(45) Date of publication and mention of the grant of the patent: 27.03.2019 Bulletin 2019/13

(21) Application number: 16729800.9

(22) Date of filing: 26.05.2016

(54) METHOD FOR IDENTIFYING SUBJECTS WITH AGGRESSIVE MELANOMA SKIN CANCER AT DIAGNOSIS

VERFAHREN ZUR IDENTIFIZIERUNG VON SUBJEKTEN MIT AGGRESSIVEM MELANOM-HAUTKREBS BEI DER DIAGNOSE

PROCÉDÉ POUR IDENTIFIER DES SUJETS ATTEINTS DE MÉLANOME AGRESSIF LORS D’UN DIAGNOSTIC DU CANCER DE LA PEAU

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR

(30) Priority: 29.05.2015 EP 15169951

(43) Date of publication of application: 11.04.2018 Bulletin 2018/15

(73) Proprietors:
• Universiteit Maastricht
6211 LK Maastricht (NL)
• Academisch Ziekenhuis Maastricht
6229 HX Maastricht (NL)

(72) Inventors:
• VAN ENGELAND, Manon
6114 EE Susteren (NL)
• VAN NESTE, Leander Pieter Jo
3320 Hoegaarden (BE)
• VAN DEN HURK, Karin
2182 JJ Hillegom (NL)

(72) Inventors:
• VAN ENGELAND, Manon
6114 EE Susteren (NL)
• VAN NESTE, Leander Pieter Jo
3320 Hoegaarden (BE)
• VAN DEN HURK, Karin
2182 JJ Hillegom (NL)

(74) Representative: Life Science Patents B.V.
Oxfordlaan 55
6229 EV Maastricht (NL)

(56) References cited:
WO-A1-2013/135830
WO-A2-2012/037128


Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).
Description

Field of the invention

[0001] The invention is in the field of molecular biology and medical diagnosis. It provides means and methods for determining the prognosis and disease outcome of a subject having a melanoma.

Background of the invention

[0002] Cutaneous melanoma is a lethal skin tumor with continuously rising incidence, resulting in a growing healthcare burden [1, 2]. Worldwide, roughly 232,000 new cases and 55,000 deaths were reported in 2012 [3]. Patients diagnosed with localized disease have a five-year survival rate of more than 95% after treatment by surgical excision alone [4]. If the cancer is more advanced, however, survival rates drop substantially, i.e. 30% to 60% after five years, primarily depending on the tumor thickness, i.e. Breslow’s depth.

[0003] Metastatic disease generally leads to poor patient outcomes, as treatment options were limited for a long time. However, rapid development of next-generation sequencing technologies has identified most genetic alterations and molecular pathways involved in melanoma development and provided the basis for novel targeted therapies [5]. Moreover, novel immunomodulatory therapies are successfully being developed for melanoma treatment [6].

[0004] Currently, the American Joint Committee on Cancer (AJCC) classifies patients predominantly based on histological features of the primary tumor, i.e. Breslow thickness, ulceration, and mitotic rate, and indicates that the initial biopsy is a critical component of both diagnosis and staging [7]. In addition, the presence of advanced disease stage (stage III/IV) and, to a lesser extent, patient age, gender, and tumor location, are prognostic melanoma factors. Breslow thickness is viewed as the most important prognostic parameter, however, 20-30% of patients diagnosed with thin melanomas (<2.0mm thickness) still die from their disease [8, 9]. Hence, improvements to the current staging system that lead to more accurate prediction of prognosis are warranted, allowing clinicians to better address prognosis of individual patients. Moreover, it is of importance to identify high-risk patients with aggressive disease at an early stage as these patients may benefit from more extensive surgery, adjuvant therapy, and closer follow-up.

[0005] In summary, cutaneous melanoma is a highly aggressive skin cancer that accounts for approximately 75% of skin cancer-related deaths. Biomarkers for melanoma based on the analysis of differential methylation have been described in the prior art [55-57]. Differential methylation of the LY75 gene has been linked to cardiac disease [58]. LY75 has been described as melanoma treatment target [59]. Despite an increased understanding of the biology of melanoma development and the identification of molecular alterations that accompany melanoma progression [10, 30], the AJCC melanoma staging and classification system has not yet incorporated potentially molecular changes [7]. However, improvements to the current staging system are necessary to more accurately identify individual patients with aggressive disease at diagnosis. These patients with a so-called poor prognosis might benefit from additional therapy leading to improved clinical management and better patient outcome.

