Journal of Molecular Biomarkers & Diagnosis

Agnarsdóttir et al., J Mol Biomark Diagn 2012, 3:4
DOI: 10.4172/2155-9929.1000129

Research Article Open Access

MITF Expression in Cutaneous Malignant Melanoma

Margrét Agnarsdóttir[1]*, Fredrik Ponten[1], Hans Garmo[2,3], Gunnar Wagenius[4], Lorelei Mucci[5], Kristina Magnusson[1], Lars Holmberg[2,3] and Sonja Eaker-Fält[3]

[1]*Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-75185 Uppsala, Sweden*
[2]*King's College London, Medical School, Division of Cancer Studies, SE1 9RT London, UK*
[3]*Regional Cancer Centre, Uppsala University Hospital, SE-75185 Uppsala, Sweden*
[4]*Section of Oncology, Department of Oncology and Radiology, Uppsala University, SE-75185 Uppsala, Sweden*
[5]*Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston MA 02115, USA*

Abstract

The MITF protein has a central role in the differentiation and survival of melanocytes. The aim of the study was to investigate whether MITF can be employed as a prognostic marker in patients operated on for cutaneous malignant melanoma. A cohort study design, based on information collected from population-based registries, was employed. For included patients (n=264) tissue microarrays were stained with immunohistochemistry to study the protein expression of MITF in primary malignant melanoma tumors by estimating the fraction of positive tumor cells and the staining intensity. Most of the tumors (84%) expressed MITF in >25% of the tumor cells and for 87% of the tumors the staining intensity was strong. Tumors with cell fraction >75% had a better prognosis compared with those with <75% (HR 0.44, 95% CI: 0.20-1.0, P=0.05). Tumors with a strong staining intensity tended to have a better prognosis compared with the weaker staining ones (HR 0.59, 95% CI: 0.26 -1.36, P=0.22). When cell fraction and intensity were combined, a high-risk group dying of malignant melanoma was identified as those patients with 25-75% of tumor cells staining with weak intensity (HR 2.9, 95% CI: 0.94-8.7, P=0.06) and those with <25% of tumor cells staining with strong intensity (HR 2.5, 95% CI: 1.1- 6.1, P=0.04). However, the majority of deaths occurred in the lower risk groups. In conclusion, a high-risk group for death in malignant melanoma was identified but MITF is not suitable as a prognostic marker due to the distribution of that particular expression in the population.

Keywords: MITF; Immunohistochemistry; Tissue microarray; Prognosis; Survival

Introduction

Microphthalmia associated transcription factor (MITF) protein has a central role in the survival and differentiation of melanoblasts and melanocytes [1-3]. MITF is also involved in the regulation of cell cycle progression and migration [4,5]. The MITF protein is a basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription factor [6] where nine different promoters are known in humans. Alternative splices produce different isoforms where MITF-M is melanocyte specific [7]. It has been demonstrated that alternative splice forms of MITF-M can have different roles [8].

One comprehensive laboratory and clinical study has indicated that amplification of the *MITF* gene is associated with progression of disease and risk of distant metastases [9] but other clinical studies have shown that high protein expression on the contrary may be beneficial [10]. *In vitro* and animal studies implicate a complex pattern where both depletion and forced expression inhibit proliferation in cell lines [11] and high levels of MITF inhibit tumor growth and decrease Ki-67 expression [12].

Immunohistochemistry (IHC) is widely used in routine histopathology and is becoming an increasingly important tool for both stratification of tumors and differential diagnostics [13]. The prognostic value of IHC for predicting the outcome of patients with cutaneous melanoma is lacking and there is no molecular method used in clinical practice that improves risk stratification [14]. Further elucidation of the role of MITF in human malignant melanoma tumors treated in standard practice is of interest, since the central role of MITF in melanoma cell survival and differentiation implicates not only a possible role as a prognostic marker, but also as a therapeutic target.

To investigate if expression of MITF gives an early indication of prognosis in localized, operable malignant melanoma, a population-based large clinical database in Uppsala/Örebro Sweden was sampled. In addition, the distribution of MITF expression in this representative sample of a whole population is described.

Materials and Methods

Setting and sources of information

This study was based on information collected from population-based registries in Sweden and from tumor tissue samples archived at 13 pathology laboratories. Linking of individual information was possible through the national registration number, which is a unique identifier, assigned to each inhabitant in Sweden at birth or immigration.

The clinical database of malignant melanoma in the Uppsala/Örebro health care region and the Regional Cancer Registry: The clinical database includes individual information on all patients in the Uppsala/Örebro health care region diagnosed with cutaneous malignant melanoma from 1996 and onwards. The Uppsala/Örebro health care region consists of seven counties in central Sweden with a total population of over 1,900,000 residents (21.4% of the total Swedish population in 2003). The main purpose of the registry is to contribute to equal health care among patients diagnosed with malignant melanoma and quality assurance of treatment. During the study period 1996-

***Corresponding author:** Margrét Agnarsdóttir, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-75185 Uppsala, Sweden, Tel: +46 18 6113934; Fax: +46 18 553354; E-mail: margret.agnarsdottir@igp.uu.se

Received May 30, 2012; **Accepted** July 12, 2012; **Published** July 17, 2012

Citation: Agnarsdóttir M, Ponten F, Garmo H, Wagenius G, Mucci L, et al. (2012) MITF Expression in Cutaneous Malignant Melanoma. J Mol Biomark Diagn 3:129. doi:10.4172/2155-9929.1000129

2003, coverage of the clinical database was over 99% compared with the Regional Cancer Registry, to which reporting of all new malignancies is mandated by Swedish law [15].

