Immunohistochemical detection of the chondroitin sulfate proteoglycan 4 protein in primary and metastatic melanoma

ANNA GROSSAUER[1,2], KAROLINA URANOWSKA[1,3], MELITTA KITZWÖGERER[4], MARGIT MOSTEGEL[2], HEIMO BREITENEDER[3] and CHRISTINE HAFNER[1,5]

[1]Department of Dermatology, University Hospital St. Poelten, Karl Landsteiner University of Health Sciences, A-3100 St. Poelten; [2]Department of Pathology, University Hospital Krems, Karl Landsteiner University of Health Sciences, A-3500 Krems an der Donau; [3]Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, A-1090 Vienna; [4]Department of Pathology, University Hospital St. Poelten; [5]Karl Landsteiner Institute of Dermatological Research, Karl Landsteiner Gesellschaft, A-3100 St. Poelten, Austria

Received March 2, 2023; Accepted June 22, 2023

DOI: 10.3892/ol.2023.13968

Abstract. Treatment of malignant melanoma, the most aggressive form of skin cancer, continues to be a major challenge for clinicians. New targeted therapies with kinase inhibitors or drugs which modify the immune response are often accompanied by the development of resistance or severe side effects. In this context, chondroitin sulfate proteoglycan 4 (CSPG4), a highly immunogenic melanoma tumor antigen, could be a potential target for alternative therapeutic approaches. The aim of the present study was to identify differences in the levels of CSPG4 protein expression in primary and metastatic melanomas as well as to analyze correlations between CSPG4 expression and histopathological data and patient characteristics. A total of 189 melanoma tissue samples from Lower Austria, including primary melanomas and melanoma metastases, were immunohistochemically stained for the expression of CSPG4 and statistical analyses were performed. A total of 65.6% of melanoma tissue samples stained positive for the expression of CSPG4. Primary nodular and primary superficial spreading melanomas demonstrated a significantly higher number of positively stained tissue samples for CSPG4 compared with primary lentigo maligna melanomas. No significant differences in the expression of CSPG4 were demonstrated between primary melanomas and melanoma metastases. The present study supports the advancement of the understanding of CSPG4 tissue expression patterns in melanoma patients and provides additional information for further investigation of CSPG4 as a potential therapeutic target.

Correspondence to: Dr Christine Hafner, Department of Dermatology, University Hospital St. Poelten, Karl Landsteiner University of Health Sciences, Dunant Platz 1, A-3100 St. Poelten, Austria
E-mail: christine.hafner@edu.kl.ac.at

Abbreviations: ALM, acral lentiginous melanoma; CAR, chimeric antigen receptor; CSPG4, chondroitin sulfate proteoglycan 4; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; LMM, lentigo maligna melanoma; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase kinase; NM, nodular melanoma; NOS, not otherwise specified; SSM, superficial spreading melanoma

Key words: CSPG4, primary melanoma, metastatic melanoma, tumor antigen, immunohistochemistry

Introduction

Malignant melanoma continues to be a major challenge for clinicians with 324,635 newly diagnosed cases and 57,043 deaths worldwide in 2020 (1). According to the GLOBOCAN 2020 database, Austria has a moderate incidence of 13.5 cases per 100,000 person years (1). The data might however be underestimated as private pathology laboratories are not required to report the data to the national registry (2). Superficial spreading melanomas (SSM) represent the most common histopathological subtype in Central Europe, including Austria, followed by not otherwise specified (NOS) malignant melanomas and nodular melanomas (NM) (3). The majority of cutaneous melanomas confirmed using histopathology in Austria are classified as Tis or T1 (2). Melanomas in a more advanced stage, such as regional (III), distant (IV) or with a Breslow thickness >4 mm (pT4), were reported to present gender-related differences and to be more common in men than in women in Austria (2,4).

It previously been reported that ~50% of metastatic melanomas harbor a BRAF V600 activating mutation (5). Therefore, current therapeutic strategies include the combination of specific BRAF and MEK inhibitors (6-8). The use of the immune-checkpoint inhibitor ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 antibody, and the programmed cell death protein 1 inhibitors nivolumab and pembrolizumab demonstrate progress in the treatment of malignant melanoma (9-13). Despite the remarkable advances in targeted therapies and immunotherapy during the last decade, ~50% of patients with metastatic melanoma do not

survive >5 years after diagnosis (13,14). Therefore, there is an urgent need for additional therapeutic targets to enable better management of melanoma patients.

In the search for new treatment approaches in melanoma therapy the chondroitin sulfate proteoglycan 4 (CSPG4) has been reported to be an important molecule involved in the oncogenic potential of melanoma (15). CSPG4, also termed human high molecular weight-melanoma associated antigen or melanoma-associated chondroitin sulfate proteoglycan, was reported as a glycoprotein-proteoglycan complex on human melanoma cells nearly 40 years ago (16). It is composed of a large extracellular domain, a transmembrane region and a short cytoplasmic tail (17). CSPG4 does not possess any catalytic function on its own but it associates with receptors that contain an intrinsic receptor tyrosine kinase activity (15). The mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and the integrin-regulated focal adhesion kinase signaling pathway were proposed as the two major signaling pathways associated with CSPG4 activity (18). The enhanced downstream signaling of these pathways can promote tumor progression via cellular functions such as adhesion, migration, proliferation and survival (18). It has been reported that the cytoplasmic domain of CSPG4 contains a phospho-acceptor site at Thr-2314, which is phosphorylated by ERK1/2, which results in the stimulation of cell proliferation (19). Moreover, the expression of full length CSPG4 has been reported to be necessary for the maximal ERK1/2 activation in melanoma cells possessing a BRAF V600E mutation (20). Inhibition of CSPG4 with short interfering RNA has been reported to lead to a reduction in constitutive ERK1/2 activation (20). Consequently, inhibition of ERK1/2 with specific MEK inhibitors reduces CSPG4-dependent growth and the motility of BRAF-mutant cells (20).

Originally, expression of CSPG4 was only associated with malignant melanomas (21). In recent years the proteoglycan has also been reported to be present in numerous other cancer entities, including triple-negative breast cancer, glioma, squamous carcinoma of the head and neck (22), certain types of leukemia (23), pancreatic tumors (24), soft-tissue sarcomas (25), anaplastic thyroid cancer (26), osteosarcomas (27,28) and ovarian cancer (29).

CSPG4 expression in malignant melanoma has been reported to vary among the different histopathological subtypes (30-32). An early study by Kageshita *et al* (30) reported that CSPG4 expression in acral lentiginous melanoma (ALM) was significantly higher in metastatic lesions compared with primary lesions. This was observed both in terms of the number of tissue samples positively stained for CSPG4 and the percentage of stained melanoma cells within each lesion (30). Furthermore, Kageshita *et al* (30) reported that CSPG4 expression was more prevalent in primary NM lesions compared with primary ALM lesions and that the percentage of positively stained cells within each lesion was significantly higher in this subtype. Notably, CSPG4 expression in primary ALM lesions has been reported to be negatively associated with survival (30,33). In NM, the expression of CSPG4 was reported to be consistently high in both primary and metastatic lesions, with >90% of tissue samples demonstrating positive staining in each group (30). Similarly, in SSM, CSPG4 was expressed in the majority of stained cases (31). However, in mucosal melanoma, the frequency of CSPG4 expression was reported to be lower in primary lesions compared with metastatic lesions (32). A high expression of CSPG4 was demonstrated in ≤95% of uveal melanoma samples (34).