Summary of the invention.

[0006] We found that methylation of the promoter of lymphocyte antigen 75 (LY75), also known as CD-205 or DEC-205, is a strong marker that predicts poor clinical outcome, independent of the currently used prognosticators in an independent melanoma series. The invention therefore relates to a method for determining whether a subject having a melanoma has a poor prognosis, the method comprising the step of determining whether the LY75 promoter is methylated and if the LY75 promoter is methylated, classifying the subject as having a poor prognosis.

Detailed description of the invention.

[0007] We examined the methylation status of the lymphocyte antigen 75 (LY75) promoter in a well-characterized series of 123 primary melanomas with follow-up data. We found that LY75 promoter methylation (HR=4.442; 95%-CI 2.307-8.553, P<.001) together with ulceration (HR=2.262; 95%-CI 1.164-4.396, P=.016), and metastatic disease at diagnosis (HR=5.069; 95%-CI 2.489-10.325, P<.001) were significant predictors of melanoma survival.

[0008] LY75, also known as CD-205 or DEC-205, is a collagen-binding mannose family receptor that is predominantly expressed on thymic cortical epithelium and myeloid dendritic cell subsets [37]. LY75 has been reported to play a role in the endocytic uptake of antigen leading to both CD4+ and CD8+ T-cell response [37-39].

[0009] LY75 is Ensemble gene ID ENSG00000054219, situated at chromosome 2, with gene description Lymphocyte antigen 75 Precursor (DEC-205)(gp200-MR6)(CD205 antigen).

[0010] LY75 gene promoter was analyzed for its methylation status in melanoma cell lines and normal human epidermal
melanocytes (NHEM), and in 20 primary melanoma samples and 20 common nevus samples. It was found that the promoter was methylated in 6 out of 6 melanoma cell lines, not in the NHEM cells, in 35% of the pilot melanomas and in 0% of the pilot nevi samples. 

LY75 promoter methylation was identified as a strong predictor of poor melanoma prognosis and identified patients with aggressive disease at diagnosis independent of current prognostic parameters. LY75 promoter methylation is therefore an important aid in the identification of patients who require more extensive surgery, adjuvant treatment, and closer follow-up which then leads to improved clinical outcome. 

The invention therefore relates to a method for determining whether a subject having a melanoma has a poor prognosis, the method comprising a step of determining in a sample from the subject whether the LY75 promoter is methylated and if the LY75 promoter is methylated, classifying the subject as having a poor prognosis. 

In the patient series examined herein, higher Breslow thickness, presence of ulceration, and presence of metastatic disease at diagnosis were the main prognostic indicators (P<.001, Table 4; Kaplan-Meier survival curves depicted in Figure 1A, B, and C, respectively). 

Additionally, a higher age at diagnosis, presence of tumor mitoses, and location on the head and neck were significant predictors of poor prognosis (Table 3), indicating that this series was suitable to study the additional prognostic value of methylation markers as it reflected the overall disease progression in a similar tendency as the general population. 

The term "poor prognosis" is used herein to indicate that patients with a methylated LY75 promoter may be expected or be predicted to have a shorter life expectancy than those having an unmethylated LY75 promoter.
Table 4. Associations of clinicopathological characteristics and methylation marker LY75 with melanoma-specific survival.

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>Multivariate analysis*</th>
<th>Final**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>P-value</td>
</tr>
<tr>
<td><strong>Current prognostic markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>1.675</td>
<td>0.900-3.116</td>
<td>.103</td>
</tr>
<tr>
<td>Age (continuous)</td>
<td>1.026</td>
<td>1.004-1.050</td>
<td>.023</td>
</tr>
<tr>
<td>Location (head/neck vs other)</td>
<td>2.875</td>
<td>1.451-5.697</td>
<td>.002</td>
</tr>
<tr>
<td>Breslow thickness (continuous)</td>
<td>1.175</td>
<td>1.114-1.239</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Ulceration (yes vs no)</td>
<td>3.355</td>
<td>1.827-6.160</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Disease stage (stage III/IV vs stage I/II)</td>
<td>6.498</td>
<td>3.372-12.520</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Mitoses (&gt;1mm² vs &lt;1mm²)***</td>
<td>30.835</td>
<td>1.405-676.773</td>
<td>.030</td>
</tr>
</tbody>
</table>

**Methylation marker; methylated vs un methylated (% methylation)**


*Multivariate Cox proportional hazards regression analyses were adjusted for gender, age, location, Breslow thickness, ulceration, and disease stage. **Final analyses were the result of backward stepwise elimination on a saturated multivariate Cox proportional hazards regression model with the current prognostic makers (except for tumor mitoses) and methylation markers as covariates.