Between 1996 and 2003, information on 3,247 patients diagnosed with cutaneous malignant melanoma was reported to the clinical database by treating clinicians and pathologists. In addition to the national registration number, the following information is reported to the registry: age at diagnosis, gender, diagnosing hospital, date of diagnosis, localization of the primary tumor, tumor thickness, clinical stage (primary tumors only, lymph node metastasis, metastasis in transit, distant metastasis), surgical treatment (primary surgery, extended surgery, type of method used, surgery of the lymph nodes), pathology laboratory and accession number of the tissue sample at the pathology laboratory as well as histopathological information on subtype of melanoma, thickness, ulceration and Clark level.

The Swedish Cause of Death Registry (held by the National Board of Health and Welfare): This nationwide registry contains information from 1961 and onwards on cause of death of all individuals registered as living in Sweden at the time of death; >99% of all deaths are included in the Cause of Death Registry. The cause of death (including underlying and contributing causes) is generally determined from the medical death certificate and coded in accordance with ICD10. In validation studies, the registered underlying cause in the registry has high validity, with approximately 94% of cases being correctly classified on the most detailed level of the ICD-code. The clinical database was matched against the Cause of Death Registry to identify deaths from malignant melanoma and other causes through 2007.

Archived tissue samples

Tissue material from primary tumors of malignant melanoma is archived as glass slides and paraffin tissue blocks at the pathology laboratory collaborating with the treating hospital or clinic. The glass slides and the corresponding paraffin blocks were identified at each laboratory by the unique identification number of the tissue sample as well as the national registration number.

Selection of patients

The selection of study subjects is detailed in (Figure 1). From the clinical database of 3,247 cases of malignant melanoma diagnosed between 1996 and 2003, a random sample of 450 patients was drawn, corresponding to 13.9% of the study base. From the sample, 9 patients were excluded; 8 (1.8%) as they were diagnosed before 1996 according to the Regional Cancer Registry and 1 (0.2%) patient that had been wrongly diagnosed with malignant melanoma. This left us with a cohort of 441 (98%) eligible patients.

Among the eligible patients, 55 (12.5%) had died from malignant melanoma and 386 (87.5%) had not. According to the decision of the Ethical Review Board all patients who were alive after 2004 had to give informed consent to participate in the study. Twelve eligible patients refused to participate, and 32 patients did not respond, leaving a total of 395 of whom 53 died of melanoma.

Collection of archived material

We sought to retrieve archival tumor tissue samples for the 395 patients from the 13 laboratories storing the archived material (Figure 1). For 103 of these cases there was not enough tissue material to construct a tissue microarray (TMA) (see below), either because there was not enough material left in the paraffin tissue block or because the tumor was originally small. In 28 cases the paraffin tissue block could not be found, either because the tissue block was missing (18 cases) or wrong tissue was registered as primary tumor (mostly the tissue registered was a metastasis and not the primary tumor, 10 cases). This left us with a total of 264 patients (of whom 45 died of malignant melanoma) with complete information from the registries and tissue samples.

Generation of tissue microarrays

TMAs were constructed as previously described [16,17]. From each paraffin donor block, 0.6 mm cylinders containing representative tumor tissue were collected and transferred into a recipient block using an automated tissue microarrayer (Beecher Instruments, Silver Springs, MD, USA). If possible three cylinders were collected from the tumors but for tumors with sparse residual tumor tissue only one or two cylinders were sampled.

Immunohistochemistry/ Scoring of immunostainings

A monospecific antibody (HPA003259, provided by Atlas Antibodies, Stockholm, Sweden) [18] generated within the Human Protein Atlas project [19-21] (www.proteinatlas.org) was used to analyze the expression of MITF. The antibody was validated according to standard working procedures within the Human Protein Atlas project including performance in protein arrays, Western blots, immunofluorescence and IHC. Automated IHC (Autostainer, Dako-Cytomation, Glostrup, Denmark) was performed essentially as previously described [22]. The primary antibody, HPA003259, was diluted 1:50.

To validate the antibody further a Western blot on a melanoma cell line (SK-MEL-30) was performed. To show that the MITF antibody specifically stained melanocytic cells with IHC, a double immunohistochemcial staining was performed using the MITF

