-NRAS Coding Mutational Load = 3

[0233] Hence, tumour lesions presenting a mutational load equal or greater to these values are indicative of a high likelihood of melanoma diagnosis.

Example 10 – The bivariate genomic model retains high performance across age, anatomical site and thickness levels

[0234] The clinical differences between melanomas and nevi in this investigative cohort prompted the evaluation of the performance of the bivariate genomic model in patients stratified by age, anatomical body site and thickness of lesion (Figure 13A-G. Across anatomical sites of varying levels of sun exposure, including trunk, head and neck and extremities, the model retained high performance (Figure 13A-C, discovery AUC range: 0.91-0.99, validation AUC range: 0.96-0.96). At a selected age threshold of 55 (close to the median age of nevi in this cohort), the model retained high performance in both younger and older patients (Figure 13D-E, discovery AUC range: 0.94-0.97, validation AUC range: 0.96-0.98). The model also retained high performance in thinner lesions (<=1 mm) as well as thicker lesions (>1 mm) (Figure 13F-G, discovery AUC range: 0.94-0.97, validation AUC range: 0.95-0.98). These results indicate the bivariate genomic model retains good performance within clinically relevant cohorts of patients.

Example 11- Clinical and pathological features of a cohort of borderline tumours

[0235] To better appreciate the utility of our genomic classifier in a real-world setting, a cohort of 110 borderline tumors were triaged into 3 tiers of clinical certainty based on the language conveyed in the expert pathologist's consultation pathology report. This cohort (N=110) was composed of young patients (median age at biopsy 28 years), with majority female (n=67, 61%) and most tumors located on the trunk (n=34, 31%) and lower limb (n=31, 28%) (Table 8). The three tiers assigned were: Tier 1 – favors nevi (n=18), Tier 2 – challenging/unresolved malignancy (n=80) – and Tier 3 – favors melanoma (n=12). Cases were further subclassified into a likely pathway of origin (9) based on their assessment at time of diagnosis. These included conventional tumors (n=55, referring to tumors typically associated, but not limited to harboring a BRAF V600 or NRAS hotspot driver mutation without an alternative or additional pathway-defining driver mutation, with acral, mucosal and congenital subtypes specifically included in this group), and non-conventional tumors that typically harbor (additional) non-BRAF/NRAS pathway-defining genomic mutations/aberrations (Spitz (n=31), blue (n=10), WNT-activated (n=9), BAP-1-inactivated tumors (n=4), and PEM, (n=1) (Table 8).

Table 8. Clinical and pathological characteristics of borderline melanocytic tumors. PEM: Pigmented Epithelioid Melanocytoma.

Variable	Category/Statistic	Borderline Tumors (n=110)
Age at Biopsy (years)	median [min – max]	28 [1 – 87]
Sex	Female - count (%)	67 (61%)
	Male - count (%)	43 (39%)
Anatomical Location	Head & Neck - count (%)	27 (25%)
	Trunk - count (%)	34 (31%)
	Upper Limb - count (%)	17 (15%)
	Lower Limb - count (%)	31 (28%)
	Unknown - count (%)	1 (1%)
Tier-Likely pathway of origin	*Tier 1* - Conventional	8 (7%)
	Tier 1 - BAP1	0 (0%)
	Tier 1 - Blue	3 (3%)
	Tier 1 - WNT-activated	3 (3%)
	Tier 1 - PEM	0 (0%)
	Tier 1 - Spitz	4 (4%)
	Tier 2 - Conventional	37 (34%)
	Tier 2 - BAP1	3 (3%)
	Tier 2 - Blue	6 (5%)
	Tier 2 - WNT-activated	6 (5%)
	Tier 2 - PEM	1 (1%)
	Tier 2 - Spitz	27 (25%)
	Tier 3 - Conventional	10 (9%)
	Tier 3 - BAP1	1 (1%)
	Tier 3 - Blue	1 (1%)
	Tier 3 - WNT-activated	0 (0%)
	Tier 3 - PEM	0 (0%)
	Tier 3 - Spitz	0 (0%)

[0236] A large proportion of cases (34%, 37/110) were of a conventional subtype with uncertainty around malignant risk (Tier 2), typically representing concern around nevoid melanoma diagnosis and subtle melanoma arising in nevi. The second largest grouping was of the Spitz subtype (25%, 27/110) in Tier 2, which reflected a diagnosis of an atypical Spitz tumor with uncertain malignant potential. Two samples did not meet QC criteria based on a coverage threshold of 300X (both of conventional type) and were excluded from further analysis. The remaining 108 samples (17 Tier 1, 79 Tier 2 and 12 Tier 3) had a median coverage of 1384X.