***The wide 95% confidence interval for mitotic rate can be explained by the fact that none of the patients died of melanoma when mitoses were absent, as a result the statistical analysis was problematic by the presence of a value equal to zero. Additionally, the AJCC staging system [7] uses the presence of mitoses only to categorize T1 melanoma (<1.0mm thickness). For these reasons we left out tumor mitoses in multivariate analysis.
Tumor ulceration and metastatic disease at diagnosis were, together with LY75 methylation, the most significant predictors of survival. Using Akaike Information Criterion (AIC) showed that LY75 methylation as single methylation marker always outperformed this panel.

Since melanoma survival largely depends on the formation of lethal metastases we wondered if LY75 methylation was a significant predictor of distant metastases formation in disease stage I and II patients. Of 95 stage I and II patients with known methylation status, a total of 26 patients developed metastatic disease. Using univariate analysis we observed a strong prognostic value of LY75 methylation to predict distant metastasis formation, HR=7.835, 95%-CI 3.554-17.274, P<.001. Applying multivariate analyses, promoter methylation of LY75 (HRLY75=7.924, 95%-CI 3.492-17.980, P<.001) remained the best predictor of metastatic disease development together with ulceration (HRulceration=3.477, 95%-CI 1.579-7.655, P=.002) and age (HRage=1.040, 95%-CI 1.009-1.072, P=.011).

To validate the observed association of LY75 methylation with distant metastasis formation we evaluated the the Cancer Genome Data Atlas (TCGA) dataset. This dataset provides clinical follow-up data for 44 primary melanomas of which 14 patients did recur. (https://tcga-data.nci.nih.gov/tcga/)

For analysis, a single representative probe region was selected (cg24478096; wherein probe A: AAACAACAAAACATAACATCAAAACCCCAACAAACTACAAAACTACA (SEQ ID NO: 7), and probe B: AAACAACAAACTATAACGTCAAAACACGACTAAGAATACG (SEQ ID NO: 8) were employed. Probe A detects unmethylated alleles and probe B detects methylated alleles.

As a cut-off value, a normalized $\beta$-value of 0.2 was chosen, wherein $\beta$ is equal to the intensities of the A and B probes according to the equation: B/(A+B). In other words, if the intensity of the B probe was more than 20% of the total intensity of probes A and B together, the sample was scored as having a methylated LY75 promoter. Samples with $\beta$-value >0.2 were thus scored as methylated and samples with $\beta$-value below or at 0.2 were scored as unmethylated.

It goes without saying that other cut-off values may advantageously employed. Depending on the desired specificity and sensitivity of the method, the cut-off value may be adjusted.

The step of determining whether a subject has a methylated LY75 promoter may thus advantageously include a step of determining whether the level of methylation of the LY75 promoter is above a predetermined reference value or cut-off value. A skilled person may be well aware of ways of obtaining such a predetermined reference value. It may for instance be the value obtained using the same probes and methods as described herein when applied to a normal individual or a panel of normal individuals. It may also be an arbitrarily chosen value or it may be determined by trial and error. A preferred reference value is a beta value of more than 0.2 as determined with a suitable probe set, such as for instance probes A and B according to SEQ ID NO: 7 and SEQ ID NO: 8 respectively.

Kaplan-Meier survival analysis showed that primary melanomas that were methylated had a significantly higher risk to develop metastatic disease (log-rank P=.020).

Backward stepwise elimination on a saturated multivariate Cox proportional hazards regression model with disease stage, Breslow thickness, tumor ulceration, gender, and age as covariates revealed that LY75 methylation was the best single marker to predict recurrence in this series (HRLY75=3.568, 95%-CI 1.142-11.149, P=.029).