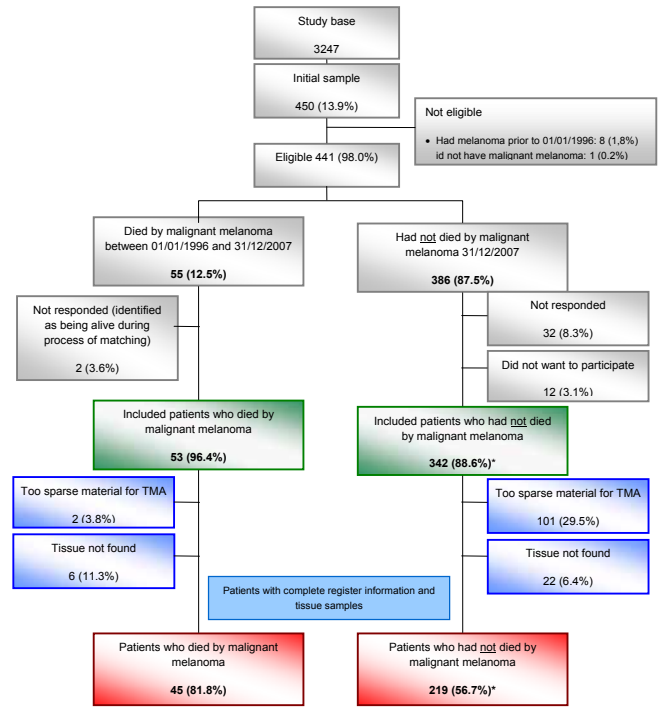

Figure 1: Sampling of patients from the population based registries and collection of paraffin-embedded tissue material. TMA: tissue microarray, * including 84 patients who had died of other causes.

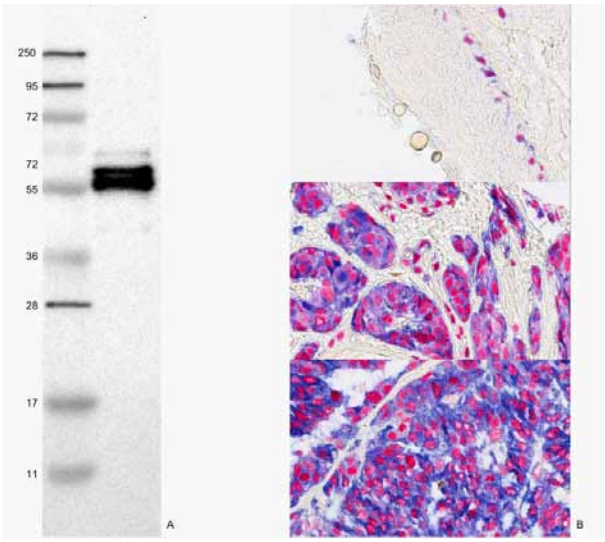

Figure 2: Characterization of the MITF antibody (HPA003259). (A) A Western blot in SK-MEL-30 melanoma cell line demonstrating a doublet 55 and 60 kDa of the expected size where one band is phosphorylated (right lane). The left lane shows the molecular weight markers (250, 130, 95, 72, 55, 36, 28, 17 and 11 kDa) (B) A double immunostaining with Melan-A staining blue in the cytoplasm and MITF staining red in the nucleus. At the top benign melanocytes in the basal layer of normal skin, in the middle a benign melanocytic naevus and at the bottom malignant melanoma. (scale bar = 50µm).

antibody together with another primary mouse monoclonal antibody (diluted 1:100) towards Melan-A (NCL-MelanA, Novocastra) (Figure 2).

The immunohistochemical results were based on nuclear staining of the tumors. The glass slides were scanned to generate corresponding high-resolution images. Aperio ImageScope (Aperio Technologies, Vista, California, USA) was used to visualize images and outcome of immunostaining was subjectively scored essentially as previously described [23]. The histopathological evaluation was done blinded to outcome status. In brief, the extent of staining was measured as the fraction of positive tumor cells and scored using a four-graded scale: >75% of tumor cells staining positively (3 points), 25-75% (2), <25% (1), and negative tumor cells (0). The intensity of immunoreactivity in tumor cells was evaluated using a three-graded scale: strong staining (2), weak (1) or negative (0). These scoring data were used to study the association between fraction of positive tumor cells, intensity of staining or a combination of the two with risk of dying of malignant melanoma. The combination was analyzed in two different ways based on a prior hypothesis: In Alternative 1 some preference for fraction was given priority for ranking the patients and in Alternative 2 intensity was given priority. The pattern of a peaking risk in those with a medium level remained more symmetric when fraction of positive tumor cells was given priority and therefore those results are presented.

Statistical methods

Cox proportional hazard models were used to study the effect of tumor characteristics on cause specific survival. In multivariate analysis we adjusted the analysis for tumor thickness by using tumor thickness in categories with *in situ* tumors in a separate category. Strata without any deaths were excluded in all models. Follow-up time was calculated from date of cancer diagnosis to death or end of follow-up (31st of December 2007) censoring for end of follow-up or death from other causes. The discriminatory capacity of MITF expression was tested using the overall C statistics [24]. All statistics were performed using the statistical program package R [25].

Results

Table 1 show the demographic and tumor characteristics in the cohort by outcome status. The median follow-up was 5.9 years (range: 8 days - 11.8 years). Patients who died from malignant melanoma had thicker tumors, more advanced Clark level and more often presence of ulceration. Only one patient reported dead from malignant melanoma had an *in situ* lesion, whereas a fourth of the survivors had such lesions. Tumor involvement of lymph nodes was considerably more common among those dead from malignant melanoma (Table 1). In 84% (223/264) of the tumors, MITF was expressed in >25% of the tumor cells and for 87% (229/264) of the tumors the staining intensity was strong (Figure 3).

