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ORIGINAL ARTICLE

G protein-coupled receptor 40 expression in human melanoma – correlation with tumour thickness, AJCC stage and survival

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Abstract

Background In melanoma, preclinical data suggest a possible role of polyunsaturated fatty acids inhibiting cell growth. A new target molecule for free fatty acids, the G protein-coupled receptor GPR40, was identified in melanoma cells.

Objectives The aim of this study was to investigate GPR40 expression in human melanocytic tissues and to evaluate its potential as a prognostic marker.

Methods and Results A total of 114 tissue sections of naevi, primary melanoma and melanoma metastasis were immunohistochemically stained with anti-GPR40. The staining was evaluated, using the immunoreactivity scoring system. Compared to naevi, primary melanoma and melanoma metastasis showed significantly higher levels of GPR40 ($P < 0.05$). In primary melanoma, GPR40 expression positively correlated with tumour thickness ($P = 0.044$) and AJCC level ($P = 0.017$) and in melanoma metastasis with AJCC level ($P = 0.035$). Primary melanoma patients with high levels of GPR40 had a significantly poorer overall survival ($P = 0.004$) and shorter disease-free survival (0.040).

Conclusion The present study identified GPR40 as a novel target molecule in melanoma. First evidence for a potential role of the receptor in tumour progression and metastases was found, and it could be demonstrated that GPR40 expression is negatively correlated with patient's survival.

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Conflict of interests

None.

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Introduction

Despite the recent therapeutic progress, advanced malignant melanoma remains one of the most aggressive cancers with poor prognosis.[1,2] In metastatic disease, immune checkpoint inhibitors and targeted therapies have significantly improved progression-free and overall survival.[3] However, after initial response, therapy resistances, leading to progression, are frequently observed.[3,4] To the present date, it is almost impossible to predict which patient will benefit from a sustained treatment response. Hence, novel therapy targets, prognostic and predictive markers are strongly needed to further improve melanoma treatment.

Medium- and long-chained free fatty acids, especially the ratio between omega-3 (n-3) and omega-6 (n-6) free fatty acids in modern diets, have been widely discussed regarding their influence on tumorigenesis.

In preclinical malignant melanoma studies, n-3 PUFAs inhibited tumour growth by pro-apoptotic activity and suppression of angiogenesis hampering invasion and metastasis of malignant melanoma.[5] Consumption of high levels of n-3 PUFAs was inversely associated with melanoma incidence.[6] Furthermore, Fat-1 transgenic mice, which convert n-6 to n-3 fatty acids and which have therefore a balanced ratio of n-6 to n-3 fatty acids compared to wild-type mice, showed considerably reduced melanoma tumour growth.[7] Underlying mechanisms explaining this observation are mainly unknown. In 2002, Briscoe, Tadayyon[8] were the first to identify medium- and long-chained free fatty acids (among them n-3, n-6 and n-9 fatty acids) as ligands of the orphan G protein-coupled receptor 40 (GPR40). As GPR40 is highly expressed in the β-cells of pancreatic islets, metabolic functions of the receptor were early investigated.[8] Activation of GPR40 strongly potentiates glucose-induced insulin secretion

and is therefore an interesting target for type 2 diabetes treatment. Several preclinical and clinical trials with GPR40 agonists (for example TAK-875, LY2922470 and SHR0534) are currently underway.[9]

Interestingly, GPR40 is also expressed in several cancer cells. In human breast cancer, Hardy, St-Onge[10] demonstrated that receptor activation with oleic acid, a n-9 fatty acid, stimulates cell proliferation.[10,11] Soto-Guzman, Robledo[12] postulated a GPR40-dependent signal transduction pathway in breast cancer cells leading to ERK1/2 activity and transactivation of the epidermal growth factor receptor (EGFR) promoting cancer cell proliferation.[12] In contrast to these findings, investigations with the n-3 fatty acid eicosapentaenoic acid (EPA) and specific synthetic GPR40 agonists (GW9508, TUG-891) revealed an inhibition of breast cancer cells.[13] In colorectal cancer, increased GPR40 and GPR120-dependent activation of the Hippo pathway by n-3 fatty acids resulted in growth inhibition.[14] More data concerning GPR40-mediated pro-tumorigenic or antitumorigenic effects were published depending on the investigated receptor agonists and the particular tumour entity. In summary, GPR40 activation inhibited cell motility and cell invasion in pancreas carcinoma, osteosarcoma and fibrosarcoma cells, whereas in lung carcinoma GPR40 activation stimulated cell motility and cell invasion.[15–18]