Certain approaches which make use of CSPG4 as a potential therapeutic target in the treatment of melanoma have already been reported, including monoclonal antibodies (35-40), mimotope vaccines (41,42), anti-idiotypic monoclonal antibodies (43-45), fusion proteins (46-48), CAR-T cells (27,49-54), bispecific T-cell engagers (55), antibody-drug conjugate (56) or targeted radioimmunotherapy (57-59). Those strategies rely on different mechanisms of action, as reviewed previously (60). Among these approaches, CAR-T cells targeting CSPG4 hold particular promise due to their ability to specifically recognize and eliminate CSPG4-expressing tumor cells, which makes them a highly attractive therapeutic option for further investigation in a clinical setting.

A more detailed analysis of CSPG4 expression in melanoma samples, including primary tumors as well as metastases, could support new approaches for melanoma therapy. Therefore, the present study assessed CSPG4 protein expression in melanoma tissue samples, both primary tumors and metastases, to evaluate the histopathological data as well as detailed patient characteristics.

Materials and methods

Patients and tissue samples. A total of 189 melanoma tissue samples were obtained from 159 Caucasian patients, who had been histologically diagnosed with primary melanoma (n=104) or a melanoma metastasis (n=85) at the Department of Pathology at the University Hospital Krems (Krems an der Donau, Austria) or at the Department of Pathology at the University Hospital St. Poelten (St. Poelten, Austria) between January 2010 and August 2018. Residual tissue samples were used for the present study. Clinicopathological data recorded with the samples included sex, age at diagnosis, histopathological subtype of primary melanomas (nodular, superficial spreading, lentigo maligna, acral lentiginous, mucosal or NOS), site of melanoma metastases (cutaneous, subcutaneous, lymph node, lung or other visceral metastases) and BRAF mutation status (wild type, V600E or V600K).

For primary melanoma tissue samples, the tumor thickness in mm (according to Breslow), the ulceration status and T classification were also evaluated (61). For routine histopathology, the formalin-fixed samples (10% buffered formalin) were placed in a Tissue-Tek VIP machine (Sakura Finetek Europe) overnight for dehydration and clearing, following the manufacturer's instructions. Samples were then embedded in paraffin, cut into 2-3 μm thick sections and stained using hematoxylin and eosin (Tissue-Tek Prisma H&E Stain Kit#1, cat. no. 6190; Sakura Finetek Europe), following the manufacturer's instruction. Routine immunohistochemistry (IHC) for HMB45, cytokeratin AE1/AE3, Melan A, S100, Vimentin, Ki-67 was performed using the fully-automated Benchmark ULTRA staining platform (Roche Tissue Diagnostics; Roche Diagnostics, Ltd) to support the histopathological diagnosis of primary malignant melanoma or melanoma metastasis. The following ready-to-use primary antibodies (Roche Diagnostics Ltd.) were used and the recommended staining

procedure (temperature and duration of incubation) was applied: Anti-HMB45 mouse mAb (cat. no. 05479282001; incubation, 8 min at 36˚C), anti-Pan Keratin mouse mAbs (cat. no. 05267145001; incubation, 8 min at 36˚C), anti-Melan A mouse mAb (cat. no. 05278350001; incubation, 32 min at 36˚C), anti-S100 mouse mAb (cat. no. 05278104001; incubation, 8 min at 36˚C), anti-Vimentin mouse mAb (cat. no. 05278139001; incubation, 16 min at 36˚C) and anti-Ki-67 rabbit mAb (cat. no. 05278384001; incubation, 16 min at 36˚C). The antibodies were visualized using an UltraView Universal Detection Kit (cat. no. 760-501; Roche Diagnostics Ltd.) according to the manufacturer's instructions. Stained sections were examined using a light microscope (Olympus BX53; Olympus Europa SE & Co. KG). NOS melanomas were mainly punch biopsies that did not allow a more detailed diagnosis of the primary melanoma. The group 'other visceral metastases' comprised different parts of the gastrointestinal tract and other organ systems that occurred in a scattered manner in the sample group and were therefore pooled. The tumor (T) classification for primary melanomas was determined according to the eighth edition of the American Joint Committee on Cancer melanoma staging system (61). Information regarding the BRAF mutation status was obtained from data files for each sample. The routine testing procedure involved utilizing either the cobas 4800 BRAF V600 mutation test (Roche Diagnostics, Ltd) or the BRAF-strip Assay (ViennaLab Diagnostics GmbH). The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Karl Landsteiner University of Health Sciences (Krems an der Donau, Austria; approval no. 1031/2018).

CSPG4 staining and scoring. All tissue samples were stained for CSPG4 expression at the Department of Pathology at the University Hospital St. Poelten. Formalin-fixed paraffin-embedded tissue samples were deparaffinized and stained using the BenchMark XT automated immune-staining platform (Roche Tissue Diagnostics; Roche Diagnostics, Ltd) and the ultraView Universal DAB detection system (Roche Tissue Diagnostics; Roche Diagnostics, Ltd) according to the manufacturer's protocol. Briefly, heat-induced antigen retrieval was performed for 10 min at 95˚C. Then the tissue sections were incubated in 3% H_2O_2 for 14 min at room temperature. Following this, Inhibitor CM (Roche Diagnostics Ltd.) was applied, and slides were incubated for 4 min at 37˚C. Next, the sections were incubated with a mouse monoclonal antibody specific to CSPG4 (1:200; cat. no. ab50009; Abcam) overnight at 4˚C. The slides were then incubated with OmniMap anti-Ms HRP for 16 min in conjunction with the ultraView Universal DAB detection system (cat. no. 760-700; Roche Diagnostics Ltd.), following the manufacturer's instructions. Between the steps, slides were washed with the Reaction buffer (Tris-based buffer, pH 7.6-7.8; cat. no. 950-300; Roche Diagnostics Ltd.). Finally, the samples were counterstained with Hematoxylin II (cat. no. 790-2208; Roche Diagnostics Ltd.) and Bluing Reagent (cat. no. 760-2037; Roche Diagnostics Ltd.) for 3 min at room temperature, and dehydrated in graded ethanol and xylene.

Scoring of tissue slides was performed independently by two investigators. Stained sections were examined using an Olympus BX53 microscope (Olympus Europa SE & Co. KG). Expression of CSPG4 was categorized visually according to a four-tiered scale as follows: i) 0, negative (a complete loss of CSPG4 expression); ii) +, weakly positive staining (<25% cells stained positive for CSPG4); iii) ++, moderately positive staining (25-50% cells stained positive for CSPG4); and iv) +++, strongly positive staining (>50% cells stained positive for CSPG4).

