[Frontiers in Bioscience E2, 13-21, January 1, 2010]

"CXCR4-CXCL12 and VEGF correlate to uveal melanoma progression"

Renato Franco[1], Gerardo Botti[1], Massimo Mascolo[2], Giovanna Loquercio[1], Giuseppina Liguori[1], Gennaro Ilardi[2], Simona Losito[1], Anna La Mura[3], Rosa Calemma[4], Caterina Ieranò [4], Jane Bryce[5], Crescenzo D'Alterio[4], Stefania Scala[4]

[1]Pathology Department, National Cancer Institute ' G. Pascale', Naples, Italy, [2]Biomorphological and Functional Sciences Department, Pathology Section, "Federico II" University, Naples, Italy, [3]Pathology Unit, "A. Cardarelli" Hospital, Naples, Italy,[4]Immunology Unit, National Cancer Institute ' G. Pascale', Naples, Italy, [5]Clinical Trials Unit, National Cancer Institute ' G. Pascale', Naples, Italy

TABLE OF CONTENTS

1. ABSTRACT

Despite improvements in early diagnosis of uveal melanoma, prognosis is still poor due to metastases development. Neoangiogenesis and migration are requisites to metastasis promotion. Cross-talking between CXCR4-CXCL12 axis and the VEGF pathway was shown to favours tumour progression. CXCR4-CXCL12-VEGF expression was evaluated by immunohistochemistry in 53 selected cases of primary uveal melanoma and in liver melanoma metastases. CXCR4 protein was detected in 41.4% cases, CXCL12 in 43.4% cases and VEGF expression in 39.6% cases. A significant correlation was found between CXCR4 and VEGF expression (p=0.011), CXCL12 and both tumour dimension and (p=0.006) and epithelioid-mixed cytotype (p=0.012). The two cases of uveal melanoma liver metastases in our series showed CXCR4 expression, weak immunoreactivity for CXCL12 and absent VEGF immunostaining. These data indicate that CXCR4-CXCL12 axis and its cross-talking with VEGF plays a role in uveal melanoma metastases and may be new prognostic markers in UMM. Moreover, these results suggest that targeted inhibition of CXCR4 could be introduced to control metastasis development in UMM.

2. INTRODUCTION

Uveal malignant melanoma (UMM) is the most common intraocular malignancy in adults. It has an incidence of 0.6 cases per 100,000 per year, accounting for 70-88% of all ocular tumours. Despite improvements in early diagnosis documented over the last decade, approximately 50% of patients with UMM still die from metastatic disease (1-4). UMM metastases occur through haematogenous dissemination in a period ranging between 1 and 40 years, mainly to the liver but also to lung, bone, kidney and brain (2-5). Survival of patient with disseminated disease is between 2 and 7 months (6,7), mainly for the absence of effective treatments (1).

The most important factors predicting biological behaviour of UMM are the cell type (epithelioid) and tumour dimension (> 1.5 cm). In addition, scleral infiltration, advanced age, vascular loops and tumour-infiltrating lymphocytes additionally affect the prognosis (8). However, the predictive accuracy of these factors does not allow reliable identification of "high risk" patients (2,3). There is still a need to investigate on the factors involved in metastasization of UMM, in order to identify

new prognostic factors and possible molecular targets for UMM patients.

Metastatic disease is a multistep process that requires neoangiogenesis in order to be successful (9). In fact, the degree of micro-vasculature is related to poor outcome in several tumours (10,11). In UMM, both microvessel count and vascular pattern are predictive of aggressive behaviour (12,13). VEGF is one of the main factors controlling angiogenesis enhancing vascular permeability and thrombus formation. VEGF expression data in UMM are extremely conflicting; expression level between 0 and 94% were reported (14-16). Although VEGF mRNA has been detected in all UMM by reverse transcription –PCR (RT-PCR), the relative protein expression has been reported only in 22% of tumours (17).

A further critical step of the metastasization process is cell migration to secondary sites. Chemokines and their specific receptors play a role in migration of cancer cells to distant sites (18,19). Chemokines are known to mediate leukocyte trafficking, hematopoiesis, inflammatory background, angiogenesis and tumourigenesis.

CXCR4 interaction with its specific ligand CXCL12 has been extensively related to the ‘‘metastatic homing’’ of neoplastic cells (20,21). The expression of several chemokine receptors has been described in cutaneous melanoma. CXCR4 was found to be expressed and functioning in melanoma cell lines (21,22), and its expression was associated with poor prognosis in primary cutaneous melanoma (23,24). CXCR4 expression was previously demonstrated in UMM, and it was found to be correlated with aggressive epithelioid cytotype. In malignant tumours, the autocrine production of chemokines has also been described. In ovarian and colon cancer, CXCL12 is constitutively produced by cancer cells and is involved in promotion of cancer growth and metastasis (21,25).

Interaction between the CXCR4/CXCL12 axis and VEGF expression was previously demonstrated in tumours. In colon cancer CXCR4 expression induces VEGF production. In colon cancer cell lines, SW620, Lovo, and HT29 cells, CXCL12/CXCR4 interaction triggers VEGF production, whilst inhibition of CXCR4/CXCL12 axis by AMD3100 reduces VEGF production (26). CXCR4 up-regulation by VEGF has been demonstrated in breast carcinoma, osteosarcoma and renal cell carcinoma (27-29).

Thus, we aim to establish the role of CXCR4/CXCL12 axis and VEGF production in metastasis development in primary UMM. A further goal is to define CXCR4 as a new prognostic marker for UMM patients.