Among stage I and II melanoma patients, LY75 methylation was the strongest predictor (HR=7.924, 95%-CI 3.492-17.980, P<.001) of distant metastasis development, together with tumor ulceration (HR=3.477, 95%-CI 1.579-7.655, P=.002) and older age at diagnosis (HR=1.040, 95%-CI 1.009-1.072, P=.011). LY75 methylation outperformed Breslow thickness, the most important clinical prognostic parameter. This is especially of interest since the incidence of patients diagnosed with thin melanoma has been rising and an increasing proportion of melanoma-related deaths occur among these patients [8, 9, 36]. Thus, notwithstanding their generally favorable prognosis, thin melanomas contribute considerably (approximately 25%) to melanoma mortality. The identification of a strong relationship between LY75 methylation and poor prognosis that was irrespective of Breslow thickness provides a promising lead to identify high-risk patients, that are not detected by traditional risk factors, who might benefit from adjuvant therapy and closer follow-up.

Without wanting to be bound by theory, we hypothesize that the poor clinical outcome of patients with LY75-methylated tumors might be partly explained by poor immune recognition, although we could not observe a significant association of methylation with the absence of tumor-infiltrating lymphocytes (TILs) in our series (data not shown). However, it might also be that the TILs are functionally defective or incompletely activated as is commonly seen during tumor progression [40]. Melanoma is increasingly treated with immunomodulatory therapies, such as anti-CTLA4, and anti-PD1 antibodies that exploit the capacity of CD8+ T-cells to kill immunogenic melanoma cells [41, 42]. Although responses can be durable, the response rate to these therapies is generally low (roughly 25% of patients) [6] and biomarkers predicting response are thus far lacking [43]. It is therefore conceivable that LY75 methylation might reflect poor response to immune therapies as well.

In summary, LY75 methylation was recognized as a strong, independent predictor of poor prognosis, both in predicting melanoma-specific death and predicting the formation of distant metastases in stage I and II melanoma patients.
Legend to the figures.

Figure 1: Kaplan-Meier survival curves of the best prognostic markers.

[A0028] (A) Kaplan-Meier curve of melanoma-specific survival of patients grouped according to primary tumor thickness divided by AJCC tumor stage. (B) Kaplan-Meier curve of melanoma-specific survival of patients grouped according to the presence or absence of tumor ulceration. (C) Kaplan-Meier curve of melanoma-specific survival of patients grouped according to localized disease (Stage I/II) and metastatic disease (Stage III/IV). (D) Kaplan-Meier curve of melanoma-specific survival of patients grouped according to LY75 promoter methylation.

Figure 2: Kaplan-Meier survival curve according to LY75 methylated promoter status

[A0029] Kaplan-Meier survival curve of recurrence-free survival of TCGA patients grouped according to LY75 methylation status at probe cg24478096.

Examples

Example 1: Cell culture and MBD-sequencing

[A0030] Methyl-binding domain (MBD)-sequencing was performed on six melanoma cell lines (WM35, WM3248, WM164, A375, M14, SK-MEL-28) and normal human epidermal melanocytes (NHEM) provided by Dr. Leon van Kempen (McGill University, Montreal, Canada). Authentication of all cell lines was performed using short tandem repeat (STR) profiling (DSMZ, Braunschweig, Germany). WM cell lines were cultured in W489 medium consisting of four parts of MCDB153 (Sigma-Aldrich, Zwijndrecht, The Netherlands) and one part of L15 (Sigma-Aldrich, Zwijndrecht, The Netherlands), A375, M14, and SK-MEL-28 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Breda, the Netherlands). Cells were supplemented with 2% or 10% heat inactivated fetal calf serum (HyClone Perbio Science, Erembodegem-Aalst, Belgium), respectively. NHEM cells were cultured in ready-to-use medium supplied by Promocell (Heidelberg, Germany). Genomic DNA was isolated using the Puregene® DNA isolation kit (Gentra systems, Minneapolis, MN) according to the manufacturer’s instructions.

Example 2: Total RNA sequencing

[A0032] RNA-sequencing on WM35, WM3248, M14, and SK-MEL-28 cells was performed to determine functional methylation, i.e. methylation associated with downregulated gene expression [26]. In brief, total ribonucleic acid (RNA) was isolated using the standard procedure for TRIzol ® RNA extraction (Invitrogen, Bleiswijk, The Netherlands) and stored at -80°C. For total RNA sequencing library preparation was carried out using a modified version of the Illumina 'Directional mRNA-sequencing Sample Preparation' protocol with total RNA instead of mRNA. Ribosomal DNA was depleted from the DNA fraction using Illumina’s Duplex-Specific Thermostable Nuclease normalization protocol for bidirectional mRNA sequencing (application note 15014673).