Statistical analysis. For statistical analysis, CSPG4 expression was dichotomized into positive (including +, ++ and +++) and negative groups. The T classification of primary melanomas was simplified to pT1-pT4 (pT1, tumor thickness according to Breslow ≤1 mm; pT2, >1-2 mm; pT3, >2-4 mm; and pT4, >4 mm) (61). The supplementary staging criterion non-ulcerated or ulcerated was considered as the separate variable 'ulceration status of primary melanomas'. The numeric parameters, age at diagnosis and tumor thickness were tested for an association with CSPG4 expression using the Mann-Whitney-U test. Pearson's χ^2 test was used to analyze associations between qualitative clinicopathological data and CSPG4 expression. When cells had an expected count of <5 in the crosstabulation, Fisher's Exact test was used instead of Pearson's χ^2. For a more detailed analysis of significant results in the χ^2 test or Fisher's Exact test the column proportion test (Z-test) and the Bonferroni method to adjust P-values for multiple comparisons were used. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS Statistics, version 24.0 (IBM Corp.).

Results

Histopathological and clinical characteristics of the sample group. A total of 196 melanoma tissue samples were stained for CSPG4. CSPG4 expression could not be evaluated in seven samples due to technical reasons or missing tumor tissues. Therefore, the final data set included 189 melanoma tissue samples. Tables I and II presented the absolute frequencies for all characteristics of the sample group. A flowchart indicated the division and classification of the melanoma tumor samples (Fig. 1).

A total of 104 of the tissue samples (55.0%) were histologically diagnosed as primary melanomas and 85 (45.0%) as melanoma metastases (Table I and Fig. 1). Ninety-two of the tissue samples (48.7%) were obtained from female patients and 97 (51.3%) from male patients (Table I). The median age at diagnosis of the sample group was 71 years (range, 25-93 years). Data for tumor thickness, ulceration status and T classification were available for 86 primary melanomas. These data were not available for mucosal melanomas and for most of the NOS primary melanomas. The median tumor thickness (according to Breslow) of primary melanoma tissue samples was 2 mm (range, 0.2-25 mm), and 36 (41.9%) presented with an ulceration and 50 (58.1%) did not (Table I). A total of 33 of the primary melanoma tissue samples (38.4%) were classified as pT1, 13 (15.1%) as pT2, 14 (16.3%) as pT3 and 26 (30.2%) as pT4 (Table I). BRAF mutation status analyses were available for 74 tissue samples, including primary melanomas and melanoma metastases, and 32 (43.2%) samples demonstrated a BRAF V600E mutation (Table I). In terms of the histopathological subtype of the primary melanomas, NM were represented with the highest number (n=41; 39.4%),

Table I. Association of CSPG4 expression and clinicopathological parameters.

Clinicopathological parameters	Samples, n	Expression of CSPG4		P-value
		Negative, n (%)	Positive, n (%)	
Sex				0.182[a]
Female	92	36 (39.1)	56 (60.9)	
Male	97	29 (29.9)	68 (70.1)	
Diagnosis				0.813[a]
Primary melanoma	104	35 (33.7)	69 (66.3)	
Melanoma metastasis	85	30 (35.3)	55 (64.7)	
Ulceration status of primary melanomas				0.742[a]
Ulcerated	36	12 (33.3)	24 (66.7)	
Non-ulcerated	50	15 (30.0)	35 (70.0)	
T classification of primary melanomas				1.000[b]
pT1	33	11 (33.3)	22 (66.7)	
pT2	13	4 (30.8)	9 (69.2)	
pT3	14	4 (28.6)	10 (71.4)	
pT4	26	8 (30.8)	18 (69.2)	
BRAF mutation status				0.214[b]
Wild type	40	14 (35.0)	26 (65.0)	
V600E	32	6 (18.8)	26 (81.3)	
V600K	2	1 (50.0)	1 (50.0)	

[a]Pearson's χ^2 test, [b]Fisher's exact test. pT1-4, classification of primary melanoma according to AJCC (61); CSPG4, chondroitin sulfate proteoglycan 4.

Table II. Association of CSPG4 expression and histopathological diagnosis.

Histopathological diagnosis	Samples, n	Expression of CSPG4		P-value
		Negative, n (%)	Positive, n (%)	
Primary melanoma subtypes				0.009[a]
Nodular	41	10 (24.4)	31 (75.6)	
Superficial spreading	28	7 (25.0)	21 (75.0)	
Lentigo maligna	8	7 (87.5)	1 (12.5)	
Acral lentiginous	3	2 (66.7)	1 (33.3)	
Mucosal	11	5 (45.5)	6 (54.5)	
Not otherwise specified	13	4 (30.8)	9 (69.2)	
Site of melanoma metastases				0.882[a]
Cutaneous	7	3 (42.9)	4 (57.1)	
Subcutaneous	25	7 (28.0)	18 (72.0)	
Lymph node	20	7 (35.0)	13 (65.0)	
Lung	14	5 (35.7)	9 (64.3)	
Other visceral metastases	19	8 (42.1)	11 (57.9)	

[a]Fisher's exact test. CSPG4, chondroitin sulfate proteoglycan 4.

followed by SSM (n=28; 26.9%) (Table II and Fig. 1). Among the melanoma metastases group, the most common were subcutaneous metastases (n=25; 29.4%), followed by lymph node metastases (n=20; 23.5%) (Table II and Fig 1).

Immunohistochemical analysis of the expression of CSPG4. After staining the samples using CSPG4-specific antibodies, 124 samples (65.6%) were demonstrated to be positive for the expression of CSPG4. Among these, 47 (24.9%) were classified

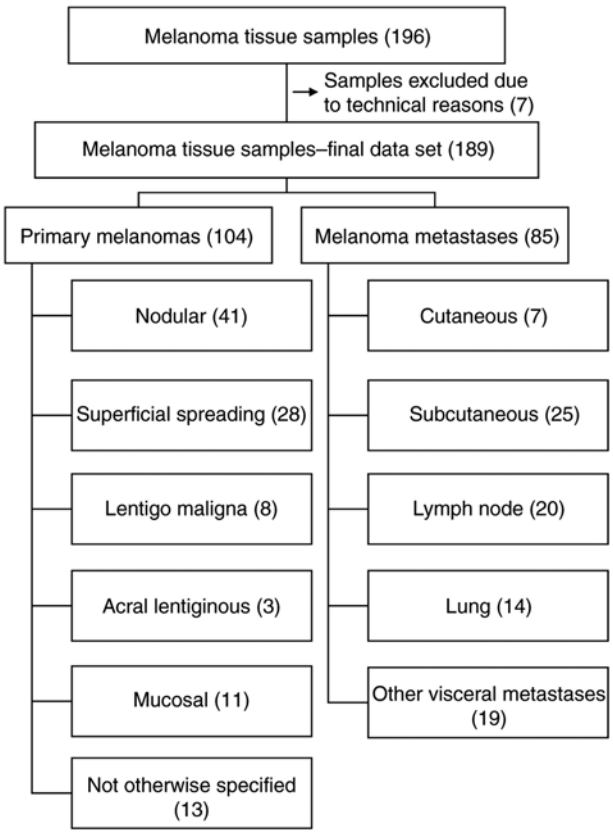

Figure 1. Flowchart illustrating the classification of melanoma tumor samples. The numbers in parentheses illustrate the respective counts for each group.

as +, 62 (32.8%) as ++, and 15 (7.9%) as +++. Sixty-five (34.4%) samples demonstrated no expression of CSPG4. No immunohistochemical staining for CSPG4 was demonstrated in the adjacent normal skin of the tumor tissues within the tissue samples, including both primary melanomas and cutaneous metastases (data not shown). Immunohistochemical analysis of CSPG4 expression in the sample group, which included a total of 189 melanoma tissue samples, was performed (Table III). Examples of H&E staining and CSPG4 staining for primary and metastatic melanomas were presented in Fig. 2.