3. MATERIALS AND METHODS

3.1.Patients

From the archives of the Pathology Section of the Department of Biomorphological and Functional Sciences, University “Federico II” of Naples, Italy, 53 cases of uveal melanoma were selected between January 1984 and December 2006. All patients in this study were treated with primary enucleation. The selection criteria was the availability of follow-up data. These consisted of clinical examination findings plus evaluation of **serum LDH level** (every 3 months), chest X-ray and hepatic ultrasound examination (every 6 months for the first year following surgery); from the second year after surgery, data concerning hepatic ultrasound and total body computed tomography (CT) examination were collected respectively every 6 months and yearly, for each patient. Patients with coexisting disease that could compromise survival were considered ineligible for the study. The follow-up time ranged from 2 to 218 months (average value: 55.7 months). As a common closing date of follow-up, 31 December 2006 was chosen. The study was performed according to the guidelines of the Institutional Ethics Committee.

3.2. Histology

For each case, a paraffin block from a representative area of each one tumour was selected, and 4-µm-thick serial sections were cut and mounted on pre-treated slides. A hematoxylin-eosin section of each tumour was re-examined by two expert pathologists (R.F. and M.M.) to confirm the original diagnosis. Bleaching of melanin was achieved by incubating the sections in a solution of 0.25% potassium permanganate, 5% oxalic acid, for 60 minutes at room temperature.

3.3. Immunohistochemistry

Deparaffinized sections were microwaved in 1 mmol/L EDTA (pH 8.0) for two cycles of 5 minutes each to unmask epitopes. After treatment with 1% hydrogen peroxidase for 30 minutes to block endogenous peroxidases, the sections were incubated with monoclonal antibodies (anti-CXCR4, clone 44716, R&D systems, Minneapolis, MN), anti-CXCL12 (MAB 530, R&D systems, Minneapolis, MN) and anti-VEGF (clone VG1; 1:30, DAKO, Denmark) for 2 hours at room temperature. The sections were then incubated with biotin-labelled secondary antibody for 20 minutes and with streptavidin-peroxidase for 20 minutes. Slides were stained for 10 minutes with AEC chromogen (DAKO, Milan, Italy), counterstained with hematoxylin, washed, and mounted in aqueous medium. All series included positive controls (cutaneous melanomas and breast invasive cancer). Negative controls were obtained by substituting the primary antibodies with a mouse myeloma protein of the same subclass, at the same concentration as the monoclonal antibody. All controls gave satisfactory results.

The results of immunohistochemical staining were recorded independently by two observers (R.F. and M.M.), both blinded with regard to the histological typing of tumours and to the follow-up data of patients. At least 10 high-power fields for each section were randomly selected for microscopic examination.

The immunostaining for CXCR4, VEGF and CXCL12 was expressed as the percentage of positive tumour cells among the total neoplastic cells present in all the selected fields and categorized into three semi

quantitative arbitrary classes: a) absence of staining; b) + <50% c) ++ > 50%.

3.4. RNA isolation and reverse transcriptase-PCR.

RT-PCR analysis was only performed on total RNA isolated from the two cases of uveal melanoma liver metastases in our series. Total cellular RNA from human frozen liver was extracted using TriPure reagent (Roche Diagnostics Corporation, Indianapolis, IN). Tissues were homogenized and RNA was extracted using a monophasic solution of guanidine thiocyanate and phenol. The RNA was quantified and assessed for purity by UV spectrophotometry.

3.5. Reverse transcriptase-PCR

Reverse transcriptase-PCR mRNA was detected by reverse transcriptase-PCR. DNase-treated RNA(2 micro gr) was reversed transcribed with Superscript II RNase H-reverse transcriptase according to the manufacturer's instructions (Invitrogen-Life Technologies, Carlsbad, CA). Reverse transcriptase-PCR was carried out using 2 microL of cDNA in a 20 microL final reaction mixture. A Robocycler gradient 96(Stratagen e, La Jolla, CA) was used for the amplification. Cycling conditions of the respective PCR were as follows: initial denaturation (4 minutes at 94C) followed by 32 cycles of denaturation (1 minute at 94C), annealing (75 seconds at 56C, CXCR4; 58C, CXCL12; 60C, CCR10; 62C, CCR7), and elongation (3 minutes at 72C). Ten microliters of the products were run on a 2% agarose gel and analyzed under UV light. The gene-specific primers used for the amplification were as follows: CXCR4, 5'-GGTGGTCTATGTTGGCGTCT- 3' (forward) and 5'-TGGAGTGTGACAGCTTGGAG-3' (reverse); CXCL12, 5V-GGGCTCCTGGGTTTTGTATT-3V (forward) and 5'-GTCCTGAGAGTCCTTTTGCG-3' (reverse).

3.6. Statistical analysis

Correlations between CXCR4, CXCL12, VEGF expression, data on patient and tumour features, and tumours were studied by Pearson's Chi-Square test where appropriate. Overall survival (OS) and disease-free survival (DFS) curves were calculated using the Kaplan-Meier method. Univariate analysis was done with the log-rank test. All statistical analyses were performed using the SPSS 98 program**.** OS was defined as the time from diagnosis (first biopsy) to death by any cause or until the most recent follow-up. FFS was measured as the time from diagnosis to the occurrence of progression, relapse or metastasis.

4. Results

4.1. Clinical Pathological features

Fifty-three specimens from uveal melanomas surgically resected from 1984 to 2006 were tested for CXCR4, CXCL12 and VEGF expression. The original histological diagnosis of uveal melanoma was confirmed for the all cases examined. Characteristics of all patients are described in Table 1. Genders were equally represented. Median age was 61.7 years; 31 patients were over 60 years of age. For each case the largest tumour diameter (LTD) was recorded: 19 patients had a melanoma with a diameter <1 cm (35,8%); in 17 patients the LTD was comprised between 1 and 1.5 cm (32%) and 15 patients showed an LTD >1.5 cm (28.3%). Sclera invasion was observed in 7 cases (13.2%). Eight cases experienced progression, in a mean period of 99.2 months (range 6 to 216 months) from diagnosis. Death was reported in 5 cases in a mean period of 93.8 months (range 19 to 115 months) from diagnosis. All reviewed cases were classified according to the modified Callender classification as: epithelioid (14 cases, 28.3%), mixed (consisting of spindle and epithelioid cells in variable percentage; 23 cases, 41.5%) and spindle cytotype (16 cases, 30.2%).