Example 3: Infinium-450K data

[A0033] Since The Cancer Genome Atlas (TCGA) has no methylation data available on control samples we performed Infinium-450K assays on 14 fresh-frozen nevi, collected from the archives of the University Hospital of Leuven, Belgium, to be able to determine melanoma-specific methylation (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Samples used for methylation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Common nevi used for Infinium-450K analyses (n=14)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
</tbody>
</table>
Genomic DNA from the 14 nevus samples was extracted as described previously [27]. DNA quantification was performed using a Qubit 2.0 plate reader (Invitrogen, Bleiswijk, The Netherlands) and PicoGreen dye (Invitrogen, Bleiswijk, The Netherlands). DNA quality was inspected on agarose gels stained with SYBR ® Safe (Invitrogen, Bleiswijk, The Netherlands). Bisulfite conversion of DNA samples was carried out using the EZ DNA methylation kit (Zymo Research, Orange, CA) and converted DNA was hybridized on Infinium-450K BeadChips, following the Illumina Infinium HD Methylation protocol as described elsewhere [28].

Example 4: Patient samples.

LY75 Promoter CpG island methylation was examined in a well-characterized series of formalin-fixed, paraffin-embedded (FFPE) common nevi (n=20) and primary melanomas (n=123) of patients diagnosed at the Maastricht University Medical Centre, The Netherlands and University Hospital Leuven, Belgium. Collection, storage and use of all tissues and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands". All of the used samples and corresponding data were de-linked and anonymized. Usage of both melanoma and healthy tissue samples was approved by the Maastricht Pathology Tissue Collection (MPTC) scientific committee. Detailed clinicopathological information of melanoma samples is shown in Table 2, characteristics of nevus samples are listed in Table 1.

Table 1. Samples used for methylation analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of Patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common nevi used for Infinium-450K analyses (n=14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.6±24.</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Trunk</td>
<td>8</td>
<td>57</td>
</tr>
<tr>
<td>Extremities</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Common nevi used for validation with MSP (n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31.7±14.3</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Trunk</td>
<td>11</td>
<td>57</td>
</tr>
<tr>
<td>Extremities</td>
<td>5</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 2. Clinicopathological characteristics of 123 primary melanoma cases with follow-up

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of Patients*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>82</td>
<td>67</td>
</tr>
<tr>
<td>Male</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>59.9 ± 16.9</td>
<td></td>
</tr>
<tr>
<td>≤50 years</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>Characteristics</td>
<td>No. of Patients*</td>
<td>%</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>------------------</td>
<td>----</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>86</td>
<td>70</td>
</tr>
<tr>
<td><strong>Disease stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized - Stage I/II</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>Metastasized - Stage III/IV</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td><strong>Breslow thickness, mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01-1.0</td>
<td>32</td>
<td>26</td>
</tr>
<tr>
<td>1.01-2.0</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>2.01-4.0</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>&gt; 4.0</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td><strong>Ulceration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>86</td>
<td>70</td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td><strong>Mitotic Rate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1/mm²</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>≥1/mm²</td>
<td>99</td>
<td>81</td>
</tr>
<tr>
<td><strong>TILs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>Non-brisk</td>
<td>69</td>
<td>57</td>
</tr>
<tr>
<td>Brisk</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>85</td>
<td>73</td>
</tr>
<tr>
<td>NM</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>LMM</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>ALM</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Trunk</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>Extremities</td>
<td>67</td>
<td>55</td>
</tr>
<tr>
<td><strong>Distant metastasis formation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td>Yes</td>
<td>43</td>
<td>35</td>
</tr>
<tr>
<td><strong>Disease-related death</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>81</td>
<td>66</td>
</tr>
<tr>
<td>Yes</td>
<td>42</td>
<td>34</td>
</tr>
<tr>
<td><strong>Mean follow-up (months)</strong></td>
<td>75.3 ± 57.0</td>
<td></td>
</tr>
</tbody>
</table>

ALM, acral lentigious melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; TILs, tumor infiltrating lymphocytes
Example 5: DNA isolation, bisulfite conversion, and promoter CpG island methylation analyses.

[0036] A 4-μm section of each FFPE tissue block was stained with haematoxylin & eosin (H&E) and reviewed by an experienced dermatopathologist. Cases that contained >50% nevus or melanoma cells were included. Subsequently, ten sections of 10μm were cut and another H&E section was made to confirm the percentage of nevus and melanoma cells. Next, slides were deparaffinised and DNA was extracted following macro dissection with the QIAamp DNA Micro Kit (Qiagen, Venlo, The Netherlands). NanoDrop quantification was used to estimate the quality and concentration of extracted DNA (NanoDrop ND-1000 Spectrophotometer). Sodium bisulphite modification of 500ng genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions.