Association between CSPG4 expression and clinicopathological characteristics. Details of the frequencies of positive and negative CSPG4 staining among the different clinicopathological parameters were presented (Table I). There was no significant association between the expression of CSPG4 in melanoma tissue samples and the demographic parameters sex (P=0.182) and age at diagnosis (P=0.121). Furthermore, no significant association with CSPG4 expression was demonstrated for tumor thickness (P=0.713), ulceration status (P=0.742) and T classification (P=1.000). The vast majority of BRAF V600E mutated tissue samples was stained positive for CSPG4 expression (n=26; 81.3%) and only few samples with a BRAF V600E mutation demonstrated no CSPG4 expression (n=6; 18.8%).

CSPG4 expression in primary melanomas and melanoma metastases. Frequencies of CSPG4 expression among the different primary melanoma subtypes and the different sites of

Table III. Immunohistochemical expression of CSPG4 in the sample group.

Expression of CSPG4	Samples, n (%)
Negative	65 (34.4)
Positive	
+	47 (24.9)
++	62 (32.8)
+++	15 (7.9)
Combined total	124 (65.6)

Percentage of the individual sample groups is relative to the total sample group (n=189). +, 1-24% of tumor cells express CSPG4; ++, 25-50% of tumor cells express CSPG4; +++, >50% of tumor cells express CSPG4. CSPG4, chondroitin sulfate proteoglycan 4.

melanoma metastases were summarized (Table II). There was a significant association between primary melanoma subtypes and CSPG4 protein expression (P=0.009). Therefore, a further evaluation was performed using the column proportion test (Z-test) in SPSS to evaluate which subtypes demonstrated significant differences. This analysis demonstrated that the number of primary nodular (P=0.009) and SSM lesions (P=0.021) stained positive for CSPG4 was significantly higher than the number of primary lentigo maligna melanomas (LMM). Primary nodular (n=31; 75.6%), primary superficial spreading (n=21; 75.0%) and mucosal (n=6; 54.5%) melanomas demonstrated positive staining for CSPG4 expression. However, only 1/8 (12.5%) primary LMM and 1/3 (33.3%) ALM demonstrated CSPG4 expression (Table II). No significant association was demonstrated (P=0.882) regarding the site of melanoma metastases. CSPG4 expression demonstrated a comparable distribution between negative and positive CSPG4 expression across different metastatic sites, including cutaneous, subcutaneous, lymph node, lung and other visceral metastases (Table II).

In total 69 (66.3%) of the primary melanoma tissue samples stained positive for the expression of CSPG4 and 35 (33.7%) samples were negative (Table I). Fifty-five (64.7%) samples in the melanoma metastases group were positive for CSPG4 expression and 30 (35.3%) were negative (Table I). There was no significant difference in the frequency of CSPG4 expression between the primary melanoma and melanoma metastases groups (P=0.813) (Table I).

Discussion

CSPG4 is a transmembrane proteoglycan involved in the oncogenic potential of malignant melanoma through numerous cellular mechanisms (15). Determining the expression of CSPG4 among a large number of primary and metastatic melanoma lesion samples may contribute to the demonstration of the clinical significance of CSPG4 and to defining its role in new treatment approaches. Previous studies have reported differences in the expression of the proteoglycan among the different histopathological subtypes of primary and metastatic malignant melanoma lesions (30-32). The present study