4.2. CXCR4, CXCL12 and VEGF expression in uveal melanoma: **pattern of expression**

Staining for CXCR4 protein was absent (0) in 31 tumours (58.4 %) and present in 22 cases (41.4%), specifically in <50% of cells (+) in 16 tumours (30.1%) and in >50% of cells (++) in 6 tumours (11.3 %). CXCR4 immunohistochemical staining was observed mainly in the cytoplasm (Figure 1A); rare positive plasmatic membranes were observed (Figure 1B). CXCL12 expression was negative (0) in 30 tumours (56.6%). Cytoplasmic positivity (Figure 1 C-D) was observed in 23 cases (43.4%); in <50% of cells (+) in 10 tumours (18.86%) and in >50% of cells (++) in 13 cases (24.52%). VEGF expression was negative (0) in 32 tumours (60.37%). VEGF immunohistochemical staining was generally weak (Figure 1E), mainly perivascular (Figure 1F) and was observed in 21 cases (39.6%); in < 50% of cells (+) in 14 cases (26.41%) and in > 50% of neoplastic cells (++) in 7 cases (13.2 %). The expression of CXCR4-CXL12 and VEGF was also evaluated in 2 liver metastases of uveal melanoma. Distribution of patient relatively to CXCR4 expression is reported in Table 2. CXCR4 was observed in the two liver metastases, but weak CXCL12 expression and no VEGF staining (0) was detected in liver metastases (Figure 2, Upper panel). CXCR4 and CXCL12 expression was also evaluated in liver metastases through RT-PCR. Figure 2 shows CXCR4 induction in uveal melanoma liver metastases compared to normal liver tissue. In liver metastases derived by cutaneous melanoma and colon cancer CXCR4 level was unmodified compared to normal liver tissue (Figure 2, Lower panel). CXCL12 expression was unaffected in metastases compared to normal liver tissue (data not shown).

4.3 Expression of CXCL12 correlated with melanoma dimension and cytotype.

The expression of CXCL12 was dramatically associated with the dimension of the uveal melanoma (p=0.006) and significantly associated with the mixed/epithelioid cytotype (p=0.012). CXCL12 was expressed in 3 tumours <1 cm, 8 tumours from 1 to 1.5 cm and 11 tumours >1.5 cm; moreover, CXCL12 was expressed in 17 cases of tumours with epithelioid cells (mixed and epithelioid cytotype) and in 7 cases of spindle cytotype. CXCR4 expression correlated to VEGF expression (p=0.011). The evaluation of the concomitant expression of CXCR4, CXCL12 and VEGF was not significant.

Table 1. Main clinico-pathological features

Patient	Cytotype	Sex	Age (yrs)	Scleral infiltration	Dimension	Follow-up(months)	Progression (time to progression)	status	VEGF	CXCL12	CXCR4
1	S	M	60	NO	1-1.5cm	36	NO	A	++	++	++
2	E	M	70	NO	1-1.5cm	100	NO	A	+	0	+
3	E	M	71	NO	ND	102	NO	A	+	0	0
4	M	F	84	NO	1-1.5cm	30	NO	A	0	++	0
5	M	F	70	NO	1-1.5cm	115	YES(106)	D	0	0	0
6	M	F	66	NO	<1cm	77	NO	A	0	0	0
7	M	F	74	NO	>1.5cm	36	NO	A	+	+	0
8	S	F	75	NO	<1cm	36	NO	A	++	0	+
9	E	F	62	NO	>1.5cm	144	NO	A	+	+	+
10	M	F	82	NO	<1cm	89	YES(80)	AWD	+	0	+
11	M	M	78	NO	>1.5cm	40	NO	A	++	++	++
12	E	F	59	NO	1-1.5cm	36	NO	A	0	++	0
13	M	F	53	NO	1-1.5cm	80	YES(80)	AWD	0	0	+
14	S	M	25	NO	<1cm	48	NO	A	0	0	0
15	S	F	56	NO	<1cm	36	NO	A	0	0	0
16	M	M	43	NO	1-1.5cm	36	NO	A	0	++	0
17	S	F	73	NO	<1cm	67	NO	A	0	+	0
18	E	M	45	NO	<1cm	39	NO	A	0	0	+
19	S	M	73	NO	>1.5cm	90	NO	A	+	++	0
20	S	M	77	NO	1-1.5cm	36	NO	A	0	+	++
21	M	F	61	NO	>1.5cm	71	NO	A	0	+	0
22	S	F	44	NO	<1cm	112	YES(108)	D	0	0	0
23	E	F	30	NO	<1cm	50	NO	A	+	0	+
24	M	F	72	NO	1-1.5cm	60	NO	A	0	0	++
25	S	F	52	NO	<1cm	95	NO	A	+	0	0
26	S	M	63	NO	1-1.5cm	48	NO	A	0	0	0
27	S	M	54	NO	1-1.5cm	75	NO	A	0	+	+
28	M	F	44	YES	>1.5cm	2	NO	A	0	++	0
29	S	M	73	YES	>1.5cm	3	NO	A	0	0	0
30	M	M	82	NO	<1cm	3	NO	A	0	++	0
31	M	M	70	YES	ND	7	NO	A	+	+	+
32	S	M	71	YES	>1.5cm	11	NO	A	0	0	0
33	M	F	46	YES	>1.5cm	15	NO	A	+	+	0
34	M	F	49	NO	<1cm	15	NO	A	+	++	++
35	S	M	68	NO	1-1.5cm	15	NO	A	0	++	0
36	S	F	70	YES	>1.5cm	19	NO	A	0	+	0
37	M	F	81	NO	1-1.5cm	20	NO	A	0	0	+
38	M	F	71	NO	>1.5cm	26	NO	A	++	++	+
39	M	F	53	NO	>1.5cm	30	NO	A	0	++	+
40	E	F	53	NO	<1cm	38	NO	A	0	0	0
41	E	F	63	NO	1-1.5cm	82	NO	A	0	0	0
42	S	M	73	NO	<1cm	91	NO	A	0	0	0
43	E	M	66	YES	>1.5cm	111	YES(105)	D	+	0	+
44	E	M	58	NO	1-1.5cm	112	YES(93)	D	0	+	+
45	E	F	47	NO	<1cm	72	NO	A	0	0	0
46	E	F	43	NO	1-1.5cm	61	NO	A	0	0	+
47	E	M	45	NO	<1cm	26	NO	A	++	0	0
48	M	F	72	NO	>1.5cm	60	NO	A	++	0	+
49	M	M	43	NO	<1cm	218	YES(216)	A	+	0	+
50	M	M	63	NO	<1cm	19	YES(6)	D	+	0	++
51	E	M	68	NO	<1cm	56	NO	A	0	0	0
52	M	F	57	NO	1-1.5cm	36	NO	A	++	0	0
53	M	F	70	NO	>1.5cm	20	NO	A	0	++	0