Example 12 - The coding mutational landscape of borderline tumors agrees with their likely pathway of origin and the non-coding mutational load is associated with the pathology-based tiers of malignancy in conventional tumors

[0237] The landscape of coding and non-coding mutations across borderline tumors reveals distinct mutational events in agreement with their likely pathway of origin and pathology-based tier of malignancy (Figure 14A). BRAF (n=27) and NRAS (n=13) were the most frequently mutated coding genes, with mutations in these genes being mutually exclusive across samples. The majority of BRAF mutations were in the V600 codon (24/27) and all NRAS mutations were in the Q61 codon. All except one BRAF V600 and NRAS Q61 mutation occurred in tumors of either conventional, WNT-activated or BAP1 morphology, with the remaining BRAF V600 mutation in the Spitz grouping (which was regarded as a BRAF-mutated and morphologically spitzoid (BAMS) tumor at diagnosis. One CDKN2A loss was identified in a Tier 2 conventional tumor (BL82). Other oncogenic mutations detected for individual tumors agreed with the likely pathway of origin assigned at diagnosis. All 7 samples with Q209L mutations in GNAQ had diagnoses favoring a blue family origin (BL10, BL23, BL62, BL63, BL71, BL86, and BL95). Two samples (BL69 and BL114) with a frameshift indel in BAP1 that results in early truncation of the protein had diagnoses favoring BAP1-inactivated tumors. Three samples with missense mutations in CTNNB1 (Beta-Catenin; T41I in BL103 and S45F in BL66, BL106) had diagnoses favouring WNT-activated tumors. Two HRAS Q61 mutant tumors (Q61K in BL1 and Q61R in BL51) had a spitzoid morphology of uncertain malignant potential. These results highlight the added value of this genomic panel of being able to reveal or confirm a variety of driver mutation targets across likely pathways of origin, particularly when no lineage immunostain is available, such as tumors with diagnoses that favor blue morphology.

[0238] The landscape of non-coding mutations showed a strong agreement with the pathology-based tiers of malignancy. Hotspot mutations in RPL13A promoter (n=11) and pTERT (n=9) were the most frequent in non-coding regions, detected only in Tier 2 (7/79 and 5/79 for RPL13A and TERT, respectively) and Tier 3 (4/12 and 4/12 for RPL13A and pTERT, respectively) tumors. For conventional borderline tumors, total mutational load was significantly associated with tiers (Figure 14B, Kruskal-Wallis p-value=0.021). Tier 1, Tier 2 and Tier 3 conventional tumors had a median total mutational load of 0 (range 0-1), 1 (range 0-7) and 2.5 (range 0-8) mutations,

respectively. Separate assessment of coding and non-coding mutational loads for conventional tumors revealed this association to be driven by the non-coding mutational load (Kruskal-Wallis p-value=0.018) as opposed to coding mutational load (Kruskal-Wallis p-value=0.16).

[0239] In contrast, no significant associations between total mutational load and tier classification were identified in non-conventional tumors (Figure 14B). Tier 1, Tier 2 and Tier 3 tumors in this group had median total mutational loads of 0 (range 0-2), 0 (range 0-7) and 1.5 (range 1-2) mutations, respectively (Kruskal-Wallis p-value=0.08). Of note, the majority of non-conventional tumors with no mutations identified were regarded as atypical Spitz tumors, with only four Tier 2 Spitz tumors haboring coding mutations, which were in BRAF (BL72 – the BAMS tumor), HRAS Q61 (BL1 and BL51) and MAP2K1 I107 (BL74 - in the vicinity of the in-frame deletion position associated with spitzoid morphology, whilst one Tier 2 Spitz tumor harbored a non-coding mutation in the promoter region of NSUN6 (BL21). Other non-Spitz non-conventional tumors harbored coding and non-coding mutations with potential significant influence over malignant risk classification. For example, BL103 with WNT-activated morphological features had, in addition to a CTNNB1 T41I mutation, coding mutations in MYC (Phe22Leu) and HRAS (Gln61Lys) as well as non-coding mutations in the promoter regions of TERT, FTH1, DHX16 and C16orf59. Whilst this tumor was negative with a PRAME immunostain, its mutational profile suggests a high potential for malignancy, which was supported by the expansile growth profile and cytomorphology that was more atypical than usually seen in benign WNT-activated tumors (Figure 14C). Overall, these results demonstrate the value of the non-coding mutations in estimating malignancy for difficult-to-diagnose conventional tumors as well as non-conventional tumors.