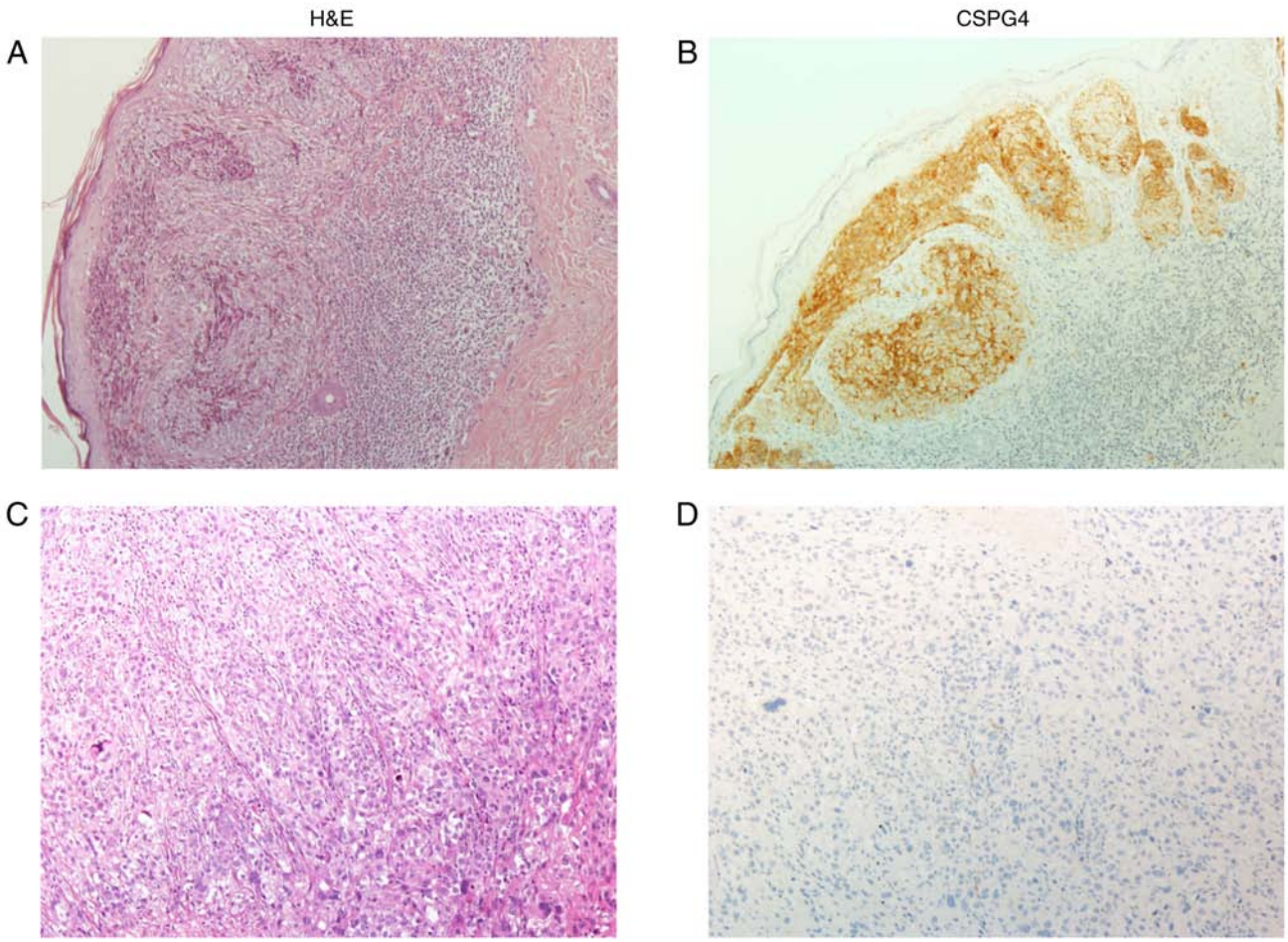

Figure 2. H&E and CSPG4 staining of representative primary melanoma and melanoma metastases. (A) Primary melanoma sample (magnification x100). (B) Positive immunohistochemical staining of a primary melanoma with a mAb specific for CSPG4 (magnification, x20). (C) Sample of a melanoma metastasis (magnification x100). (D) Negative immunohistochemical staining of a melanoma metastasis with a mAb specific for CSPG4 (magnification, x20). CSPG4, chondroitin sulfate proteoglycan 4; H&E, hematoxylin and eosin; mAb, monoclonal antibody.

demonstrated significant differences in CSPG4 protein expression in an analysis of a large number of archived melanoma tissue samples, including primary and metastatic lesions, from different histopathological subtypes.

As it has been reported that melanoma represents an underestimated disease burden in Austria, research on this topic is of importance (2). To the best of our knowledge, the present study represents the largest collection of melanoma samples systematically analyzed for the expression of a melanoma antigen in the region of Lower Austria to date. It should be noted that patients who underwent primary surgical intervention in a hospital setup tended to have thicker primary melanomas. Consequently, there was a higher frequency of nodular melanomas observed in these cases (Table II). It is important to acknowledge, that the data presented in the present study represents the collection of tumor materials from a specific group of institutions within a defined period of time.

The majority of tissue samples included in the present study demonstrated positive staining for the expression of CSPG4, both in the primary melanoma and melanoma metastases groups (Table I). An early study reported by Natali *et al* (21) in 1983 reported higher (>75%) percentages of melanoma tissue samples which stained positive for the expression of CSPG4. The reported differences in the number of tissue samples staining positive for the expression of CSPG4 may be attributed to the use of different antibodies that target distinct determinants of the proteoglycan. The monoclonal antibody used in the present study was validated for its suitability to stain formalin-fixed paraffin-embedded tissue samples specifically for CSPG4.

There was no significant difference in the frequency of CSPG4 expression between primary melanoma and melanoma metastasis lesions in the study population (Table I), which supported the previous hypothesis that CSPG4 already has an impact in the formation process of metastases (15). A higher frequency of cells which expressed CSPG4 in metastatic lesions than in primary ones has, to the best of our knowledge, only previously been described for ALM and mucosal melanomas (30,32). Given that the sample cohort in the present study primarily included NM and SSM, no statistically significant differences were demonstrated between primary and metastatic tumor samples. Further studies examining the detailed mechanisms of up- and down-regulation of CSPG4 expression in primary and metastatic lesions would therefore be of interest.

No correlation was demonstrated between CSPG4 expression and clinicopathological parameters, such as sex, age,

BRAF mutation status, tumor thickness, ulceration status and T-classification of primary melanomas in the present study (Table I). A previous study by Kageshita *et al* (33) which also tested for an association between the factors such as age and stage of disease, and CSPG4 expression among primary ALM tissue samples, reported a significance between these clinical parameters and CSPG4 expression in this subtype of primary melanomas. The same study also reported an inverse correlation between CSPG4 expression and survival in primary ALM lesions, which emphasized the prognostic significance of CSPG4 (33). A significant finding in survival analysis has only been observed thus far in primary ALM lesions for CSPG4-expressing melanomas. Unfortunately, due to limitations of the study protocol, no information on the treatment status of the patients from whom samples were collected was available; as a result, it was not possible to calculate the correlation between the expression of CSPG4 and treatment modalities or overall survival within the study cohort.

The BRAF mutational status was available for 74 (39.1%) tissue samples in the present study (Table I). As analysis of the BRAF mutational status has been available only in the recent years, it has not been performed for all tissue samples in the present study, some of which date back to the year 2010.

The present study demonstrated that the vast majority of tissue samples with a confirmed BRAF V600E mutation also expressed CSPG4 (Table I). This finding supported the observations made in an earlier study which reported that CSPG4 seemed to function with an important role in a strong and sustained activation of ERK1,2 in BRAF-mutant melanoma cell lines (20). A detailed analysis of CSPG4 expression in BRAF-mutant melanomas is of importance when defining the role of this proteoglycan in tumor advancement and regarding it as a potential therapeutic target. Indeed, Yu *et al* (36) reported that a combination therapy of the BRAF-selective inhibitor vemurafenib with the CSPG4-specific monoclonal antibody (mAb) 225.28 resulted in a more effective inhibition of CSPG4-positive melanoma cells with a BRAF V600E mutation. The addition of mAb 225.28 to vemurafenib was also reported to delay the development of resistance to this therapy (36), which further supported the oncogenic potential of CSPG4 in BRAF mutant melanomas. It has also been reported that CSPG4-specific anti-225D9+-TT polyclonal antibodies enhanced the anti-proliferative effects of the BRAF inhibitor, PLX4032, in melanoma cells (62). In addition, a recent study reported that when combined with PLX4032, the anti-CSPG4 mAb 9.2.27 contributed to a significant, additional inhibition of melanoma cell viability, compared with cells treated with BRAF inhibitor alone (37).

The present study demonstrate a high prevalence of CSPG4 expression in primary NM and primary SSM (Table II), and in line with previous reports (30,31), CSPG4 was expressed in a distinct majority of stained tissue samples in these subtypes of primary melanomas. In primary LMM the frequency of CSPG4 expression was significantly lower than that in NM and SSM (Table II) and, to the best of our knowledge, the present study is the first to describe this significant difference. Previous studies had only reported a significantly lower frequency of cells which expressed CSPG4 in primary ALM tissue samples (30,31). In this retrospective analysis of CSPG4 expression in primary melanomas a low frequency of primary ALM tissue samples that stained positive for CSPG4 were observed. However, this result was not significant, which might be due to the small sample number of primary ALMs included in the present study (Table II). As ALMs are not that common in the Caucasian population (63), only a limited number of samples were available for inclusion in the present study.