M=Male; F=Female; ND=Not Disposable;A=Alive; AWD=Alive With Disease; D= Death with disease; 0=staining comprise between 0 and 10% of positive cells; + (from 10% to < 50% of cell); c) ++ > 50%

4.4. CXCR4, VEGF and SDF1/CXCL12 expression: correlation to outcome

At the time of this analysis, after a median follow-up for living patients of 55.66 months (range 2-216 months), 5 patients died (two cases expressing both VEGF and CXCR4, 1 case expressing CXCR4 and CXCL12 and remaining cases with no expression of three examined proteins), and 8 developed progression (4 cases expressing both VEGF and CXCR4, 1 case expressing CXCR4 and CXCL12, 1 case expressing only CXCR4 and remaining cases with no expression of three examined proteins). Expression of CXCR4, VEGF and CXCL12 failed to significantly correlate to DFS and OAS. However, significant correlation between CXCR4 expression and recurrences (p=0.050) was demonstrated by Pearson's Chi Square test. The primary statistically significant correlations are summarized in Table 3.

5. DISCUSSION

Despite of improvement of early diagnosis of uveal malignant melanoma (UMM), related mortality

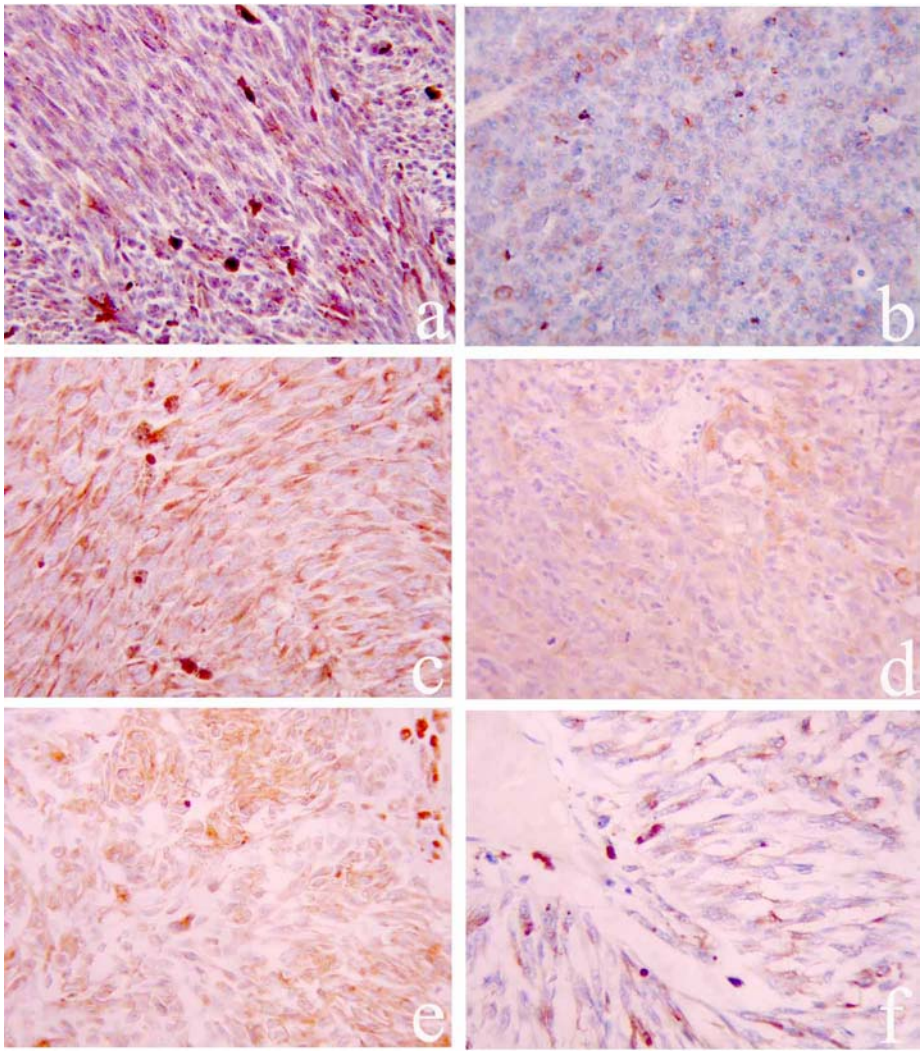

Figure 1. CXCR4-CXCL12 and VEGF expression in primary uveal melanoma A) High CXCR4 staining (40x); B) Low CXCR4 staining with presence of membrane positive cells(40x); C) High CXCL12 staining (40x); D) Low CXCL12 staining (40x); E) High VEGF staining (40x); F) Prevalent VEGF staining around a vessel (63x)

remains unchanged. In fact 31%, 45%, 49% and 52% of patients still die from UMM, respectively within 5, 15, 25 and 35 years from diagnosis (30).