[0037] Following bisulfite conversion, nested, multiplex methylation-specific polymerase chain reaction (MSP) analyses were performed as described elsewhere [19]. Primer sequences and conditions are shown in Table 3. The number of PCR cycles performed was 30.

[0038] PCR conditions were as follows: The PCR mixture contains 1 x PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl2/10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 50 μl. Reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of Taq polymerase (BRL). Amplification was carried out in a thermocycler for 30 cycles (30 sec at 95°C, 30 sec at the annealing temperature of 64°C, and 30 sec at 72°C), followed by a final 4-min extension at 72°C. The flanking PCR was carried at 56°C for 35 cycles.

[0039] PCR reactions were performed with controls for unmethylated alleles (for example unmethylated human control DNA, EpiTect Control DNA, Qiagen, Cat. no. 59568), methylated alleles (normal lymphocyte DNA treated in vitro with Sssl methyltransferase [New England Biolabs]), and a no-template DNA control.

[0040] Ten μl of each MSP reaction was loaded onto 2% agarose gels containing GelStar Nucleic Acid Gel Stain (Cambrex, New Jersey, USA), and visualized under UV illumination. The presence of a PCR product performed with the methylated primers indicates the presence of methylated DNA and predicts a poor prognosis of the melanoma patient.

[0041] Nested MSP reactions were performed with controls for unmethylated alleles (unmethylated human control DNA, EpiTect Control DNA, Qiagen, Cat. no. 59568), methylated alleles (normal lymphocyte DNA treated in vitro with Sssl methyltransferase [New England Biolabs]), and a no-template DNA control.

[0042] To ensure reproducibility, MSP reactions were performed in duplicate starting from DNA amplification with flanking primers. Discordant results were analyzed a third time, and the majority vote principle was used to determine the methylation status.

Example 6: Statistical analyses

[0043] Cox proportional hazards regression was used to evaluate the effect of gene methylation and clinicopathological variables on melanoma-specific survival, resulting in hazard ratios (HRs) and their corresponding 95% confidence intervals (95%-CI). For LY75 methylation, the Cox proportional hazards model was used to evaluate the effect of methylation on distant metastasis formation (melanoma-free survival).

[0044] Survival time was defined as the time between first diagnosis and the first date of diagnosis of a distant metastasis. Akaike Information Criterion (AIC) was used to assess the predictive capacity of models with single and multiple methylation markers. The model with the lowest AIC was chosen as the best model. All reported P-values were two-sided, and P<.05 was considered statistically significant. Analyses were performed using the statistical package IBM SPSS Statistics 21 (IBM, New York, USA) and R (R Foundation for Statistical Computing, Vienna, Austria).
References

27. van den Hurk K, Balint, B., Toomey S., O'Leary, P.C., Unwin, L., Sheahan, K., McDermott, E.W., Murphy, I.,


SEQUENCE LISTING

[0046]

<110> Universiteit Maastricht and Academisch Ziekenhuis Maastricht

<120> METHOD FOR IDENTIFYING SUBJECTS WITH AGGRESSIVE MELANOMA SKIN CANCER AT DIAGNO- SIS

<130> 309 WO

<160> 8

<170> PatentIn version 3.5

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 1
ttaggatgag gataggttgg g 21

<210> 2
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 2
ggataggttg ggtgattttt tgtt 24

<210> 3
<211> 19
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 3
ggttggcga ttttcgtc 19

<210> 4
<211> 27
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 4
caactaaaa aacaacaaaa ctataac 27

<210> 5
<211> 26
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 5
aaactataac atcaaaacac ccaaca 26

<210> 6
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 6
tataacgtcg aaacacccaa cg 22

<210> 7
<211> 50
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 7
aaacaacaaa actataacat caaaacacc aacaaactac aaactaaca 50

<210> 8
<211> 50
<212> DNA
<213> artificial sequence

<220>
<223> Probes and primers for detection of methylated LY75 promoter

<400> 8
aaacaacaaa actataacgt caaaacacc aacgaactac gaaactaacg 50

Claims

1. Method for determining whether a subject having a melanoma has a poor prognosis, the method comprising the step of determining in a sample from the subject whether the LY75 promoter is methylated and if the LY75 promoter
is methylated, classifying the subject as having a poor prognosis, wherein the sample is a melanoma sample.