[0240] The bivariate genomic model is complementary to routinely used ancillary tests and it is associated with the pathology-based tier of malignancy and clinical outcome in patients with borderline conventional tumors.

[0241] In agreement with the good association between mutational landscape and pathology-based tiers of malignancy for conventional tumors, the distribution of genomic scores was significantly different across tiers for this group (Figure 15A, Kruskal-Wallis test p-value= 0.02). Notably, all 7 conventional Tier 1 tumors had a probability of melanoma below 0.5. with a median genomic score of 0.07 (range = [0.07-0.19]). The 36 conventional tumors in Tier 2 had a median genomic score of 0.19 (range=[0.07 to

1]), with 8/36 and 6/36 tumors having a probability of melanoma greater than 0.5 and 0.65 (the Youden index threshold), respectively. The 10 conventional tumors in Tier 3 had a median genomic score of 0.57 (range=[0.07-1]), with 5/10 having a score greater than 0.5 and 0.65.

[0242] Importantly, seven of the 8 tumors with a probability of melanoma greater than 0.5 in the Tier 2 group were negative for either PRAME, FISH, and/or had p16 retained (Figure 15B). This highlights its complementary value to that of routinely used ancillary assays. The majority of these tumors were located on the trunk (3/8) or head and neck (3/8). Six of the 8 patients were younger than 50 years, with three patients younger than 30 years. Assessment of these and other ancillary tests including staining for BRAF V600E, RAS Q61R, BAP1 and CTNNB1 available across the borderline cohort further confirmed the complementary value of the genomic panel and the genomic model to that of those routinely used in clinic.

[0243] Eight patients with borderline conventional tumors had available clinical follow-up across tiers (1 Tier 1, 6 Tier 2 and 1 Tier 3, median follow-up = 8 months. No events associated with malignant behavior were observed in this group, although for 4 cases the follow-up duration was less than 4 months. The other four cases had more than one year follow-up, including 2 with more than 4 years follow-up. Consistent with their positive clinical outcomes, these 4 cases had a low likelihood of melanoma predicted with the genomic model.

CLAIMS

1. A method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;

- detecting in the sample the presence of gene alterations in each of the following genes: noncoding mutations in *pTERT, RPL13A and SMUG1,*

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma) optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus).

2. The method of claim 1, further comprising detecting the presence of gene non-coding alterations in one or more of the following genes: *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SWI5, SYF2, TERT, UBXN8, YAE1D1 and ZNF778.*

3. The method of claim 1 or claim 2, further comprises detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 31, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2, to 31, 3 to 5, 3 to 10, 3 to 15, 3 to 20, 3 to 31, 5 to 31, 10 to 31, 15 to 31 or 20 to 31 of these additional genes.

4. The method of any one of claims 1 to 3, further comprising detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *TP53, CDKN2A, RAC1, PTEN.*

5. The method of any one of claims 1 to 4, the method further comprising detecting the presence of gene coding alterations in one or more of the following genes: *BAP1, BRAF, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, RAF1, RB1, SF3B1, TERT,* and *TYRP1.*

6. The method of any one of claims 1 to 5, the method further comprising detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15, 3 to 25, 3 to 25, 10 to 25, or 20 to 25 of these additional genes.

7. The method of any one of claims 1 to 6, further comprising detecting the presence of copy number alterations in one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53*.

8. The method of any one of claims 1 to 7, the method further comprising detecting the presence of copy number alterations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

9. The method of any one of claims 1 to 8, wherein the gene alterations are: coding mutations in *TP53, CDKN2A, RAC1* and *PTEN*; copy number alterations in *CDKN2A* and *CDK4; and* non-coding mutations in *pTERT, RPL13A, and SMUG1*

10. A method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;

- detecting in the sample the presence of gene alterations in each of the following genes : non-coding mutations in C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, ZNF778,

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma) optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus)..