Uveal melanoma metastasizes hematogenously. It is assumed that neoplastic dissemination often occurs prior to starting primary therapy (31). Therefore, the early identification of UMM has been the most important factor influencing the start of an adequate treatment before metastases develop (30,32). There is a need to better understand the metastatic molecular pathway of UMM in order to improve treatment of these tumours (32).

Neovascularization and cell migration are critical events in uveal melanoma metastasis (14,18). Cross-talk between the CXCR4/CXCL12 axis and VEGF has been demonstrated in neoangiogenesis for several malignancies (25,26). Moreover, in a model of neovascularization in normal human retina, the expression of *Cxcr4* and *Sdf1* genes has been shown to be induced by VEGF expression. On the other hand, RNA silencing-induced CXCR4 reduction in retinal microvascular endothelial cells has been shown to reduce invasivity and the overall network of retinal microvasculature (33).

In tumours, VEGF has been reported as a major regulator of angiogenesis (14-16). VEGF expression correlates with tumour vascularity, metastasis and tumour proliferation; thus, high expression of VEGF in tumour tissue may be an indication of poor prognosis (14-17). The *Vegf* gene is located on chromosomal region 6p21.1 and contains 8 exons. Alternative splicing drives to six different VEGF protein isoforms: VEGF189 (full length), VEGF183(with a deletion of 18 pb in exon 6), VEGF206 (alternate splice site selection in exon 7 with insertion of 17 amino acids), VEGF 121(lacking exons 6 and 7), VEGF165 (lacking exon 6), and VEGF145 (lacking exon7), with different expression patterns as well as biochemical and

Table 2. Distribution of patients' series in relation to CXCR4 expression

		CXCR4 0 (N=30)	CXCR4 + (N=17)	CXCR4 ++ (N=6)	Significance
Sex	***M***	12	7	4	NS
	F	18	10	2	
Age	***≥60 yrs***	18	9	5	NS
	<60 yrs	12	8	1	
Scleral Infiltration	***Y***	5	2	0	NS
	N	25	15	6	
LTD	***<1.5***	12	4	3	NS
	1-1.5	9	6	2	
	>1.5	9	5	1	
	ND	0	2	0	
VEGF expression	***0***	24	7	2	**0.011**
	+	5	7	2	
	+	1	4	2	
CXCL12 expression	***0***	17	11	2	NS
	+	5	4	1	
	+	8	2	3	
Progression	***Y***	2	5	1	**0.050**
	N	28	12	6	
Status	***A***	28	13	6	NS
	AWD	0	2	0	NS
	D	2	2	1	

0=staining comprise between 0 and 10% of positive cells; + (from 10% to < 50% of cell); c) ++ > 50%; M=Male; F=Female; NS=not significant; Y=yes; N=no; LTD= Largest tumour dimension; ND=not determined

Table 3. Main statistically significant association (Pearson's Chi Square)

Evaluated parameters	p-value
CXCL12- dimension	0.006
CXCL12-mixed/epithelioid cytotype	0.012
CXCR4-VEGF	0.011
CXCR4-Progression	0.050

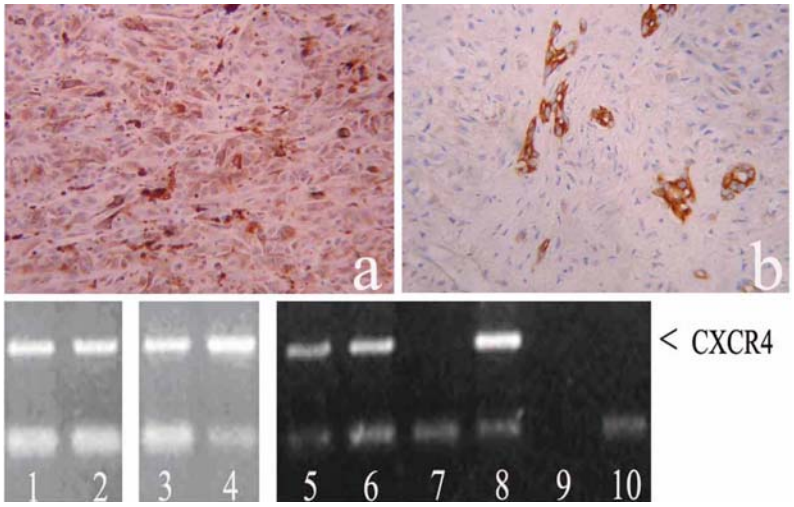

Figure 2. CXCR4-CXCL12 expression in liver metastases from uveal melanoma. Upper panel: Immunohistochemical staining in the A) CXCR4 staining in liver uveal melanoma metastasis (40x); B) CXCL12 staining in liver uveal melanoma metastasis surrounding intensively positive biliar ducts Lower panel: RT-PCR for CXCR4 expression in normal liver and relative colon carcinoma metastasis(1-2); in normal liver and relative cutaneous melanoma metastasis (3-4); in normal liver and relative uveal melanoma metastasis (case 49; 5-6); in normal liver and relative uveal melanoma metastasis (case 50; 7-8); 9 empty well and 10 water control.

biological properties (14,34). VEGF189, VEGF165 and VEGF121 are detected in the majority of cells and tissue expressing the *Vegf* gene (34). VEGF stimulates and induces migration and proliferation of endothelial cells and enhances vascular permeability (14). There are several promising therapies targeting the expression of VEGF in ocular diseases associated with florid angiogenesis (14).