2. Method according to claim 1 wherein the sample is obtained from a biopsy taken from the subject.

3. Method according to claims 1 or 2 wherein an additional step is performed selected from the group consisting of determining tumor ulceration, determining metastatic disease at diagnosis, disease stage, and Breslow thickness of the melanoma.

4. Method according to any one of claims 1 - 3 wherein the step of determining in a sample from the subject whether the LY75 promoter is methylated is performed by multiplex methylation-specific polymerase chain reaction analysis.

5. Method according to claim 4 wherein the multiplex methylation-specific polymerase chain reaction is a nested multiplex methylation-specific polymerase chain reaction.

Patentansprüche

1. Verfahren zum Bestimmen, ob für eine ein Melanom aufweisende Testperson eine schlechte Prognose besteht, wobei das Verfahren den Schritt des Bestimmens umfasst, ob der Promotor LY75 in einer von der Testperson stammenden Probe methyliert ist, und, wenn der Promotor LY75 methyliert ist, das Einstufen der Testperson umfasst, dass für sie eine schlechte Prognose besteht, wobei es sich bei der Probe um eine Melanomprobe handelt.

2. Verfahren nach Anspruch 1, wobei die Probe aus einer bei der Testperson durchgeführten Biopsie stammt.


5. Verfahren nach Anspruch 4, wobei es sich bei der mehrfachen methylierungsspezifischen Polymerase-Kettenreaktion um eine verschachtelte mehrfache methylierungsspezifische Polymerase-Kettenreaktion handelt.

Revendications

1. Procédé pour déterminer si un sujet atteint d’un mélanome a un mauvais pronostic, le procédé comprenant l’étape consistant à déterminer dans un échantillon provenant du sujet si le promoteur de LY75 est méthylé et si le promoteur de LY75 est méthylé, à classer le sujet comme ayant un mauvais pronostic, où l’échantillon est un échantillon de mélanome.

2. Procédé selon la revendication 1, dans lequel l’échantillon est obtenu à partir d’une biopsie prélevée sur le sujet.

3. Procédé selon la revendication 1 ou 2, dans lequel une étape supplémentaire est réalisée qui est choisie dans le groupe constitué de la détermination d’une ulceration tumorale, de la détermination d’une maladie métastatique au moment du diagnostic, du stade de la maladie et de l’épaisseur de Breslow du mélanome.

4. Procédé selon l’une quelconque des revendications 1 à 3, dans lequel l’étape consistant à déterminer dans un échantillon provenant du sujet si le promoteur de LY75 est méthylé est réalisée par une analyse par réaction en chaîne par po-lymérase spécifique de la méthylation multiplexe.

5. Procédé selon la revendication 4, dans lequel la réaction en chaîne par polymérase spécifique de la méthylation multiplexe est une réaction en chaîne par po-lymérase spécifique de la méthylation multiplexe nichée.
Figure 1A

A. Breslow thickness

Number at risk

<table>
<thead>
<tr>
<th>T1 (≤1.0 mm)</th>
<th>32</th>
<th>25</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (1.0-2.0 mm)</td>
<td>29</td>
<td>19</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>T1 (2.0-4.0 mm)</td>
<td>29</td>
<td>19</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>T1 (&gt;4.0 mm)</td>
<td>33</td>
<td>11</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>
B. Ulceration status

Cumulative survival

Time (months)

Number at risk

<table>
<thead>
<tr>
<th>Status</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>86</td>
<td>59</td>
<td>28</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
C.

*Figure 1C*

**Disease stage**

![Graph showing disease stage over time](image)

**Number at risk**

<table>
<thead>
<tr>
<th>Stage</th>
<th>103</th>
<th>71</th>
<th>33</th>
<th>19</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I/II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>20</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1D

D. 