It would be valuable to perform a prospective analysis of CSPG4 expression in a substantial number of melanoma tissue samples both before and after treatment with kinase inhibitors, as well as other treatment approaches such as immunotherapy. Our group has previously reported that CSPG4 expression is downregulated after treatment with a BRAF/MEK inhibitor by retrospectively analyzing a small number of patient-derived tumor samples (64). It has been reported that shedding of tumor antigens, such as carcinoembryonic antigen into the blood circulation could potentially suppress antitumor CD8+ T-cell function (65). It could be hypothesized that a similar phenomenon might occur for CSPG4.

In summary, the present study utilized a cohort of melanoma tissue samples from Lower Austria, which provided valuable insights into the expression profile of CSPG4 in this specific Caucasian population. By correlating CSPG4 expression with both histopathological characteristics and patient characteristics, w the clinical relevance and potential implications of CSPG4 expression in melanoma subtypes was demonstrated, which laid the foundation for personalized treatment strategies. Notably, the present study demonstrated a previously unreported finding of low CSPG4 expression in LMM within this particular population, which adds to our understanding of the heterogeneity of CSPG4 expression in melanoma.

Acknowledgements

The authors wish to acknowledge Mag. Konrad Kogler and Dipl. Ing. Alfred Zens (NÖ Landesgesundheitsagentur, the legal entity of University Hospitals in Lower Austria), for their contribution in providing the organizational framework for conducting this research.

Funding

The present study was funded by the NÖ Forschungs-und Bildungsges.m.b.H. (grant no. LSC15-007). The authors also acknowledge the support of the Open Access Publishing Fund of the Karl Landsteiner University of Health Sciences.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

AG and CH conceived and designed the study, and analyzed and interpreted the data. KU and HB participated in designing the study and in analyzing the data. MK and MM analyzed and interpreted the data. CH and HB confirm the authenticity of all the raw data. AG, CH, KU and HB wrote the manuscript. All

authors have read and revised the manuscript, and approved the final version.

Ethics approval and consent to participate

All histopathological samples and clinical data were obtained from the collection of patient samples at the Department of Pathology at the University Hospital St. Poelten, Karl Landsteiner University of Health Sciences and the Department of Pathology at the University Hospital Krems, Karl Landsteiner University of Health Sciences. The collection and storage of archival tissue samples and data were performed according to local ethical guidelines. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Karl Landsteiner University (Krems an der Donau, Austria; approval no. 1031/2018). Informed patient consent was not required due to the retrospective nature of this study, in accordance with local ethical guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. World Health Organization, International Agency for Research on Cancer (IARC): GLOBOCAN 2020: Estimated incidence, mortality and prevalence rates in 2020, melanoma of skin. Available from: http://gco.iarc.fr/today/home [Accessed Feb 15, 2022].
2. Monshi B, Vujic M, Kivaranovic D, Sesti A, Oberaigner W, Vujic I, Ortiz-Urda S, Posch C, Feichtinger H, Hackl M and Rappersberger K: The burden of malignant melanoma-lessons to be learned from Austria. Eur J Cancer 56: 45-53, 2016.
3. Crocetti E, Mallone S, Robsahm TE, Gavin A, Agius D, Ardanaz E, Lopez MC, Innos K, Minicozzi P, Borgognoni L, *et al*: Survival of patients with skin melanoma in Europe increases further: Results of the EUROCARE-5 study. Eur J Cancer 51: 2179-2190, 2015.
4. Duschek N, Skvara H, Kittler H, Delir G, Fink A, Pinkowicz A and Waldhor T: Melanoma epidemiology of Austria reveals gender-related differences. Eur J Dermatol 23: 872-878, 2013.
5. Platz A, Egyhazi S, Ringborg U and Hansson J: Human cutaneous melanoma; a review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site. Mol Oncol 1: 395-405, 2008.
6. Larkin J, Ascierto PA, Dreno B, Atkinson V, Liszkay G, Maio M, Mandala M, Demidov L, Stroyakovskiy D, Thomas L, *et al*: Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. N Engl J Med 371: 1867-1876, 2014.
7. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, Lichinitser M, Dummer R, Grange F, Mortier L, *et al*: Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med 372: 30-39, 2015.
8. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, Garbe C, Jouary T, Hauschild A, Grob JJ, *et al*: Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: A multicentre, double-blind, phase 3 randomised controlled trial. Lancet 386: 444-451, 2015.
9. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, *et al*: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363: 711-723, 2010.
10. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, Hamid O, Robert C, Ascierto PA, Richards JM, *et al*: Adjuvant ipilimumab versus placebo after complete resection of high-risk stage III melanoma (EORTC 18071): A randomised, double-blind, phase 3 trial. Lancet Oncol 16: 522-530, 2015.
11. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, Dalle S, Schenker M, Chiarion-Sileni V, Marquez-Rodas I, *et al*: Adjuvant Nivolumab versus ipilimumab in resected stage III or IV melanoma. N Engl J Med 377: 1824-1835, 2017.
12. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, Daud A, Carlino MS, McNeil C, Lotem M, *et al*: Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 372: 2521-2532, 2015.
13. Schachter J, Ribas A, Long GV, Arance A, Grob JJ, Mortier L, Daud A, Carlino MS, McNeil C, Lotem M, *et al*: Pembrolizumab versus ipilimumab for advanced melanoma: Final overall survival results of a multicentre, randomised, open-label phase 3 study (KEYNOTE-006). Lancet 390: 1853-1862, 2017.
14. Ribas A, Lawrence D, Atkinson V, Agarwal S, Miller WH Jr, Carlino MS, Fisher R, Long GV, Hodi FS, Tsoi J, *et al*: Combined BRAF and MEK inhibition with PD-1 blockade immunotherapy in BRAF-mutant melanoma. Nat Med 25: 936-940, 2019.
15. Price MA, Wanshura LE, Yang J, Carlson J, Xiang B, Li G, Ferrone S, Dudek AZ, Turley EA and McCarthy JB: CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. Pigment Cell Melanoma Res 24: 1148-1157, 2011.
16. Bumol TF and Reisfeld RA: Unique glycoprotein-proteoglycan complex defined by monoclonal antibody on human melanoma cells. Proc Natl Acad Sci USA 79: 1245-1249, 1982.
17. Pluschke G, Vanek M, Evans A, Dittmar T, Schmid P, Itin P, Filardo EJ and Reisfeld RA: Molecular cloning of a human melanoma-associated chondroitin sulfate proteoglycan. Proc Natl Acad Sci USA 93: 9710-9715, 1996.
18. Yang J, Price MA, Neudauer CL, Wilson C, Ferrone S, Xia H, Iida J, Simpson MA and McCarthy JB: Melanoma chondroitin sulfate proteoglycan enhances FAK and ERK activation by distinct mechanisms. J Cell Biol 165: 881-891, 2004.
19. Makagiansar IT, Williams S, Mustelin T and Stallcup WB: Differential phosphorylation of NG2 proteoglycan by ERK and PKCalpha helps balance cell proliferation and migration. J Cell Biol 178: 155-165, 2007.
20. Yang J, Price MA, Li GY, Bar-Eli M, Salgia R, Jagedeeswaran R, Carlson JH, Ferrone S, Turley EA and McCarthy JB: Melanoma proteoglycan modifies gene expression to stimulate tumor cell motility, growth, and epithelial-to-mesenchymal transition. Cancer Res 69: 7538-7547, 2009.
21. Natali PG, Giacomini P, Russo C, Steinbach G, Fenoglio C and Ferrone S: Antigenic profile of human melanoma cells. Analysis with monoclonal antibodies to histocompatibility antigens and to melanoma-associated antigens. J Cutan Pathol 10: 225-237, 1983.
22. Wang X, Wang Y, Yu L, Sakakura K, Visus C, Schwab JH, Ferrone CR, Favoino E, Koya Y, Campoli MR, *et al*: CSPG4 in cancer: Multiple roles. Curr Mol Med 10: 419-429, 2010.
23. Fenton M, Whiteside TL, Ferrone S and Boyiadzis M: Chondroitin sulfate proteoglycan-4 (CSPG4)-specific monoclonal antibody 225.28 in detection of acute myeloid leukemia blasts. Oncol Res 22: 117-121, 2015.
24. Keleg S, Titov A, Heller A, Giese T, Tjaden C, Ahmad SS, Gaida MM, Bauer AS, Werner J and Giese NA: Chondroitin sulfate proteoglycan CSPG4 as a novel hypoxia-sensitive marker in pancreatic tumors. PLoS One 9: e100178, 2014.
25. Hsu SC, Nadesan P, Puviindran V, Stallcup WB, Kirsch DG and Alman BA: Effects of chondroitin sulfate proteoglycan 4 (NG2/CSPG4) on soft-tissue sarcoma growth depend on tumor developmental stage. J Biol Chem 293: 2466-2475, 2018.
26. Egan CE, Stefanova D, Ahmed A, Raja VJ, Thiesmeyer JW, Chen KJ, Greenberg JA, Zhang T, He B, Finnerty BM, *et al*: CSPG4 is a potential therapeutic target in anaplastic thyroid cancer. Thyroid 31: 1481-1493, 2021.
27. Beard RE, Zheng Z, Lagisetty KH, Burns WR, Tran E, Hewitt SM, Abate-Daga D, Rosati SF, Fine HA, Ferrone S, *et al*: Multiple chimeric antigen receptors successfully target chondroitin sulfate proteoglycan 4 in several different cancer histologies and cancer stem cells. J Immunother Cancer 2: 25, 2014.