11. The method of claim 10, further comprising detecting the presence of gene non-coding alterations in one or more of the following genes: *ARHGEF18, BLCAP,*

CHCHD2, ERGIC3, NFKBIE, RALY/RP5-1125A11.1, RPL13A, RPS27, SMUG1, SWI5, SYF2, TERT and YAE1D1.

12. The method of claim 10 or claim 11, the method further comprising detecting the presence of coding mutations in 1 to 3, 1 to 5, 1 to 10, 1 to 13, 2 to 3, 2 to 5, 2 to 10, 2 to 13, 3 to 5, 3 to 10, 3 to 13 or 5 to 13 of these additional genes.

13. The method of any one of claims 10 to 12, further comprising detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT and TYRP1.*

14. The method of any one of claims 10 to 13, the method further comprising detecting the presence of gene coding alterations in one or more of the following genes: *BRAF, CDKN2A, CDK4, NRAS, PLCB4, PTEN, RAC1 and TP53.*

15. The method of anyone of claims 10 to 14, the method further comprising detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15, 3 to 25, 3 to 25, 10 to 25, or 20 to 25 of these additional genes.

16. The method of any one of claims 10 to 15, further comprising detecting the presence of copy number alterations in one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT,* KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT and TP53.

17. The method of any one of claims 10 to 16, the method further comprising detecting the presence of copy number alterations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

18. The method of any one of claims 10 to 17, wherein the gene alterations are: coding mutations in *BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT* and *TYRP1*; copy number alterations in *MDM2, MAP2K1 and RAC1; and* non-coding mutations in *C16orf59, AP3D1, PES1, CDC20, FTH1, RPL18A, SLC30A6, OXNAD1, DHX16, HSBP1,*

KBTBD8, MRPS31, MRPS33, NSUN6, RNF185, RPL29, RPL34, RPS14, UBXN8, and ZNF778.

19. A method of determining whether a lesion in a subject is melanoma or a melanocytic naevus (or determining that a lesion is likely to be a melanoma or a melanocytic naevus), the method comprising:

- receiving a genetic sample from the lesion;

- detecting in the sample the presence of gene alterations in each of the following genes : non-coding mutations in *ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE* and *SWI5*,

wherein the presence of gene alterations in at least these genes indicates that the individual has melanoma (or the lesion is a melanoma), optionally, the absence of the gene alterations in at least these genes indicates the individual has a melanocytic naevus (or the lesion is a melanocytic naevus)..

20. The method of claim 19, further comprising detecting the presence of gene non-coding alterations in one or more of the following genes: *AP3D1, C16ORF59, CDC20, DHX16, DPH3/OXNAD1, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NSUN6, PES1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, SLC30A6, SMUG1, TERT, UBXN8,* and *ZNF778.*

21. The method of claim 19 or claim 20, the method further comprising detecting the presence of coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15, 3 to 20, 3 to 23, 5 to 23, 10 to 23 or 15 to 23 of these additional genes.

22. The method of any one of claims 19 to 21, further comprising detecting in the sample the presence of gene alterations in each of the following genes: coding mutations in *PLCB4, BRAF, NRAS, CDK4.*

23. The method of any one of claims 19 to 22, the method further comprises detecting the presence of gene coding alterations in one or more of the following genes: *TP53, CDKN2A, RAC1, PTEN, BAP1, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, RAF1, RB1, SF3B1, TERT,* and *TYRP1.*

24. The method of any one of claims 19 to 23, the method further comprising detecting the presence of non-coding mutations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 25, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 25, 5 to 25, 10 to 25, 15 to 25 or 20 to 25 of these additional genes.

25. The method of any one of claims 19 to 24, further comprising detecting the presence of copy number alterations in one or more of the following genes: *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT* and *TP53*.

26. The method of any one of claims 19 to 25, the method further comprises detecting the presence of copy number alterations in 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 23, 2 to 5, 2 to 10, 2 to 15, 2 to 20, 2 to 23, 3 to 5, 3 to 10, 3 to 15 or 3 to 20, 3 to 23, 10 to 20 or 15 to 23 of these additional genes.