Univariate models

The distribution of the characteristics among patients dead from malignant melanoma and survivors as seen in Table 1 was reflected in the hazard ratios obtained in Cox regression models shown in Table 2. Thus, a thicker tumor, a higher Clark level, presence of ulceration and positive lymph node status were all strongly correlated with risk of death (Table 2). Tumor thickness of 4 mm and over was associated with a 8 times higher risk of death (P<0.001), while presence of ulceration yielded a hazard ratio of 5.8 (P<0.001) and the presence of positive nodes was associated in the non-adjusted models with a 12 times higher risk of death (P<0.001) from malignant melanoma.

Patients with cell fraction >75% had a better prognosis compared with those with <75% (HR 0.39, 95% CI: 0.18-0.85, P=0.02). Those with a strong staining intensity tended to have a better prognosis than those with a weaker staining (HR 0.44, 95% CI: 0.20-0.98, P=0.05) (Table 2). When fraction of positive tumor cells and intensity were combined those with a medium expression (fraction 25-75%, weak intensity) had the highest risk followed by those with a cell-fraction of >75% stained with weak intensity. Their HR was 4.9 (95% CI: 1.7-14, P=0.004) with all the other HRs varying between 1.9 and 2.1 when compared to those with the highest levels of expression.

Multivariate models

To exclude the possibility that the MITF expression was only an indicator of advancement of disease and not an inherent tumor property, the analyses in Table 2 were also adjusted for tumor thickness. Essentially the same pattern was demonstrated with the exception that

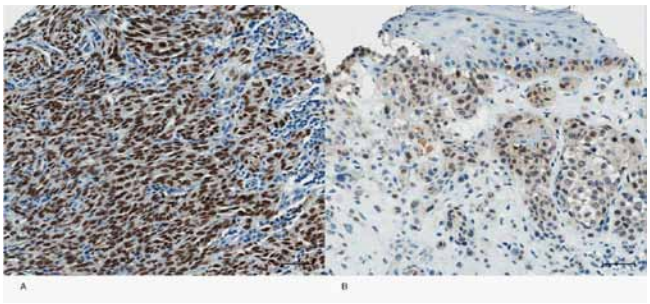

Figure 3: Examples of the protein expression of MITF in two different tumors employing immunohistochemistry. (A) A strong nuclear staining in >25% of the tumor cells. (B) A weak nuclear staining in 25-75% of the tumor cells. (scale bar = 50µm).

	Dead	%	Not-dead	%	All	%
Age group						
20-49	7	15.6	41	18.7	48	18.2
50-64	16	35.6	67	30.6	83	31.4
65-74	9	20.0	49	22.4	58	22.0
75+	13	28.9	62	28.3	75	28.4
Sex						
Male	26	57.8	110	50.2	136	51.5
Female	19	42.2	109	49.8	128	48.5
Year of diagnosis						
1996-1999	23	51.1	92	42.0	115	43.6
2000-2003	22	48.9	127	58.0	149	56.4
Tumor thickness[1]						
0-0.99 mm	5	11.4	42	24.6	47	21.9
1-1.99 mm	4	9.1	51	29.8	55	25.6
2-2.99 mm	7	15.9	24	14.0	31	14.4
3-3.99 mm	6	13.6	13	7.6	19	8.8
4+ mm	19	43.2	29	17.0	48	22.3
Missing data	3	6.8	12	7.0	15	7.0
Clark level						
I	1	2.2	56	25.6	57	21.6
II	1	2.2	30	13.7	31	11.7
III	14	31.1	63	28.8	77	29.2
IV	20	44.4	56	25.6	76	28.8
V	7	15.6	9	4.1	16	6.1
Missing data	2	4.4	5	2.3	7	2.7
Ulceration						
No ulceration	14	31.1	125	57.1	139	52.7
Ulceration	27	60.0	43	19.6	70	26.5
Missing data	4	8.9	51	23.3	55	20.8
Histopathology						
NM	22	48.9	47	21.5	69	26.1
SSM	17	37.8	77	35.2	94	35.6
In situ	1	2.2	48	21.9	49	18.6
Others	1	2.2	25	11.4	26	9.8
Missing data	4	8.9	22	10.0	26	9.8
Node status						
N0	29	64.4	212	96.8	241	91.3
N+	15	33.3	5	2.3	20	7.6
NX	1	2.2	2	0.9	3	1.1
Fraction (%)						
0 (0)	1	2.2	8	3.7	9	3.4
1-25 (1)	9	20.0	18	8.2	27	10.2
25-75 (2)	11	24.4	36	16.4	47	17.8
75-100 (3)	24	53.3	152	69.4	176	66.7
Missing data	0		5	2.3	5	1.9
Intensity of staining						
Negative (0)	1	2.2	8	3.7	9	3.4
Weak (1)	7	15.6	14	6.4	21	8.0
Strong (2)	37	82.2	192	87.7	229	86.7
Missing data	0		5	2.3	5	1.9
MITF Fraction/ Intensity						
3/2	22	48.9	146	66.7	168	63.6
2/2	7	15.6	31	14.2	38	14.4
3/1	2	4.4	6	2.7	8	3.0
2/1	4	8.9	5	2.3	9	3.4
1/2	8	17.8	15	6.8	23	8.7
1/1	1	2.2	3	1.4	4	1.5
0/0	1	2.2	8	3.7	9	3.4
Missing data	0		5	2.3	5	1.9