The reported differences in VEGF expression in UMM is probably due to different fixation and processing techniques. In our study, the samples were collected in one institution over a period of 25 years, during which fixation and processing have not substantially changed. VEGF expression was observed in 21/53 cases (39.6%) in agreement to previously published frequencies. The

specificity of the antibody to different VEGF splice variants may account for variability in VEGF expression. The clone VG1 antibody used in our study recognizes the most expressed VEGF isoforms, 121, 165 and 189 isoforms.

Since new vessels have been formed, migration of cancer cells from the primary site is favored by signalling or "homing" mechanisms, shared with organogenesis, development, hematopoiesis and immune responses. According to this mechanism, cancer cells reach specific metastatic sites through an interchange of signals, whose mediators are chemokines and their specific ligands (20). Müller et al. and Zeelenberg et al. have demonstrated in murine models that chemokine receptors are implicated in metastatic progression 35,36). Such chemokines and receptors are typically quiescent in many normal tissues, with the notable exception of immune cells, and appear to be activated or up-regulated in cancer (20). Activation of chemokine receptors promotes the growth, adhesion and, most importantly, directional migration of immune cells during antigen-specific inflammatory responses (20). Recent evidence indicates that the possibility of cancer cells to re-activate innate signalling mechanisms through alternative chemokine receptors allows yielding of specific targeted metastasis (20). For example, CCR7 is highly expressed in nodal metastasis of colorectal carcinoma and it is highly expressed also in dermal and nodal metastasis of melanoma (22,24,37).

Among chemokine receptors, the role of CXCR4 in metastasis development has been widely described in at least 20 different cancer types, such as breast cancer, glioblastoma, pancreas, prostate, colon, thyroid and lung cancers (21,38). The chemokine receptor CXCR4 was identified as a co-receptor for T-tropic HIV-1 and HIV-2 (37). In particular, CXCR4 overexpressing cells migrate toward secondary organs, such as the liver and lung, which are a rich source of CXCL12, the specific ligand for CXCR4 (22,24,37).

The role of CXCR4 was studied in *in vitro* systems of cutaneous melanoma and in patients in whom CXCR4 expression correlated with a poor prognosis in terms of DFS and OS (21-24). Our group demonstrated that CXCR4 expression in cutaneous melanoma characterizes cases that have developed liver metastasis. Recently, CXCR4 expression in uveal melanoma was described and correlated to the epithelioid phenotype, considered to be the most important pathological adverse prognostic factor (18). In the present study, the concomitant expression of CXCR4 with the ligand, CXCL12 and VEGF, was evaluated in uveal melanoma. Although a robust correlation was detected between CXCR4 expression and occurrence of progression by Pearson's Chi-Square test, single protein and concomitant expression failed to detect correlation with DFS and OAS, probably due to the limited number of patients who could be evaluated. CXCR4 expression also correlated with VEGF expression, reinforcing the interaction between the two pathways reported in colorectal cancer (26). Links between the VEGF and CXCR4 pathways were previously described in human breast cancer, osteosarcoma and glioma cells, where VEGF increased CXCR4 expression and migration towards CXCL12 (27-29,39). Moreover, the unique CXCR4 expression observed in UMM liver metastasis suggests its critical role in tumour progression, whilst other proteins seem only to support metastasis occurrence. In fact, malignant tumours modulate the microenvironment producing factors, such as CXCL12, CCL17, CCL11, CCL22 and CXCL1 in an autocrine manner, in order to favor their survival, growth, invasion and metastasis (25). CXCR4 expression in metastasis and its continue activation through its ligand CXCL12 favors local growth and invasion within the liver (37).

High levels of VEGF and CXCL12 produced by malignant cells may promote proliferation and vascularization. CXCL12 production has been demonstrated in ovarian carcinoma, acute myelogenous leukemia cells, prostate cancer, colon and in renal cell carcinoma (25-29,40). An impact on DFS for CXCL12 expression was previously described in low grade oligodendroglioma and oligoastrocytomas (41). To the best of our knowledge, this is the first report of CXCL12 in uveal melanoma. In a previous study of chemokines, CXCL1, CXCL8 and HGF expression was found in UMM cell lines, while CXCL12 was not detected (9). Instead, in our study, 22 of 53 uveal melanoma cases (41%) expressed CXCL12. Our finding is probably due to the high variability of neoplastic tissue with respect to cell lines. Moreover, CXCL12 expressing uveal melanoma correlated to the melanoma diameter and to the epitheliod cytotype, features of a more aggressive phenotype.

In conclusion, the CXCR4-CXCL12 axis and VEGF in uveal melanoma affected tumour progression and add valid prognostic information, since neovascularization and migration are particularly critical for metastasis development in this group of tumours. In particular, activated CXCR4 seems to play a relevant role in liver metastasis development and in its growth. Moreover, its role is amplified through promoting VEGF-mediated angiogenesis. These observations call for further study into the control of metastasis development or treatment of liver metastases by targeting this receptor. Recent studies have shown that specific inhibitors to CXCR4 were effective in arresting specific pathways regulated by CXCR4 activation, representing a potentially developing therapy against molecules promoting liver metastasis (42,43).

6. ACKOWLEDGMENTS

This study was supported by a grant from AIRC (Associazione Italiana per la Ricerca sul Cancro, IG 1107).