*LY75* methylation status

![Kaplan-Meier curve for *LY75* methylation status](image)

**Cumulative survival**

**Time (months)**

**Number at risk**

<table>
<thead>
<tr>
<th></th>
<th>Unmethylated</th>
<th>Methylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>58</td>
<td>29</td>
</tr>
<tr>
<td>29</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

**LY75 methylation status TCGA**

Cumulative survival

Time (months)

Number at risk

<table>
<thead>
<tr>
<th></th>
<th>25</th>
<th>21</th>
<th>5</th>
<th>1</th>
<th>1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>19</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader’s convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2012037128 A [0045]
- WO 2013135830 A [0045]
- WO 2015052537 A [0045]

Non-patent literature cited in the description

- FERLAY J; SOERJOMATARAM I; ERLIK M; DIKSHIT R; ESHER S; MATHERS C; REBELO M; PARKIN DM; FORMAN D; BRAY, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase. International Agency for Research on Cancer, 2013 [0045]
-WHITEMAN DC; BAÄDE PD; OLSEN CM. More People Die from Thin Melanomas (1 mm) than from Thick Melanomas (>4 mm) in Queensland. J Invest Dermatol, 2014 [0045]
- THOMAS NE; SLATER NA; EDMISTON SN et al. DNA methylation profiles in primary cutaneous melanomas are associated with clinically significant pathologic features. Pigment Cell Melanoma Res, 2014, vol. 27 (6), 1097-1105 [0045]
• LI L; YING J; LI H et al. The human cadherin 11 is a pro-apoptotic tumor suppressor modulating cell stemness through Wnt/beta-catenin signaling and silenced in common carcinomas. Oncogene, 2012, vol. 31 (34), 3901-3912 [0045]

• SONG YH; SHIOTA M; KUROIWA K et al. The important role of glycine N-methyltransferase in the carcinogenesis and progression of prostate cancer. Mod Pathol, 2011, vol. 24 (9), 1272-1280 [0045]

• HUANG YC; CHEN M; SHYR YM et al. Glycine N-methyltransferase is a favorable prognostic marker for human cholangiocarcinoma. J Gastroenterol Hepatol, 2008, vol. 23 (9), 1384-1389 [0045]


• VAN VLODROP IJ; NIJSENN HE; DERKS S et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics, 2011, vol. 6 (6), 692-702 [0045]


• COLABROY KL; ZHAHI H; LI T et al. The mechanism of inactivation of 3-hydroxyanthranilate-3,4-dioxygenase by 4-chloro-3-hydroxyanthranilate. Biochemistry, 2005, vol. 44 (21), 7823-7831 [0045]


• HUANG YW; LUO J; WENG YI et al. Promoter hypermethylation of CIDEA, HAAO and RXFP3 associated with microsatellite instability in endometrial carcinomas. Gynecol Oncol, 2010, vol. 117 (2), 239-247 [0045]

• WELCH HG; WOLOSHIN S; SCHWARTZLM. Skin biopsy rates and incidence of melanoma: population based ecological study. BMJ, 2005, vol. 331 (7515), 481 [0045]


• BOZZACCO L; TRUMPFHELLER C; SIEGAL FP et al. DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. Proc Natl Acad Sci U S A, 2007, vol. 104 (4), 1289-1294 [0045]


• TUNGEKAR MF; GATTER KC; RITTER MA. Bladder carcinomas and normal urothelium universally express gp200-MR6, a molecule functionally associated with the interleukin 4 receptor (CD 124). Br J Cancer, 1996, vol. 73 (4), 429-432 [0045]

• AL-TUBULY AA; SPUKER R; PIGNATELLI M et al. Inhibition of growth and enhancement of differentiation of colorectal carcinoma cell lines by MAB MR6 and IL-4. Int J Cancer, 1997, vol. 71 (4), 605-611 [0045]


• GIRIDHAR PV; FUNK HM; GALLO CA et al. Interleukin-6 receptor enhances early colonization of the murine omentum by upregulation of a mannose family receptor, LY75, in ovarian tumor cells. Clin Exp Metastasis, 2011, vol. 28 (8), 887-897 [0045]

• CHAPMAN EJ; KELLY G; KNOWLES MA. Genes involved in differentiation, stem cell renewal, and tumorigenesis are modulated in telomerase-immortalized human urothelial cells. Mol Cancer Res, 2008, vol. 6 (7), 1154-1168 [0045]

• HORN S; FIGL A; RACHAKONDA PS et al. TERT promoter mutations in familial and sporadic melanoma. Science, 2013, vol. 339 (6122), 959-961 [0045]


• GRIEWANK KG; MURALI R; PUIG-BUTILLE JA et al. TERT promoter mutation status as an independent prognostic factor in cutaneous melanoma. J Natl Cancer Inst, 2014, vol. 106 (9) [0045]


• DE ARAUJO E. Biomed Research International, 2015, vol. 94 (4), 1115-8 [0045]