[1]Applies to invasive tumors
NM: Nodular malignant melanoma, SSM: Superficial spreading melanoma
Table 1: Characteristics and distribution of MITF expression in patients dead from malignant melanoma (dead) and those alive or dead from other causes (not-dead) at end of follow-up (number and percentage).

the estimates for the two combinations of medium or high cell fractions stained with weak intensity was mitigated with the highest HR now being 2.9 (95% CI: 0.94-8.7, P=0.06) and the confidence intervals wide throughout. The HR for the tumors with cell fraction 1-25% and strong staining intensity however remained statistically significant with a HR 2.5 (95% CI: 1.1-6.1, P=0.04).

Population distribution of MITF expression

Since levels of MITF expression may be of prognostic value, it is of interest to study the distribution of the different levels of expression in

	Non adjusted HR (95% CI) P-value			Adjusted for tumor thickness/*in situ* HR (95% CI) P-value		
Age group						
20-49	1.0	ref.		1.0	ref.	
50-64	1.5	(0.61-3.5)	0.39	1.1	(0.45-2.8)	0.82
65-74	1.1	(0.43-3.1)	0.78	0.6	(0.19-1.6)	0.26
75+	1.6	(0.65-4.1)	0.29	0.9	(0.36-2.4)	0.89
Sex						
Male	1.0	ref.		1.0	ref.	
Female	0.76	(0.42-1.4)	0.35	0.89	(0.49-1.6)	0.7
Year of diagnosis						
1996-1999	1.0	ref.		1.0	ref.	
2000-2003	0.82	(0.46-1.5)	0.52	1.12	(0.61-2.1)	0.71
Tumor thickness[1]						
0-0.99 mm	1.41	(0.38-5.2)	0.61	-	-	0.61
1-1.99 mm	ref.			-	-	
2-2.99 mm	3.56	(1.0-12)	0.04	-	-	0.04
3-3.99 mm	5.05	(1.4–18)	0.01	-	-	0.01
4+ mm	8.43	(2.9-25)	<0.001	-	-	<0.001
Missing data	0.89	(0.22-3.6)	0.87	-	-	0.87
Clark level						
I-II	0.12	(0.03-0.51)	0.004	Excluded[2]	NA	
III	1.0	ref.		1.0	ref.	
IV	1.7	(0.84-3.3)	0.14	1.19	(0.56-2.6)	0.65
V	4.1	(1.6-10)	0.003	1.71	(0.61-4.8)	0.31
Missing data	3.0	(0.67-13)	0.15	Excluded[2]	NA	
Ulceration						
No ulceration	1.0	ref.		1.0	ref.	
Ulceration	5.8	(3.0-11)	<0.001	3.9	(1.9-7.9)	<0.001
Missing data	0.73	(0.24-2.2)	0.57	0.94	(0.23-3.9)	0.93
Histopathology						
NM	ref.			-	-	
SSM	0.45	(0.24-0.85)	0.01	0.76	(0.38-1.5)	0.43
In situ	0.05	(0.01-0.35)	0.003	Excluded[3]	NA	
Others	0.10	(0.01-0.78)	0.03	0.12	(0.02-0.93)	0.04
Missing data	0.38	(0.13-1.1)	0.07	0.41	(0.10-1.7)	0.22
Node status						
N0	ref.			ref.		
N+	12.03	(6.3-23)	<0.001	6.48	(3.3-13)	<0.001
NX	Excluded[4]	NA		Excluded[4]	NA	
Fraction (%)						
0 (0)	0.34	(0.04-2.6)	0.3	0.27	(0.03-2.2)	0.22
1-25 (1)	ref.			ref.		
25-75 (2)	0.89	(0.37-2.2)	0.81	0.67	(0.27-1.7)	0.39
75-100 (3)	0.39	(0.18-0.85)	0.02	0.44	(0.20-1.0)	0.05
Missing data	Excluded[4]	NA		Excluded[4]	NA	
Intensity of staining						

Negative (0)	0.30	(0.04-2.5)	0.26	0.31	(0.04-2.55)	0.27
Weak (1)	1.0	ref.		1.0	ref.	
Strong (2)	0.44	(0.20-0.98)	0.05	0.59	(0.26-1.36)	0.22
Missing data	Excluded[4]	NA		Excluded[4]	NA	
MITF Fraction/Intensity						
3/2	1.0	ref.		1.0	ref.	
2/2	1.9	(0.79-4.3)	0.16	1.2	(0.5-3.1)	0.64
3/1	2.1	(0.49-8.9)	0.31	1.5	(0.35-6.7)	0.58
2/1	4.9	(1.7-14)	0.004	2.9	(0.94-8.7)	0.06
1/2	2.9	(1.3-6.4)	0.01	2.5	(1.1-6.1)	0.04
1/1	1.7	(0.23-13)	0.61	1.6	(0.21-12)	0.65
0/0	0.89	(0.12-6.6)	0.91	0.62	(0.08-4.7)	0.65
Missing data	Excluded[4]	NA		Excluded[4]	NA	