Regarding the role of GPR40 in malignant melanoma, only limited data are available. Recently, Nehra, Pan[19] demonstrated elevated levels of GPR40 expression in melanoma cell lines. Treatment with DHA, a n-3 fatty acid or the specific GPR40 agonist TAK-875 inhibited tumour cell growth both *in vitro* and in a murine subcutaneous xenograft model.[19] In contrast, results from Fukushima, Takahashi[20] suggested enhanced melanoma cell migration by GPR40 activation.[20]

Concerning the limited information on the relevance of GPR40 expression and function in human melanoma tissue, we investigate the expression and prognostic potential of GPR40 in 114 primary melanoma, melanoma metastases and naevi. In addition, we analysed the statistic correlation between distinct prognostic factors (e.g. AJCC stage, Breslow depth, BRAF status) and GPR40 expression and performed Kaplan–Meier analysis.

Methods

Ethics statement

This study was conducted according to the Declaration of Helsinki Principles. Tissue samples and patient data used in this study were provided by the Dermatopathologic Department of the Clinic of Dermatology, Allergy and Venereology (Frankfurt/Main, Germany) in cooperation with the University Cancer Center Frankfurt. Written informed consent was obtained from all patients, and the study was approved by the Ethical Committee at the University Hospital Frankfurt (project number: SDO-04-2014).

Case selection

This study was carried out on 114 tissue samples consisting of 39 primary melanoma, 43 melanoma metastasis and 32 naevi, which were obtained from 2009 to 2015. The cases were retrospectively selected from the dermatopathologic unit of the Department of Dermatology, Allergy and Venereology, University Hospital Frankfurt (Frankfurt/M., Germany). Diagnosis of the original histological reports was re-evaluated and reconfirmed by two of the authors. Clinical data (sex, age, Breslow thickness, BMI, BRAF status, tumour localization and patient survival) were obtained from the patient's charts.

Immunohistochemistry

For immunohistochemistry, sections with 4 μm thickness were made from paraffin-embedded tissue. Sections were deparaffinized and rehydrated through a descending series of ethanol by routine procedure. Heat-induced epitope retrieval was achieved by incubation with sodium citrate buffer (pH 6.0) for 20 min. After cooling the slides for 20 min, washing steps with distilled water and Tris-wash Buffer B pH 7.2 (TBS; Zytomed Systems, Berlin, Germany) were performed.

Primary antibody binding was conducted at 37°C for 45 min with rabbit anti-human polyclonal GPR40 antibody (orb84975 Biorbyt, Cambridge, UK). After three TBS washing steps, the slides were incubated for 30 min with Histofine Simple Stain (Medac, Wedel, Germany), an amino acid polymer containing alkaline phosphatase and goat anti-rabbit IgG. Remaining Histofine Simple Stain was eliminated by two washing steps with TBS. Alkaline phosphatase reaction was visualized by Permanent AP Red (Zytomed). The sections were finally counterstained with Mayer's haematoxylin. Negative control was performed with polyclonal rabbit IgG isotype antibody (LS-A2004-50; LifeSpan Biosciences, Seattle, WA, USA) according to the above-described protocol. Pancreas tissue served as external positive control.