7. REFERENCES

1. Carlring J., M. Shaif-Muthana, K. Sisley, I.G. Rennie & A.K. Murray: Apoptotic cell death in conjunction with CD80 costimulation confers uveal melanoma cells with the ability to indice immune responses. *Immunology.* 109, 41-48 (2003)

2. Schmittel A., N. E. Bechrakis, P. Martus & D. Mutlu: Independent prognostic factors or distant metastases and survival in patients with primary uveal melanoma. *Eur J Cancer* 16, 2389–2389 (2004)

3. Zuidervaart W., P. J. Hensbergen, M. Wong, A. M. Deelder, C. P. Tensen, M. J. Jager, & N. A: Gruis. Proteomic Analysis of Uveal Melanoma Reveals Novel Potential Markers Involved in Tumour Progression. *Invest Ophthalmol Vis Sci* 47, 786–793 (2006)

4. Gamel J. W., I. W. McLean & J. B. McCurdy: Biologic distinctions between cure and time to death in 2892 patients with intraocular melanoma. *Cancer* 71, 2299–2305 (1993)

5. Diener-West M., B. S. Hawkins, J. A. Markowitz & A. P. Schachat: A review of mortality from choroidal melanoma. II. A meta-analysis of 5-year mortality rates following enucleation, 1966 through 1988. *Arch Ophthalmol* 110, 245-250 (1992)

6. Seddon J. M., D. M. Albert, P. T. Lavin & N. Robinson: A prognostic factor study of disease-free interval and survival following enucleation for uveal melanoma. *Arch Ophthalmol* 101, 1894–1899 (1983)

7. Kath R., J. Hayungs, N. Bornfeld, W. Sauerwein, K. Hoffken & S. Seeber: Prognosis and treatment of disseminated uveal melanoma. *Cancer* 72, 2219–2223 (1993)

8. McLean I.W., V.S. Saraiva & M.N. Burnier: Pathological and prognostic features of uveal melanoma. *Can J Ophthalmol* 39, 343-350 (2004)

9. Di Cesare S., J. C. Marshall, P. Logan, E. Antecka, D. Faingold, S.C. Maloney & M. N. Burnier: Expression and migratory analysis of 5 human uveal melanoma cell lines for CXCL12, CXCL8, CXCL1, and HGF. *Journal of Carcinogenesis* 6, 2-8 (2007)

10. Nakamura M, Y. Abe and T. Tokunaga: Pathological significance of vascular endothelial growth factor A isoform expression in human cancer. *Pathol Int* 52, 331–339 (2002)

11**.** Turley H., P. A. Scott, V. M. Watts, R. Bicknell, A. L. Harris & K. C. Gatter: Expression of VEGF in routinely fixed material using a new monoclonal antibody VG1. *J Pathol* 186, 313–318 (1998)

12. Foss A.J.E., R.A. Alexander, L.W. Jefferies, J. L. Hungerford, A.L. Harris & S. Lightman: Microvessel count predicts survival in uveal melanoma. *Cancer Res* 56, 2900-2903 (1996)

13. Foss A.J.E. Alexander, J. L. Hungerford, A. L Harris, I. A Cree & . Lightman:Reassessment of the PAS patterns in uveal melanoma. *Br J Ophthalmol* 81, 240-246 (1997)

14. M. H. Abdel-Rahman, E. L. Craig, F. H. Davidorf, and C. Eng: Expression of Vascular Endothelial Growth Factor in Uveal Melanoma Is Independent of 6p21-Region Copy Number. *Clin Cancer Res* 11, 73–78 (2005)

15. Kvanta A., B. Steen & S. Seregard: Expression of vascular endothelial growth factor (VEGF) in retinoblastoma but not in posterior uveal melanoma. *Exp Eye Res* 63, 511– 518 (1996)

16. Speicher M.R., G. Prescher, S. du Manoir**,** A. Jauch, B. Horsthemke, N. Bornfeld, R. Becher & T. Cremer: Chromosomal gains and losses in uveal melanomas detected by comparative genomic hybridization. *Cancer Res* 54, 3817– 3823 (1994)

17. Boyd S.R., D. S. Tan, L. de Souza, M. H. Neale, N. E. Myatt, R. A. Alexander, M. Robb, J. L. Hungerford &I. A. Cree**:** Uveal melanomas express vascular endothelial growth factor and basic fibroblast growth factor and support endothelial cell growth. *Br J Ophthalmol* 86, 440–447 (2002)

18. Scala S., C. Ieranò, A. Ottaiano, R. Franco, A. La Mura, G. Liguori, M. Mascolo, S. Staibano, P. A. Ascierto, G. Botti, G. De Rosa & G. Castello: CXC chemokine receptor 4 is expressed in Uveal Malignant melanoma and correlates to the epithelioid-mixed cell type. *Cancer Immunol Immunother* 56:1589-1595 (2007)

19. Muller A., B. Homey & H. Soto: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, 50-56 (2001)

20. Kulbe H., N. R. Levinson, F. Balkwill & J. L. Wilson: The chemokine network in cancer-much more than dircting cell movement. Int J Dev Biol 48, 489-496 (2004)

21. Payne A.S. & L. A. Cornelius: The role of chemokines in melanoma tumour growth and metastasis. *J Invest Dermatol* 118, 915-922 (2002)

22. Wiley H.E., E. B. Gonzalez & W. Maki: Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *J Natl Cancer Inst* 93, 1638-1643 (2001)

23. Scala S., A. Ottaiano, P. A. Ascierto, C. Ieranò, R. Franco, M. Napolitano, A. Ottaiano, M. L. Lombardi, M. Luongo, E. Simeone, D. Castiglia, F. Mauro, I. De Michele, R. Calemma, G. Botti, C. Caracò, G. Nicoletti, R. A. Satriano & G. Castello: Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clin Cancer Res* 11, 1835-1841 (2005)

24. Scala S., P. Giuliano, P. A. Ascierto, M. Cavalli, E. Simeone, P. Giuliano, M. Napolitano, R. Franco, G. Botti & G. Castello: Human Melanoma Metastases Express Functional CXCR4. *Clin Cancer Res* 12, 2427-2433 (2006)

25. Kulbe H., R. Thompson, J. L. Wilson, S. Robinson, T. Hagemann, R. Fatah, D. Gould, A. Ayhan & F. Balkwill: The inflammatory cytokine tumour necrosis factor-alpha generates an autocrine tumour-promoting network in

epithelial ovarian cancer cells. *Cancer Res* 67, 585-592 (2007)