[1]Applies to invasive tumors
[2]Excluded due to no events in the categories Clark level I-II and missing Clark level for any non-missing level of tumor thickness/*in situ*
[3]Excluded since the *in situ* tumors are nested within the variable tumor thickness/*in situ*.
[4]Excluded due to no events in the category
NA: Not applicable, NM: Nodular malignant melanoma, ref: Reference, SSM: Superficial spreading melanoma

Table 2: Hazard ratios (HR) with 95% confidence intervals (CI) for death in malignant melanoma estimated in Cox Proportional Hazards models. One model adjusted for tumor thickness/*in situ*.

our sample representative of a broader population. Given the findings in Table 2, we studied the distribution of levels indicating a low-risk (fraction >75% and strong intensity or no staining), a medium high-risk (fraction >75% with weak intensity or 25-75% staining with high intensity or fraction <25% and low intensity) and those with high-risk (fraction <25% with strong intensity or 25-75% staining with weak intensity) (Table 3).

Overall, patients in the low-risk group dominated greatly encompassing 67% of the patients, followed by the medium high-risk group 19% and the high-risk group 12%. Because of this distribution, most of the deaths in our cohort still occurred in the lower risk group. Twenty-three of the deaths occurred in the low-risk group, 10 in the medium high-risk group and 12 in the high-risk group. However, within the high-risk group 12 out of 32 patients died. The C-statistica, which corresponds to an area under a ROC curve, was 0.64 (95% CI: 0.47-0.81, P=0.16) indicating a weak discriminatory ability of MITF to identify patients to die from malignant melanoma.

Discussion

In a representative sample from a large, population-based cohort of patients treated for malignant melanoma we found that a medium expression of MITF was associated with worse prognosis as measured by risk of dying from malignant melanoma than a low or a high expression of MITF. This association was strong in un-adjusted models, but was attenuated by adjusting for tumor thickness, although still associated with a hazard ratio of nearly 3. The high-risk group as defined by expression of MITF was less than 15% of all patients and identified 12 of 45 deaths in the cohort.

Our results diverge from those of Garraway et al. [9], who demonstrated a regular trend for worse prognosis with amplification of the *MITF* gene. However, the patient selection in their study is different from our classical cohort design. First, some of the *MITF* analyses, which form the basis of the prognostic study, were not done on primary tumors, but on samples from metastases. Secondly, the patient series was probably a group of referred patients, possibly with at an average more advanced tumor and may have undergone treatments previously. Thus, their *MITF* status may be a reflection of events both during tumor progression and treatment. However, the study by Garraway et al. [9] focuses on the gene but this study on the protein, gene amplification does not necessarily indicate high protein expression.

The findings in our study are more in line with those of Salti et al. [10], who found that high protein expression of MITF was associated with good prognosis in patients with melanomas of intermediate thickness (1.0-4.0 mm). As in our study, their analysis was based on IHC on primary tumors. However, they only included 63 patients in a hospital-based series and a selection is indicated by that 46% of their patients had lymph node positive disease.

We had hypothesized to find a more linear and "simple" relationship between protein expression and survival, but found, interestingly a pattern which is more in line with experimental cell line research that both low and high levels may be beneficial [11] or with the suggestion that *MITF* amplification is a late event in melanoma progression and different subsets of melanomas exist, with fundamentally different biological activities of MITF [26]. Previously a dual role of MITF has been proposed where the protein enhances apoptosis or survival depending on the signal pathways the protein is participating in [27]. At transcriptional level, MITF expression is regulated by many proteins including BETA-CATENIN/LEF-1, SOX10, PAX3, CREB, ONE-CUT 2 and BRN-2 [28-33].

Our results illustrate a common dilemma in the clinical utility for biomarkers: MITF expression can aid in identifying a group of patients at higher risk. However, the strength of the association between MITF expression and risk, and the distribution of the expression in the population are such that the prospective discriminatory power to use MITF expression as decision tool in a population-based cohort will be weak or modest. The bi-phasic relation between MITF expression and prognosis differs from many other prognostic markers that have a linear correlation with outcome but should not *per se* be an obstacle for clinical use.

We utilized a population-based clinical database for patients treated in routine clinical practice. Thus, there was no selection bias as compared to a specialist centre study. Virtually all patients dying from malignant melanoma have a known history of recurrence and have undergone active or palliative treatments, the end-point thus being highly reliable.

There was no prior information about the distribution of expression of MITF and its relation to prognosis in this cohort. Therefore we decided to sample the whole cohort to reveal representative information that could raise valid hypotheses and form the basis of more detailed and directed studies. Furthermore, we aimed for a straightforward cohort study design with little chance for bias, as other studies have been based on hybrid designs or designs susceptible to selection bias. However, what we gained in internal and external validity, we lost in that some groups of interest were small with a limited statistical precision as a result. By using an alternative strategy with sampling from those with advanced melanomas and thus more end-points, we would have lost our aim of looking at prognostic information from MITF in early melanoma and thus not been able to avoid some of the problems with other studies in the field.