Interpretation and evaluation of GPR40 expression

The expression of GPR40 was evaluated in 114 tissue sections histologically confirmed as naevi (n = 32), primary melanoma (n = 39) and melanoma metastasis (n = 43). GPR40 staining was independently scored in a blinded manner by three trained investigators (IH, JK and PK) on the bases of the well-established immunoreactivity scoring system (IRS).[21] The staining intensity was evaluated on a scoring scale ranging from no signal (0), over a weak (1) and over a moderate (2) to a strong signal (3). The quantitative expression was defined by the percentage of positive cells: 0% (0), <10% (1), 10–50% (2), 51–80% (3) and >80% (4). The IRS score was calculated by multiplying the expression intensity with the quantitative expression, giving rise to a maximum range from 0 to 12. A score ranging from 0 to 2 corresponds to no expression, a score ranging from 3 to 5 corresponds to a weak positive expression, a score from 6 to 8 corresponds to a moderate expression, and a score from 9 to 12

corresponds to a strong expression. Means were formed of the three assessments, and results were mathematically rounded.

Statistical analysis

Statistical significance was tested by comparing the staining results using the Kruskal–Wallis and Mann–Whitney *U*-test (BIAS, Frankfurt, Germany). Survival analysis was conducted for all primary melanomas. The follow-up period was carried out until November 2018. From the 39 patients with primary melanoma, four patients were excluded from the analysis because the available tissue samples were secondary melanomas. For disease-free survival analysis, detailed data on tumour progression were only available for 29 patients. Overall survival and disease-free survival rates were determined by the Kaplan–Meier method, and statistical significance was tested with the log-rank test.

Results

Patient characteristics

In this study, a total number of 114 cases consisting of 32 melanocytic naevi, 39 primary melanoma and 43 metastasis were evaluated. Naevi, primary melanoma and melanoma metastasis were separately reviewed, due to the different clinical features of the tumours. Out of the 32 included naevi, 19 were from male patients and 13 from female patients. Mean age was 58 years, and mean BMI was 28.7 kg/m^2 (Table 1a). Concerning the histological subtype, 53% (17/32) were compound naevi, 43% (14/32) were junctional naevi, and 3% (1/32) were dermal naevi. 84% (27/32) of all naevi showed mild-to-moderate signs of dysplasia, and 16% (5/32) had a common histological pattern.

From the 39 primary melanomas included, 19 were nodular malignant melanomas, and 20 were superficial spreading melanomas. The mean age was 67 years (range: 29–92). Mean BMI was 25.9 kg/m^2, which corresponds to a slight overweight according to WHO definitions. Gender distribution was balanced with 21 male and 18 female patients. Primary melanoma was classified according to Breslow tumour thickness, ulceration status, localization, BRAF status and the 8th edition of AJCC tumour classification at the date of diagnosis (Table 1b).

In the melanoma metastasis group, 29 male and 14 female patients were included. The mean age was 70 years (range: 43–92). Mean BMI was 26.8 kg/m^2. Eighteen metastases were from patients in AJCC stage III, and 25 metastases were from patients in AJCC stage IV. BRAF status was available in 27 patients. Twelve patients were BRAF-positive, and 15 were BRAF-negative (Table 1c).

Immunohistochemistry

The expression of GPR40 was analysed at protein level by immunohistochemistry. GPR40 immunoreactivity was found in the cytosol and the membranes of all evaluated types of melanocytic cells. The human epidermis showed a moderate staining intensity and served as internal control. Connective tissue was devoid of GPR40. β-cells of pancreatic isles in human pancreatic tissue, which are well known to express high levels of GPR40, served as external positive control.

Primary malignant melanoma and melanoma metastasis showed significantly higher levels of GPR40 expression in all investigated samples (Fig. 1). In detail, high expression of GPR40 was observed in 38.5% (15/39) of all primary melanomas and in 65.1% (28/43) of melanoma metastasis, whereas only 3.1% (1/32) of naevi had high levels of GPR40 expression (Fig. 2).

Interestingly, GPR40 expression was significantly higher in primary melanoma with elevated Breslow tumour thickness (T3–T4) compared to those with lower tumour thickness (T1–T2; $P = 0.044$; Table 2a). In the correlation assessment, we found a moderate correlation between GPR40 expression in primary melanoma and Breslow tumour thickness (Spearman correlation coefficient $r = 0.43$, $P = 0.0062$). In accordance with these data, primary melanomas and melanoma metastases showed significantly higher GPR40 expression values, the higher the AJCC levels were, indicating a possible association of GPR40 expression with the metastatic potential of melanoma cells (Table 2a,b). Moreover, no differences in GPR40 expression were observed in the context of histological subtype for naevi and primary melanoma, BMI, Age, sex, BRAF status or localization of the primary tumour (Table 2a,b).