27. The method of any one of claims 19 to 26, wherein the gene alterations are: coding mutations in *PLCB4, BRAF, and NRAS*; copy number alterations in *BRAF, BAP1, CCND1, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, MAP2K2, MYC, NRAS, PTEN, RAF1, RB1, TERT, and TP53; and* non-coding mutations in *ARHGEF18, RPS27, CHCHD2, BLCAP, ERGIC3, RALY, YAE1D1, SYF2, NFKBIE, SWI5.*

28. A method of determining that a lesion is (or is likely to be) a melanoma or a melanocytic naevus, the method comprising:

- receiving a genetic sample from the lesion;

- determining the presence of gene alterations in genes selected from: coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1*, non-coding mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778*; and copy number alterations in *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2,*

GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN, RAC1, RAF1, RB1, TERT and *TP53,*

wherein the presence of gene alterations in one or more genes indicates that the individual has melanoma,

or

wherein clinical indicators of a melanocytic lesion in the absence of gene alterations in one or more genes indicates that the individual likely has a melanocytic naevus.

29. The method of claim 28, wherein the gene alterations are:

coding mutations in BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53 and TYRP1; and non-coding mutations in AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1 and ZNF778,

wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

30. The method of any one of claims 1 to 29, further comprising:

- determining a tumour mutational burden score (TMB) from the sample, wherein the TMB score is the number of gene alterations within the tested genes,

- comparing the TMB score with a reference TMB score,

wherein a TMB score from the sample that is at or above the reference TMB score indicates that the melanocytic lesion is (or is likely to be) melanoma,

or

wherein a TMB score from the sample that is below the reference TMB score indicates that the lesion is (or is likely to be) a melanocytic naevus.

31. The method of claim 29 or 30, wherein the number of tested genes is at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20.

32. The method of claim 30 or 31, wherein the TMB score is a coding mutation TMB score, optionally wherein the coding mutations does not include a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

33. The method of claim 30 or 31, wherein the TMB score is a non-coding mutation TMB, optionally wherein the non-coding mutation does not include a mutation in the promoter region of the *TERT* gene.

34. The method of claim 30 or 31, wherein the TMB score is a copy number alteration TMB.

35. The method of claim 30 or 31, wherein the TMB score is a promoter region TMB.

36. The method of any one of claims 30 to 35, wherein the reference TMB score is a preassigned score.

37. The method of any one of claims 30 to 36 wherein the reference TMB score is between 1 and 50, between 1 and 40, between 1 and 30, between 1 and 20, between 1 and 10, between 1 and 9, between 1 and 8, between 1 and 7, between 1 and 6, between 1 and 5, between 1 and 4 or between 1 and 3.

38. The method of any one of claims 30 to 36, wherein the reference TMB score is at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9.5 or 10, preferably at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 or 7.5.

39. The method of any one of claims 30 to 38, wherein the TMB score is the number of gene alterations that are coding mutations and/or copy number alterations, the reference TMB score is a reference number of coding mutations and/or copy

number alterations within the tested genes, preferably, the reference TMB score is at least 3.

40. The method of any one of claims 30 to 38, wherein the TMB score is the number of gene alterations that are non-coding mutations, the reference TMB score is a reference number of coding mutations within the tested genes, preferably, the reference TMB score is at least 6.

41. The method of any one of claims 30 to 38, wherein the TMB score is the number of gene alterations that are non-coding mutations, and the non-coding mutation(s) are not in the promoter region of the *TERT* gene, the reference TMB score is a reference number of non-coding mutations within the tested genes, preferably, the reference TMB score is at least 5.

42. The method of any one of claims 30 to 38, wherein the TMB score is the number of gene alterations that are coding mutations, and the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation, the reference TMB score is a reference number of non-coding mutations within the tested genes, preferably, the reference TMB score is at least 3.

43. The method of any one of claims 1 to 42, wherein the method is for determining whether a lesion in a subject is melanoma or a melanocytic nevi.

44. The method of claim 43, wherein the clinical indicators of a melanocytic lesion in the absence of gene alterations in the one or more genes indicates that the individual has a melanocytic naevus.