The evaluation of immunohistochemical staining is subjective and has been criticized as a semi-quantitative approach, in particular

	Low (#3/2+#0/0)	(%)	Medium (#3/1+#2/2+#1/1)	(%)	High (#2/1+#1/2)	(%)	Missing data	(%)
Age group								
<65	86	(65.6)	23	(17.6)	18	(13.7)	4	(3.1)
65+	91	(68.4)	27	(20.3)	14	(10.5)	1	(0.8)
Sex								
Male	91	(66.9)	26	(19.1)	15	(11.0)	4	(2.9)
Female	86	(67.2)	24	(18.8)	17	(13.3)	1	(0.8)
Node status								
N0	165	(68.5)	43	(17.8)	28	(11.6)	5	(2.1)
N+	10	(50.0)	6	(30.0)	4	(20.0)	0	(0.0)
NX	2	(66.7)	1	(33.3)	0	(0.0)	0	(0.0)
Tumor thickness[1]								
0-0.99 mm	39	(81.2)	4	(8.3)	4	(8.3)	1	(2.1)
1-1.99 mm	37	(67.3)	11	(20.0)	5	(9.1)	2	(3.6)
2-2.99 mm	26	(83.9)	3	(9.7)	2	(6.5)	0	(0.0)
3-3.99 mm	9	(47.4)	3	(15.8)	7	(36.8)	0	(0.0)
4+ mm	20	(41.7)	19	(39.6)	9	(18.8)	0	(0.0)
Missing data	46	(73.0)	10	(15.9)	5	(7.9)	2	(3.2)
Clark level								
II	42	(73.7)	8	(14.0)	5	(8.8)	2	(3.5)
III	24	(77.4)	3	(9.7)	3	(9.7)	1	(3.2)
IV	57	(74.0)	13	(16.9)	6	(7.8)	1	(1.3)
V	46	(60.5)	16	(21.1)	13	(17.1)	1	(1.3)
Missing data	3	(18.8)	8	(50.0)	5	(31.2)	0	(0.0)
Histopathology	5	(71.4)	2	(28.6)	0	(0.0)	0	(0.0)
NM								
SSM	44	(63.8)	15	(21.7)	9	(13.0)	1	(1.4)
In situ	64	(68.1)	15	(16.0)	13	(13.8)	2	(2.1)
Others	35	(71.4)	7	(14.3)	5	(10.2)	2	(4.1)
Missing data	15	(57.7)	10	(38.5)	1	(3.8)	0	(0.0)
Ulceration	19	(73.1)	3	(11.5)	4	(15.4)	0	(0.0)
No ulceration								
Ulceration	96	(69.1)	21	(15.1)	20	(14.4)	2	(1.4)
Missing data	44	(62.9)	16	(22.9)	9	(12.9)	1	(1.4)

Table 3: Distribution (number and percentage) of risk groups dying from malignant melanoma as defined by MITF expression (#fraction/intensity) in the whole sample.

regarding issues concerning the intrinsic lack of reproducibility of manual scoring. Automated scoring systems for IHC has a potential to further advance this well-established and clinically useful assay to accurately quantify both staining intensity and the subcellular localization of protein expression in a reproducible fashion [34]. However, subjective assessment of IHC remains a golden standard. Subjective scoring shows a tendency for false negatives at low levels of expression, so it is possible that some negatives in our study may rather have belonged to a group with a small cell fraction stained with low intensity. The IHC results are also dependent on the specificity of the antibody, fixation of the tissue and on what antigen retrieval method and detection system is employed. A majority of malignant melanomas are small tumors and thus it is not possible to sample a preferred number of 2-4 cores for TMAs [35]. It cannot be ruled out that TMA-based results can be affected by the well-known problems of obtaining accurately representative tumor material, both as a consequence of small sized tumors and for large tumors possible heterogeneity within a tumor.

Our findings implicate that other biomarkers must be considered simultaneously with MITF if it is to be interpreted as a prognostic marker. However, the low and high extremes of levels of MITF expression are more common in patients with good prognosis. To that end, the biological mechanism underlying the observed association with a moderate expression with more advanced stage is relevant to explore. Our population-based design also illustrates that knowing the distribution of a possible therapeutic target in an unselected patient population is important for setting priorities for drug development.

Acknowledgement

This study was supported by grants from the Knut and Alice Wallenberg Foundation and the Swedish Cancer Society (no. 09 0687).

References

1. Steingrimsson E, Copeland NG, Jenkins NA (2004) Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet 38: 365-411.
2. Levy C, Khaled M, Fisher DE (2006) MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol Med 12: 406-414.
3. Haq R, Fisher DE (2011) Biology and clinical relevance of the micropthalmia family of transcription factors in human cancer. J Clin Oncol 29: 3474-3482.
4. Vance KW, Goding CR (2004) The transcription network regulating melanocyte development and melanoma. Pigment Cell Res 17: 318-325.
5. Carreira S, Goodall J, Aksan I, La Rocca SA, Galibert MD, et al. (2005) Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 433: 764-769.
6. Hodgkinson CA, Moore KJ, Nakayama A, Steingrímsson E, Copeland NG, et al. (1993) Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 74: 395-404.
7. Fuse N, Yasumoto K, Suzuki H, Takahashi K, Shibahara S (1996) Identification

of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. Biochem Biophys Res Commun 219: 702-707.