Survival analysis

To evaluate the impact of GPR40 expression on patient outcome in malignant melanoma, we calculated Kaplan–Meier curves for overall survival and disease-free survival. By comparing patients with no and low (IRS score: 0–5) expression to those with moderate to high levels of GPR40 expression in primary malignant melanoma, we found significant differences concerning overall survival ($P = 0.004$) and disease-free survival ($P = 0.040$; Fig. 3). Patients with moderate and high GPR40 expression showed a significantly poorer disease-free and overall survival than those with low or no GPR40 expression.

For the overall survival analysis, 35 patients were included. The median survival was not reached in the low expression group; for the group with high GPR40 expression, it was 35 months. Median follow-up for all patients was 38 months. In the disease-free survival analysis, 29 patients were included. The median survival was also not reached in the low expression group; for the high expression group, it was 18 months. Median follow-up for all patients was 27 months.

Discussion

In order to identify new target molecules and potential prognostic markers in malignant melanoma, we investigated GPR40 protein expression in 114 paraffin-embedded tissues from

Table 1 (a–c) Clinical features in naevi (a), primary melanoma (b) and melanoma metastasis (c).

a) Clinical features in naevi	
Characteristics	**Naevi (*n* = 32)**
Mean age – years	58
Sex – no. (%)	
Male	19 (59.4)
Female	13 (40.6)
BMI	
Mean BMI – kg/m^2 (range)	28.7 (20.6–52.9)
b) Clinical features in primary melanoma	
Characteristics	**Primary melanoma (*n* = 39)**
Mean age – years	67
Sex – no. (%)	
Male	21 (53.8)
Female	18 (46.2)
BMI	
Mean BMI – kg/m^2 (range)	25.9 (17.6–43.2)
Melanoma type – no. (%)	
Nodular malignant melanomas	19 (48.7)
Superficial spreading melanoma	20 (51.3)
Tumour thickness – no. (%)	
T1a	6 (15.4)
T1b	3 (7.7)
T2a	8 (20.5)
T2b	2 (5.1)
T3a	6 (15.4)
T3b	3 (7.7)
T4a	6 (15.4)
T4b	5 (12.8)
Ulceration status– no. (%)	
Ulcerated	11 (28.2)
Non-ulcerated	28 (71.8)
AJCC stage – no. (%)	
AJCC stage I	17 (43.6)
AJCC stage II	13 (33.3)
AJCC stage III	7 (17.9)
AJCC stage IV	2 (5.1)
Localization – no. (%)	
Head	3 (7.7)
Lower extremities	11 (28.2)
Trunk	20 (51.3)
Upper extremities	5 (12.8)
BRAF status – no. (%)	
BRAF +	4 (10.3)
BRAF –	4 (10.3)
Unknown	31 (79.5)

Table 1 *Continued*

c) Clinical features in melanoma metastasis	
Characteristics	**Metastasis (*n* = 43)**
Mean age – years	70
Sex – no. (%)	
Male	29 (67.4)
Female	14 (32.6)
BMI	
Mean BMI – kg/m^2 (range)	26.8 (21.2–33.8)
AJCC stage – no. (%)	
AJCC stage I	0 (0)
AJCC stage II	0 (0)
AJCC stage III	18 (41.9)
AJCC stage IV	25 (58.1)
BRAF status – no. (%)	
BRAF+	12 (27.9)
BRAF–	15 (34.9)
Unknown	16 (37.2)

Clinical characteristics of each group are listed in absolute numbers and in percentages.