45. The method of any one of claims 1 to 44, further comprising collecting a genetic sample from the lesion.

46. The method of claims 1 to 45, wherein the lesion has clinical indicators of a melanocytic lesion.

47. A method of determining that an individual has (or is likely to have) melanoma or a melanocytic naevus, the method comprising:

- receiving a sample from a lesion (ie a lesion suspected of being a melanocytic lesion);

- determining two or more tumour mutational burden scores (TMB) from the sample selected from a coding mutation TMB, a non-coding mutation TMB, a copy number alteration TMB, a promoter regions TMB, and TMBs based on combinations of these;

- wherein each TMB score is the number of gene alterations within the tested genes, wherein the gene alterations are selected from one of the following groups consisting of:

  (a) for a coding mutation TMB: coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1*, optionally wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation;

  (b) for a non-coding TMB: non-coding hotspot mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778*, optionally wherein the non-coding mutation is not within the promoter region of *TERT*;

  (c) for a copy-number TMB: copy number alterations in *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN RAC1, RAF1, RB1, TERT* and *TP53;* and

  (d) for a promoter region TMB: promoter regions mutations in *TERT*,

- comparing the two or more TMB scores from the sample with a reference data set using a multivariate statistical model to generate a multivariate score,

- comparing the multivariate score to a reference multivariate score,

  wherein a multivariate score from the sample that is at or above the reference multivariate score indicates that the individual has melanoma,

or

wherein a multivariate score from the sample that is below the reference multivariate score indicates that the individual has a melanocytic naevus.

48. The method of claim 47, wherein a TMB score is determined for each of (a), (b) and (d); or (a), (b) and (c).

49. The method of claim 48, wherein at least a first and a second TMB score is determined from the sample.

50. The method of claim 49, wherein the first TMB score is determined from (b) and (c) and the second TMB score is determined from (a), preferably wherein the coding mutations of (a) does not include a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation.

51. The method of any one of claims 47 to 50, wherein the reference data set is constructed using a multivariate statistical model based on a plurality TMB scores determined from a plurality of reference melanoma and melanocytic samples.

52. The method of any one of claims 47 to 51, wherein the TMB scores for the sample and reference data set are determined from testing for the presence of the same groups of gene alterations.

53. The method of any one of claims 47 to 52, wherein the reference multivariate score is a pre-assigned score.

54. The method of claim 53, wherein the reference multivariate score is between about 0.4 to 1, between about 0.5 to 1, between about 0.55 to 1, between about 0.6 to 1, between about 0.65 to 1, between about 0.7 to 1, between about 0.75 to 1, between about 0.8 to 1, between about 0.85 to 1, between about 0.9 to 1 or between about 0.95 to 1. In some embodiments, the reference multivariate score is between 0.4 to 1, between 0.5 to 1, between 0.55 to 1, between 0.6 to 1, between 0.65 to 1, between 0.7 to 1, between 0.75 to 1, between 0.8 to 1, between 0.85 to 1, between 0.9 to 1 or between 0.95 to 1.

55. The method of any one of claims 47 to 54, wherein the multivariate statistical model comprises multivariate linear regression (MLR) or multivariate generalised linear regression (GLM), preferably multivariate GLM.

56. The method of any one of claims 1 to 55, further comprising using one or more additional diagnostic methods, preferably histology.

57. A method comprising detecting in a sample the presence of gene alterations in one or more genes selected from:

(a) coding mutations in *BAP1, BRAF, CDKN2A, CDK4, CTNNB1, CYSLTR2, EIF1AX, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MYC, NRAS, PLCB4, PTEN, RAC1, RAF1, RB1, SF3B1, TERT, TP53* and *TYRP1,* optionally wherein the coding mutation is not a *BRAF* V600 mutation and/or a *NRAS* Q61 mutation;

(b) non-coding hotspot mutations in *AP3D1, ARHGEF18, BLCAP, C16ORF59, CDC20, CHCHD2, DHX16, DPH3/OXNAD1, ERGIC3, FTH1, HSBP1, KBTBD8, MRPS31, MRPS33, NFKBIE, NSUN6, PES1, RALY/RP5-1125A11.1, RNF185, RPL13A, RPL18A, RPL29, RPL34, RPS14, RPS27, SLC30A6, SMUG1, SWI5, SYF2, TERT, UBXN8, YAE1D1* and *ZNF778,* optionally wherein the non-coding mutation is not within the promoter region of *TERT*;

(c) copy number alterations in *BAP1, BRAF, CCND1, CDKN2A, CDK4, CTNNB1, CYSLTR2, GNA11, GNAQ, HRAS, KIT, KRAS, MAP2K1, MAP2K2, MDM2, MYC, NRAS, PTEN RAC1, RAF1, RB1, TERT* and *TP53;* and

(d) promoter regions mutations in *TERT*,

or any combination thereof.