26. Ottaiano A., R. Franco, A. AielloTalamanca, G. Liguori, F. Tatangelo, P. Delrio, G. Nasti, E. Barletta, G. Facchini, B. Daniele, A. Di Blasi, M. Napolitano, C. Ierano, R. Calemma, E. Leonardi, V. Albino, V. De Angelis, M. Falanga, V. Boccia, M. Capuozzo, V. Parisi, G. Botti, G. Castello, R. V. Iaffaioli, S. Scala: Overexpression of both CXC Chemokine Receptor 4 and Vascular Endothelial Growth Factor proteins predicts early distant relapse in stage II-III colorectal cancer patients. *Clin Cancer Res* 12, 2795-2803 (2006)

27. Bachelder R.E., M. A. Wendt & A. M. Mercurio: Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res* 62, 7203–7206 (2002)

28. Oda Y., H. Yamamoto, S. Tamiya, S. Matsuda, K. Tanaka, R. Yokoyama, Y. Iwamoto & M. Tsuneyoshi: CXCR4 and VEGF expression in the primary site and the metastatic site of human osteosarcoma: analysis within a group of patients, all of whom developed lung metastasis. *Modern Pathology* 19, 738–745 (2006)

29. Zagzag D., B. Krishnamachary, H. Yee, Hiroaki Okuyama, L. Chiriboga, M. A. Ali, J. Melamed and G. L: Semenza. Stromal Cell–Derived Factor-1α and CXCR4 Expression in Hemangioblastoma and Clear Cell-Renal Cell Carcinoma: von Hippel-Lindau Loss-of-Function Induces Expression of a Ligand and Its Receptor. *Cancer Res* 65, 6178-6188 (2005)

30. Bell D.J. & M.W. Wilson: Choroidal melanoma: natural history and management options. *Cancer Control* 11, 296-303 (2004)

31. Anastassiaou G., S.E. Coupland, A. Stang, R. Boeloeni, H. Shilling & N. Bornfeld: Expression of Fas and Fas ligand in uveal melanoma: biological implications and prognostic value. *J Pathol* 194, 466-472 (2001)

32. Margo C.E: The collaborative ocular melanoma study: an overview. *Cancer Control* 11, 304-309 (2004)

33. Yu K., J. Zhuang, J.M. Kaminski, B. Ambati, Q. Gao, P. Ma, D. Liao, F. Li, X. Liu & J. Ge: CXCR4 down-regulation by small interfering RNA inhibits invasion and tubule formation of human retinal microvascular endothelial cells. *Biochem Biophys Res Commun* 358, 990-996 (2007)

34. Ferrara N., H.P. Gerber & J. LeCouter: The biology of VEGF and its receptors. *Nat Med* 9, 669-676 (2003)

35. Müller A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W.Yuan, S.N. Wagner, J. L. Barrera, A. Mohar, E. Verástegui & A. Zlotnik: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, 50 –56 (2001)

36 Zeelenberg I.S., L. Ruuls-Van Stalle & E. Roos: The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res* 63, 3833–3839 (2003)

37. Kim J., T. Mori, S. L. Chen, F.F. Amersi, S. R. Martinez, C. Kuo, R.R. Turner, X. Ye, A.J. Bilchik, D. L. Morton, & D. S. B. Hoon: Expression in Patients With Melanoma and Colorectal Cancer Liver Metastases and the Association With Disease Outcome. *Ann Surg* 244, 113–120 (2006)

38. Burger J.A. & T.J. Kipps. CXCR4: a key receptor in the crosstalk between tumour cells and their microenvironment. *Blood* 107, 1761-1767 (2006)

39. Hong X., F. Jiang, S.N. Kalkanis, X.P. Zhang, A.C. De Carvalho, M. Bobbitt, T. Mikkelsen & M. Chopp: SDF-1 and CXCR4 are up-regulated by VEGF and contribute to glioma cell invasion. *Cancer Lett* 236, 39-45 (2006)

40. Ao M., O. E. Franco, D. Park, D. Raman, K. Williams & S. W. Hayward. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Res* 67, 4244-4253 (2007)

41. Calatozzolo C., E. Moderna, B. Pollo, M. Gelati, C. Marras, A. Silvani, D. Croci, A. Boiardi & A. Salmeggi: Prognostic Value of CXCL12 Expression in 40 Low-grade Oligodendrogliomas and Oligoastrocytomas. *Cancer Biology and Therapy* 7, 827-832 (2006)

42. Devine S.M., N. Flomenberg, D.H. Vesole, J. Liesveld, D. Weisdorf, K. Badel, G. Calandra & J. F. DiPersio: Rapid mobilization of CD34_ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol* 22,1095–1102 (2004)

43. Mori T., R. Doi, M. Koizumi, E. Toyoda, D. Ito, K. Kami, T. Masui, K. Fujimoto, H.Tamamura, K. Hiramatsu, N. Fujii & M. Imamural: CXCR4 antagonist inhibits stromal cell-derived factor 1-induced migration and invasion of human pancreatic cancer. *Mol Cancer Ther* 3,29–37 (2004)

Abbreviations: UMM: Uveal melanoma; VEGF: Vascular Endothelial Growth Factor; CXCR 4: CXC chemokine receptor 4; CXCL 12: chemokine stromal cell-derived factor-1; SDF-1 alpha: alpha-chemokine stromal cell-derived factor.

Key Words: CXCR4, CXCL12, VEGF, Uveal Melanoma

Send correspondence to: Renato Franco, Pathology Department, National Cancer Institute "Fondazione G. Pascale", Via Mariano Semmola, Naples, Tel.360815903471, Fax:360815903718, E-mail: renfr@yahoo.com

http://www.bioscience.org/current/vol2E.htm