melanocytic lesions using immunohistochemistry. In this study, we demonstrated first time in human tissue that primary melanoma and melanoma metastases express significantly higher levels of GPR40 compared to benign naevi suggesting a possible role of GPR40 in melanoma tumorigenesis. These findings are in accordance with earlier reports showing higher GPR40 mRNA expression in several human malignant melanoma cell lines compared to fibroblast and other malignancies namely neuroblastoma and breast cancer cells.[19,20] In our study, GPR40 expression positively correlates with Breslow tumour thickness, the most important prognostic marker in primary melanoma. Fitting the image GPR40 expression is also associated with the AJCC stage in primary melanoma and melanoma metastasis, providing first evidence for a contribution of GPR40 in the metastatic process. The possible prognostic value of GPR40 is sustained for the first time by the here done survival analysis. With a median follow-up of 38 months, high expression of GPR40 is associated with an inferior median overall survival. In accordance with this, the disease-free survival for patients with strong GPR40 expression was also significantly shortened. However, it has to be taken into account that the sample size of 35 patients in overall survival analysis and 29 patients in disease-free survival analysis is limiting factor. Further studies with a larger cohort considering disease-specific survival are needed to substantiate our observations.

Regarding possible functional aspects, Fukushima, Takahashi[20] and Kita, Kadochi[18] were able to show that activation of GPR40 with the synthetic GPR40 agonist GW9508 enhanced cell motility in melanoma and lung cancer cells, whereas selective GPR40 inhibition reduced cell motility in melanoma cells.[18,20] However, in contrast to these pro-tumorigenic effects, Nehra, Pan[19] demonstrated decreased melanoma cell proliferation *in vitro* and

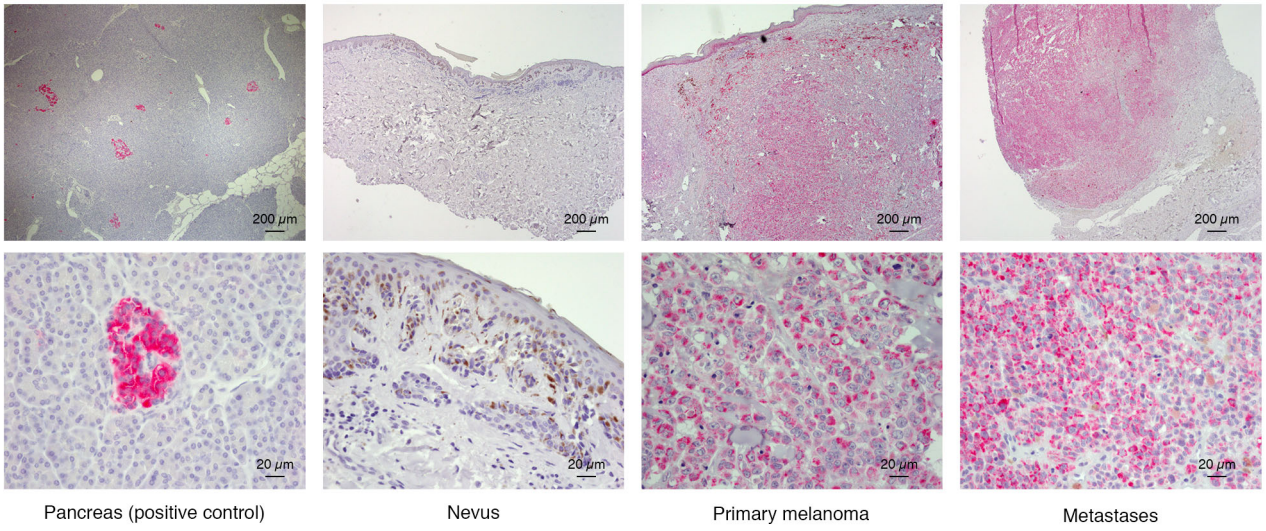

Figure 1 GPR40 Immunohistochemistry. GPR40 expression is increased in melanoma tissues. Representative tissue sections of human naevi, primary melanoma and melanoma metastasis were stained with the haematoxylin and eosin (HE) staining technique and with a polyclonal rabbit anti-GPR40 antibody as described in Materials and Methods. The magnification is indicated by scale bars. A moderate GPR40 staining in red colour is observed throughout the epidermis with weak staining of naevus cells and connective tissue. Cells of primary melanoma and melanoma metastasis show strong staining signals. Pancreas tissue shows strong immunoreactivity and served as external positive control.

decreased tumour growth in a murine tumour model in response to DHA or the synthetic GPR40 agonist TAK-875.