28. Riccardo F, Tarone L, Iussich S, Giacobino D, Arigoni M, Sammartano F, Morello E, Martano M, Gattino F, De Maria R, *et al*: Identification of CSPG4 as a promising target for translational combinatorial approaches in osteosarcoma. Ther Adv Med Oncol 11: 1758835919855491, 2019.
29. Yang J, Liao Q, Price M, Moriarity B, Wolf N, Felices M, Miller JS, Geller MA, Bendzick L, Hopps R, *et al*: Chondroitin sulfate proteoglycan 4, a targetable oncoantigen that promotes ovarian cancer growth, invasion, cisplatin resistance and spheroid formation. Transl Oncol 16: 101318, 2022.
30. Kageshita T, Nakamura T, Yamada M, Kuriya N, Arao T and Ferrone S: Differential expression of melanoma associated antigens in acral lentiginous melanoma and in nodular melanoma lesions. Cancer Res 51: 1726-1732, 1991.
31. Nishi H, Inoue Y, Kageshita T, Takata M and Ihn H: The expression of human high molecular weight melanoma-associated antigen in acral lentiginous melanoma. Biosci Trends 4: 86-89, 2010.
32. Kageshita T, Kimura T, Yoshi A, Hirai S, Ono T and Ferrone S: Antigenic profile of mucosal melanoma lesions. Int J Cancer 56: 370-374, 1994.
33. Kageshita T, Kuriya N, Ono T, Horikoshi T, Takahashi M, Wong GY and Ferrone S: Association of high molecular weight melanoma-associated antigen expression in primary acral lentiginous melanoma lesions with poor prognosis. Cancer Res 53: 2830-2833, 1993.
34. Li Y, Madigan MC, Lai K, Conway RM, Billson FA, Crouch R and Allen BJ: Human uveal melanoma expresses NG2 immunoreactivity. Br J Ophthalmol 87: 629-632, 2003.
35. Hafner C, Breiteneder H, Ferrone S, Thallinger C, Wagner S, Schmidt WM, Jasinska J, Kundi M, Wolff K, Zielinski CC, *et al*: Suppression of human melanoma tumor growth in SCID mice by a human high molecular weight-melanoma associated antigen (HMW-MAA) specific monoclonal antibody. Int J Cancer 114: 426-432, 2005.
36. Yu L, Favoino E, Wang Y, Ma Y, Deng X and Wang X: The CSPG4-specific monoclonal antibody enhances and prolongs the effects of the BRAF inhibitor in melanoma cells. Immunol Res 50: 294-302, 2011.
37. Uranowska K, Samadaei M, Kalic T, Pinter M, Breiteneder H and Hafner C: A chondroitin sulfate proteoglycan 4-specific monoclonal antibody inhibits melanoma cell invasion in a spheroid model. Int J Oncol 59: 70, 2021.
38. Schroff RW, Woodhouse CS, Foon KA, Oldham RK, Farrell MM, Klein RA and Morgan AC Jr: Intratumor localization of monoclonal antibody in patients with melanoma treated with antibody to a 250,000-dalton melanoma-associated antigen. J Natl Cancer Inst 74: 299-306, 1985.
39. Schroff RW, Morgan AC Jr, Woodhouse CS, Abrams PG, Farrell MM, Carpenter BE, Oldham RK and Foon KA: Monoclonal antibody therapy in malignant melanoma: Factors effecting in vivo localization. J Biol Response Mod 6: 457-472, 1987.
40. Oldham RK, Foon KA, Morgan AC, Woodhouse CS, Schroff RW, Abrams PG, Fer M, Schoenberger CS, Farrell M and Kimball E: Monoclonal antibody therapy of malignant melanoma: In vivo localization in cutaneous metastasis after intravenous administration. J Clin Oncol 2: 1235-1244, 1984.
41. Wagner S, Hafner C, Allwardt D, Jasinska J, Ferrone S, Zielinski CC, Scheiner O, Wiedermann U, Pehamberger H and Breiteneder H: Vaccination with a human high molecular weight melanoma-associated antigen mimotope induces a humoral response inhibiting melanoma cell growth in vitro. J Immunol 174: 976-982, 2005.
42. Wagner S, Krepler C, Allwardt D, Latzka J, Strommer S, Scheiner O, Pehamberger H, Wiedermann U, Hafner C and Breiteneder H: Reduction of human melanoma tumor growth in severe combined immunodeficient mice by passive transfer of antibodies induced by a high molecular weight melanoma-associated antigen mimotope vaccine. Clin Cancer Res 14: 8178-8183, 2008.
43. Mittelman A, Chen ZJ, Yang H, Wong GY and Ferrone S: Human high molecular weight melanoma-associated antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: Induction of humoral anti-HMW-MAA immunity and prolongation of survival in patients with stage IV melanoma. Proc Natl Acad Sci USA 89: 466-470, 1992.
44. Wang X, Ko EC, Peng L, Gillies SD and Ferrone S: Human high molecular weight melanoma-associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: Enhancement of immunogenicity of anti-idiotypic monoclonal antibody MK2-23 by fusion with interleukin 2. Cancer Res 65: 6976-6983, 2005.
45. Mittelman A, Chen ZJ, Kageshita T, Yang H, Yamada M, Baskind P, Goldberg N, Puccio C, Ahmed T and Arlin Z: Active specific immunotherapy in patients with melanoma. A clinical trial with mouse antiidiotypic monoclonal antibodies elicited with syngeneic anti-high-molecular-weight-melanoma-associated antigen monoclonal antibodies. J Clin Invest 86: 2136-2144, 1990.