58. The method of claim 57, wherein the method detects at least 3 genes with coding mutations from list (a), optionally at least 5 genes with non-coding mutations from list (b) and at least 3 genes with copy alteration mutations from list (c).

59. A method of treating an individual that has, or is likely to have, melanoma, the method comprising:

- selecting an individual having a lesion that has been genetically tested in accordance with the method of any one of claims 1 to 58, wherein the gene alterations identified in that method indicate melanoma,

- providing wide local excision or resection of the lesion.

60. A method of treating an individual that has, or is likely to have, melanocytic naevus, the method comprising:

- selecting an individual having a lesion that has been genetically tested in accordance with the method of any one of claims 1 to 58, wherein the gene alterations (or lack thereof) identified in that method indicate melanocytic naevus,

- providing intralesional or marginal excision of the lesion.

61. The method of claim 60, wherein the excision is elective, pre-emptive and/or undertaken for cosmetic reasons.

62. A method of treating melanocytic lesions, the method comprising:

- selecting at least a first melanocytic lesion that has been genetically tested in accordance with any one of claims 1 to 58, wherein the gene alterations indicate melanoma, and
- selecting at least a second melanocytic lesion that has been genetically tested in accordance with any one of claims 1 to 58, wherein the gene alterations (or lack thereof) indicate melanocytic naevus;
- treating the first lesion with wide local excision or resection of the melanoma, and

either not treating the second lesion or treating the second lesion with intralesional or marginal excision of the naevus.

63. A method of treating a melanocytic lesion, the method comprising:

- selecting a lesion that has been genetically tested in accordance with the method of any one of claims 1 to 58;
- if the gene alterations identified in that method indicate the lesion is melanoma, providing wide local excision or resection of the lesion;

- if the gene alterations (or lack thereof) identified in that method indicate the lesion is melanocytic naevus; providing intralesional or marginal excision of the lesion.

64. The method of any one of claims 7 to 9, 16 to 18, 25 to 31, 34, 36 to 39 and 43 to 63, wherein the copy number alterations are copy number amplifications.

65. The method of any one of claims 1 to 64, wherein the sample is a skin sample, preferably a skin lesion sample, more preferably a melanocytic lesion sample.

66. The method of any one of claims 1 to 65, wherein the individual is human or the lesion is a human lesion.

67. The method of any one of claims 1 to 66, wherein the lesion has been previously diagnosed as a melanocytic lesion, a melanoma or a melanocytic naevus, preferably wherein the diagnosis was performed on the same skin sample or another sample from the same lesion.

68. The method of any one of claims 1 to 66, wherein the lesion has been previously diagnosed as a borderline or indeterminate melanocytic lesion, preferably wherein the diagnosis was performed on the same skin sample or another sample from the same lesion.

69. The method of any one of claims 1 to 68, wherein the method is an *in vitro* method.

70. The method of any one of claims 1 to 69, wherein the sample is collected by biopsy.

71. The method of any one of claims 1 to 70, wherein the melanocytic lesion, melanoma or melanocytic naevus is a superficial spreading melanoma, a nodular melanoma, a lentigo maligna melanoma, a desmoplastic melanoma, a mitotically active naevus in pregnancy (MANP) or a naevoid melanoma.

72. The method of any one of claims 1 to 70, wherein the melanocytic lesion, melanoma or melanocytic naevus is not a BAP1-deficient tumour, a uveal melanoma, a blue naevus, a melanoma arising from blue naevus, a Spitz naevus, a Spitz melanoma or a pigmented epithelioid melanocytoma.

73. The method of any one of claims 1 to 72, wherein the melanocytic lesion, melanoma or melanocytic naevus is negative for one or more of PRAME, 6p25 (RREB1), 6q23 (MYB), Cep6 (centromere 6), 11q13 (CCND1), 9p21 (CDKN2A) and 8q24 (c-MYC).

74. The method of any one of claims 1 to 73, wherein the melanocytic lesion, melanoma or melanocytic naevus is positive for p16.

75. A kit, panel or microarray comprising reagents that detect gene alterations according to the method of any one of claims 1 to 74.