In breast cancer cells both, tumour-promoting and tumour-inhibiting effects were described with different GPR40 receptor agonists. Oleic acid led to GPR40-mediated tumour cell proliferation whereas treatment with the n-3 fatty acid EPA or with the synthetic GPR40 agonists (GW9508, TUG-891) inhibited tumour growth. The cause for these conflicting data could be due to the not yet fully understood ligand–receptor interactions of G protein-coupled receptors. For a long time, it was believed that activation of one GPR leads to recruitment of a unique G protein as a primary effector, which in turn would activate a particular signalling pathway. GPR activation would therefore lead to a single defined ligand–effector response. In recent years, GPRs have been identified to interact not only with G proteins but also with arrestin proteins to transmit signalling. Different ligands for one GPR showed functional selectivity, activating preferentially one pathway over another. This paradigm shift could explain ligand and entity-specific diverse responses in GPR signalling.

Notably, the here investigated GPR40 binds an enormous variety of different free fatty acids and chemical diverse synthetic agonists which leads to the assumption that the receptor has various binding sites. Corroboratively, radioligand binding assays identified at least three allosterically linked GPR40 binding sites.[22]

Diverse cellular responses may be due to differential cellular signalling. Even though GPR40 couples predominantly Gq/11,

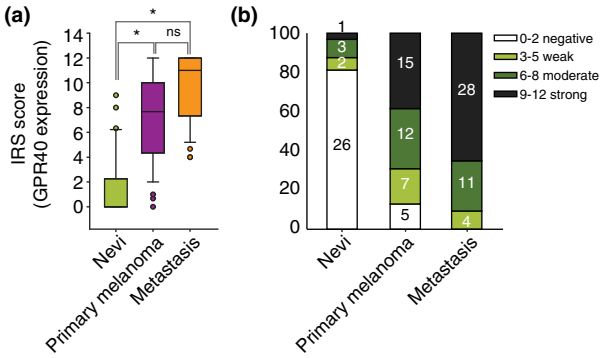

Figure 2 GPR40 expression in melanocytic lesions. The intensity of GPR40 staining was evaluated using IRS (immunoreactivity scoring). Melanoma and melanoma metastasis showed a significantly stronger GPR40 expression compared to histologically confirmed naevi. (a) Data are presented as Box plot. Statistical significance of the data was calculated by the Kruskal–Wallis test and Dunn's post hoc test. *P*-values are indicated (*<0.05). (b) The relative distribution of GPR40 expression by groups is specified in percentage. Absolute numbers are shown in white colour.

new data suggest also a Gs and a β-arrestin signal transmission in a ligand-specific manner.[23] In particular, it was shown that the synthetic GPR40 agonist TAK-875 and the free fatty acids palmitic acid and oleic acid differ in recruitment of ß-arrestin 1 and ß-arrestin 2 and Gq/11 activation.[23] Moreover, Hauge,

Table 2 (a–b) GPR40 expression in primary melanoma (a) and melanoma metastasis (b).

(a)

Primary melanoma	Total	No of expression (IRS: 0–2)	Weak expression (IRS: 3–5)	Moderate expression (IRS: 6–8)	Strong expression (IRS: 9–12)	*P*-values
Age						0.520
<60	11	2	2	4	3	
≥60	28	3	5	8	12	
Sex						0.396
Male	21	2	4	4	11	
Female	18	3	3	8	4	
BMI	17	2	3	3	9	0.232
<25 kg/m^2	20	3	4	8	5	
≥25 kg/m^2	2	0	0	1	1	
Melanoma type						0.123
Nodular malignant melanomas	19	2	1	7	9	
Superficial spreading melanoma	20	3	6	5	6	
Tumour thickness						**0.044**
T1–T2 ≤1–2 mm	19	4	4	6	5	
T3–T4 ≥ 2.01 mm	20	1	3	6	10	
Ulceration						0.168
Ulcerated	11	0	1	4	6	
Non-ulcerated	28	5	6	8	9	
AJCC stage						**0.017**
AJCC stage I–II	30	5	6	10	9	
AJCC stage III–IV	9	0	1	2	6	
Localization						0.376
Head	3	0	1	1	1	
Lower extremities	11	2	2	4	3	
Trunk	20	1	3	6	10	
Upper extremities	5	2	1	1	1	
BRAF status						0.663
BRAF+	4	0	2	0	2	
BRAF−	4	0	1	1	2	
Unknown	31	5	4	11	11	