8. Bismuth K, Maric D, Arnheiter H (2005) MITF and cell proliferation: the role of alternative splice forms. Pigment Cell Res 18: 349-359.
9. Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, et al. (2005) Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 436: 117-122.
10. Salti GI, Manougian T, Farolan M, Shilkaitis A, Majumdar D, et al. (2000) Micropthalmia transcription factor: a new prognostic marker in intermediate-thickness cutaneous malignant melanoma. Cancer Res 60: 5012-5016.
11. Kido K, Sumimoto H, Asada S, Okada SM, Yaguchi T, et al. (2009) Simultaneous suppression of MITF and BRAF V600E enhanced inhibition of melanoma cell proliferation. Cancer Sci 100: 1863-1869.
12. Lekmine F, Chang CK, Sethakorn N, Das Gupta TK, Salti GI (2007) Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem Biophys Res Commun 354: 830-835.
13. Ponten F, Jirstrom K, Uhlen M (2008) The Human Protein Atlas--a tool for pathology. J Pathol 216: 387-393.
14. Gould Rothberg BE, Bracken MB, Rimm DL (2009) Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. J Natl Cancer Inst 101: 452-474.
15. Barlow L, Westergren K, Holmberg L, Talback M (2009) The completeness of the Swedish Cancer Register: a sample survey for year 1998. Acta Oncol 48: 27-33.
16. Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, et al. (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4: 844-847.
17. Kampf C, Andersson AC, Wester K, Björling E, Uhlen M, et al. (2004) Antibody-based tissue profiling as a tool for clinical proteomics. Clin Proteomics 1: 285-299.
18. Nilsson P, Paavilainen L, Larsson1 K, Ödling J, Sundberg M, et al. (2005) Towards a human proteome atlas: high-throughput generation of mono-specific antibodies for tissue profiling. Proteomics 5: 4327-4337.
19. Uhlen M, Oksvold P, Fagerberg L,Lundberg E, Jonasson K, et al. (2010) Towards a knowledge-based Human Protein Atlas. Nat Biotechnol 28: 1248-1250.
20. Berglund L, Björling E, Oksvold P, Fagerberg L, Asplund A, et al. (2008) A Genecentric Human Protein Atlas for Expression profiles Based on Antibodies. Mol Cell Proteomics 7: 2019-2027.
21. Uhlen M, Björling E,Agaton C, Szigyarto CAK, Bahram A, et al. (2005) A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 4: 1920-1932.
22. Paavilainen L, Wernérus H, Nilsson P, Uhlén M, Hober S, et al. (2008) Evaluation of monospecific antibodies: a comparison study with commercial analogs using immunohistochemistry on tissue microarrays. Applied Immunohistochemistry & Molecular Morphology 16: 493-502.
23. Bjorling E, Lindskog C, Oksvold P, Linné J ,Kampf C, et al. (2008) A web-based tool for in silico biomarker discovery based on tissue-specific protein profiles in normal and cancer tissues. Mol Cell Proteomics 7: 825-844.
24. Pencina MJ, D'Agostino RB (2004) Overall C as a measure of discrimination in survival analysis: model specific population value and confidence interval estimation. Stat Med 23: 2109-2123.
25. Ihaka R, Gentleman R (1996) A language for data analysis and graphics. J Comput Graph Stat 5: 299-314.
26. Selzer E, Wacheck V, Lucas T, Heere-Ress E, Wu M, et al. (2002) The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. Cancer Res 62: 2098.
27. Larribere L, Hilmi C, Khaled M, Gaggioli C, Bille K, Auberger P, et al. (2005) The cleavage of microphthalmia-associated transcription factor, MITF, by caspases plays an essential role in melanocyte and melanoma cell apoptosis. Genes Dev 19: 1980-1985.
28. Verastegui C, Bille K, Ortonne JP, Ballotti R (2000) Regulation of the microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, SOX10. J Biol Chem 275: 30757-30760.
29. Goodall J, Carreira S,Denat L,Kobi D,Davidson I, et al. (2008) Brn-2 represses microphthalmia-associated transcription factor expression and marks a distinct subpopulation of microphthalmia-associated transcription factor-negative melanoma cells. Cancer Res 68: 7788.
30. Takeda K, Yasumoto K,Takada R,Takada S, Watanabe K, et al. (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. J Biol Chem 275: 14013-14016.
31. Potterf SB, Furumura M,Dunn KJ,Arnheiter H,Pavan WJ, et al. (2000) Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. Hum Genet 107: 1-6.
32. Jacquemin P, Lannoy VJ, O'Sullivan J, Read A, Lemaigre FP, et al. (2001) The transcription factor onecut-2 controls the microphthalmia-associated transcription factor gene. Biochem Biophys Res Commun 285: 1200-1205.
33. Bondurand N, Pingault V, Goerich DE, Lemort N, Sock E, et al. (2000) Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Hum Mol Genet 9: 1907-1917.
34. Brennan DJ, O'Connor DP, Rexhepaj E, Ponten F, Gallagher WM (2010) Antibody-based proteomics: fast-tracking molecular diagnostics in oncology. Nat Rev Cancer 10: 605-617.
35. Rimm DL, Camp RL, Charette LA, Costa J, Olsen DA, et al. (2001) Tissue microarray: a new technology for amplification of tissue resources. Cancer J 7: 24-31.