46. de Bruyn M, Rybczynska AA, Wei Y, Schwenkert M, Fey GH, Dierckx RA, van Waarde A, Helfrich W and Bremer E: Melanoma-associated chondroitin sulfate proteoglycan (MCSP)-targeted delivery of soluble TRAIL potently inhibits melanoma outgrowth in vitro and in vivo. Mol Cancer 9: 301, 2010.
47. Jordaan S, Chetty S, Mungra N, Koopmans I, van Bommel PE, Helfrich W and Barth S: CSPG4: A target for selective delivery of human cytolytic fusion proteins and TRAIL. Biomedicines 5: 37, 2017.
48. Schwenkert M, Birkholz K, Schwemmlein M, Kellner C, Kugler M, Peipp M, Nettelbeck DM, Schuler-Thurner B, Schaft N, Dörrie J, *et al*: A single chain immunotoxin, targeting the melanoma-associated chondroitin sulfate proteoglycan, is a potent inducer of apoptosis in cultured human melanoma cells. Melanoma Res 18: 73-84, 2008.
49. Geldres C, Savoldo B, Hoyos V, Caruana I, Zhang M, Yvon E, Del Vecchio M, Creighton CJ, Ittmann M, Ferron S and Dotti G: T lymphocytes redirected against the chondroitin sulfate proteoglycan-4 control the growth of multiple solid tumors both in vitro and in vivo. Clin Cancer Res 20: 962-971, 2014.
50. Abken H, Hombach A, Heuser C and Reinhold U: A novel strategy in the elimination of disseminated melanoma cells: Chimeric receptors endow T cells with tumor specificity. Recent Results Cancer Res 158: 249-264, 2001.
51. Burns WR, Zhao Y, Frankel TL, Hinrichs CS, Zheng Z, Xu H, Feldman SA, Ferrone S, Rosenberg SA and Morgan RA: A high molecular weight melanoma-associated antigen-specific chimeric antigen receptor redirects lymphocytes to target human melanomas. Cancer Res 70: 3027-3033, 2010.
52. Wang Y, Geldres C, Ferrone S and Dotti G: Chondroitin sulfate proteoglycan 4 as a target for chimeric antigen receptor-based T-cell immunotherapy of solid tumors. Expert Opin Ther Targets 19: 1339-1350, 2015.
53. Krug C, Birkholz K, Paulus A, Schwenkert M, Schmidt P, Hoffmann N, Hombach A, Fey G, Abken H, Schuler G, *et al*: Stability and activity of MCSP-specific chimeric antigen receptors (CARs) depend on the scFv antigen-binding domain and the protein backbone. Cancer Immunol Immunother 64: 1623-1635, 2015.
54. Wiesinger M, Marz J, Kummer M, Schuler G, Dorrie J, Schuler-Thurner B and Schaft N: Clinical-scale production of CAR-T cells for the treatment of melanoma patients by mRNA transfection of a CSPG4-specific CAR under full GMP compliance. Cancers (Basel) 11: 1198, 2019.
55. Torisu-Itakura H, Schoellhammer HF, Sim MS, Irie RF, Hausmann S, Raum T, Baeuerle PA and Morton DL: Redirected lysis of human melanoma cells by a MCSP/CD3-bispecific BiTE antibody that engages patient-derived T cells. J Immunother 34: 597-605, 2011.
56. Hoffmann RM, Crescioli S, Mele S, Sachouli E, Cheung A, Chui CK, Andriollo P, Jackson PJM, Lacy KE, Spicer JF, *et al*: A novel antibody-drug conjugate (ADC) delivering a DNA mono-alkylating payload to chondroitin sulfate proteoglycan (CSPG4)-expressing melanoma. Cancers (Basel) 12: 1029, 2020.
57. Allen BJ, Singla AA, Rizvi SM, Graham P, Bruchertseifer F, Apostolidis C and Morgenstern A: Analysis of patient survival in a phase I trial of systemic targeted alpha-therapy for metastatic melanoma. Immunotherapy 3: 1041-1050, 2011.
58. Allen BJ, Raja C, Rizvi S, Li Y, Tsui W, Graham P, Thompson JF, Reisfeld RA and Kearsley J: Intralesional targeted alpha therapy for metastatic melanoma. Cancer Biol Ther 4: 1318-1324, 2005.
59. Raja C, Graham P, Rizvi SM, Song E, Goldsmith H, Thompson J, Bosserhoff A, Morgenstern A, Apostolidis C, Kearsley J, *et al*: Interim analysis of toxicity and response in phase 1 trial of systemic targeted alpha therapy for metastatic melanoma. Cancer Biol Ther 6: 846-852, 2007.
60. Ilieva KM, Cheung A, Mele S, Chiaruttini G, Crescioli S, Griffin M, Nakamura M, Spicer JF, Tsoka S, Lacy KE, *et al*: Chondroitin sulfate proteoglycan 4 and its potential as an antibody immunotherapy target across different tumor types. Front Immunol 8: 1911, 2017.

61. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, Lazar AJ, Faries MB, Kirkwood JM, McArthur GA, *et al*: Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin 67: 472-492, 2017.
62. Pucciarelli D, Lengger N, Takacova M, Csaderova L, Bartosova M, Breiteneder H, Pastorekova S and Hafner C: Anti-chondroitin sulfate proteoglycan 4-specific antibodies modify the effects of vemurafenib on melanoma cells differentially in normoxia and hypoxia. Int J Oncol 47: 81-90, 2015.
63. Wang Y, Zhao Y and Ma S: Racial differences in six major subtypes of melanoma: Descriptive epidemiology. BMC Cancer 16: 6911, 2016.
64. Uranowska K, Kalic T, Valtsanidis V, Kitzwögerer M, Breiteneder H and Hafner C: Expression of chondroitin sulfate proteoglycan 4 (CSPG4) in melanoma cells is downregulated upon inhibition of BRAF. Oncol Rep 45: 14, 2021.
65. Hochst B and Diehl L: Antigen shedding into the circulation contributes to tumor immune escape. Oncoimmunology 1: 1620-1622, 2012.