(b)

Melanoma metastasis	Total	No of expression (IRS: 0–2)	Weak expression (IRS: 3–5)	Moderate expression (IRS: 6–8)	Strong expression (IRS: 9–12)	*P*-values
Age						0.131
<60	11	0	1	4	6	
≥60	32	0	3	7	22	
Sex						0.832
Male	29	0	3	7	19	
Female	14	0	1	4	9	
BMI						0.925
<25 kg/m^2	13	0	1	3	9	
≥25 kg/m^2	25	0	3	6	16	
Unknown	5	0	0	2	3	
AJCC stage						**0.035**
AJCC stage III	18	0	4	6	8	
AJCC stage IV	25	0	0	5	20	

Table 2 *Continued*

Melanoma metastasis	Total	No of expression (IRS: 0–2)	Weak expression (IRS: 3–5)	Moderate expression (IRS: 6–8)	Strong expression (IRS: 9–12)	*P*-values
BRAF status						0.243
BRAF+	12	0	1	4	7	
BRAF−	15	0	1	3	11	
Unknown	16	0	2	4	10	

The GPR40 expression by IRS score in absolute numbers in relation to clinical features. Statistically significant values are marked in bold. In primary melanoma, GPR40 expression is associated with Breslow tumour thickness ($P = 0.044$) and AJCC stage (0.017). In melanoma metastasis, GPR40 expression is associated with AJCC stage ($P = 0.035$).

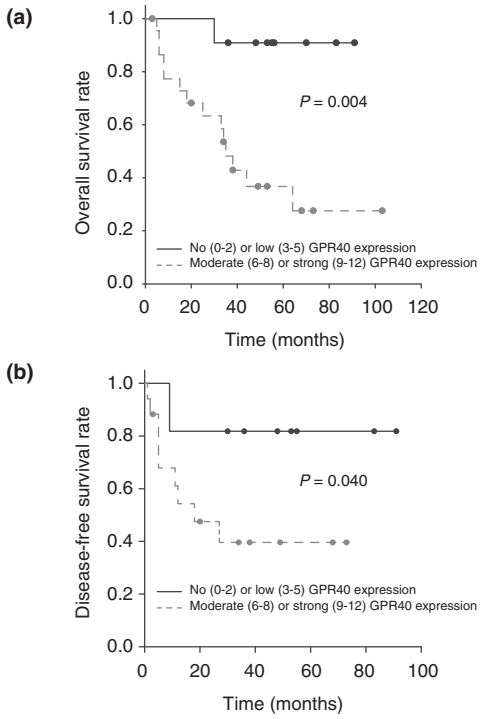

Figure 3 Survival analysis. Moderate and strong expression of GPR40 (IRS score: 6–12) is associated with poorer overall survival (a) and shorter disease-free survival (b) in patients with primary malignant melanoma. The log-rank test achieved significance with $P = 0.004$ for overall survival and $P = 0.040$ for disease-free survival.

Vestmar[24] demonstrated Gs coupling and activation of cAMP-dependent pathways by GPR40 agonists.[24] These observations might explain the contradictory effects of different GPR40 agonists in tumorigenesis.

Recently, it was shown that melanin can affect melanoma behaviour and its role in therapy.[25–27] Interestingly, GPR40 agonists like palmitic acid may regulate melanin biosynthesis in melanocytes and melanoma cells.[28] Therefore, further investigations should consider melanin levels in primary melanoma and in melanoma metastases.

In conclusion, our data demonstrate distinct expression of GPR40 in primary melanoma and melanoma metastases in contrast to naevi. GPR40 expression correlates with higher Breslow tumour thickness and AJCC levels, as well as with significantly lower overall survival. Therefore, GPR40 is a new promising receptor in melanoma research and might be useful as a target for pharmacological intervention.

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