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Tumoural expression and circulating level of VEGFR-3 (Flt-4) in metastatic melanoma patients: Correlation with clinical parameters and outcome

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ABSTRACT

Purpose: The presence of metastases in regional lymph nodes is a strong indicator of poor patient survival in many types of cancer. It has recently been shown that vascular endothelial growth factor-C (VEGF-C), and its receptor VEGFR-3, may play a pivotal role in the promotion of metastasis to regional lymph nodes. This study was designed to detect and evaluate whether the expression of VEGFR-3 or its soluble form plays a role in metastatic malignant melanoma and to determine the relationship with clinicopathological parameters and patients outcome.

Experimental design: VEGFR-3 expression on melanoma tumour was evaluated by immunohistochemical study. Using a sensitive enzyme-linked immunosorbent assay, sVEGFR-3 was measured in sera of 60 metastatic melanoma patients in comparison with 30 healthy controls.

Results: Immunohistochemical study demonstrated a high expression of VEGFR-3 in melanoma cells. Median level of pre-treatment sVEGFR-3 was significantly higher ($p = 0.00001$) in melanoma patients as compared to healthy donors. No association was noted between VEGFR-3 in situ or in sera and gender, age or LDH level. Median serum VEGFR-3 levels were significantly higher in patients with high tumour burden as compared to those with low tumour burden ($p = 0.013$) as well as in non-responding patients ($n = 33$) as compared to responding ones ($n = 27$). Finally, low level of VEGFR-3 was also related positively to disease free survival ($X^2 = 3.85$, $p = 0.022$).

Conclusion: These results suggest that the expression and high pre-treatment sVEGFR-3 level are significantly correlated to poorer prognosis, and may be promising targets for new therapeutic strategies in melanoma disease.

1. Introduction

The number of melanoma cases worldwide is increasing faster than any other cancer. The dissemination of cancer cells to distant sites is known to occur through both lymphatic and blood vessels. During the last few years, there has been a dramatic increase in the number of studies of the mechanisms of associated lymphangiogenesis and lymphatic

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metastasis. It has been recognised that lymphangiogenic growth factors promote cancer cell spreading to regional lymph nodes[1–3] and, among them, one of the most important is vascular endothelial growth factors (VEGFs) and its receptors (VEGFRs).

Five known members of VEGF family are described as VEGFs A–D and placenta growth factor (PlGF).[4] These factors interact with 3 membrane receptors belonging to the subfamily of tyrosine kinase protein receptors: VEGFR-1 (Flt1), VEGFR-2 (KDR or flk-1) and VEGFR-3 (Flt4).[5] All three VEGFRs undergo alternative splicing to generate more than one form of the receptor. The truncated form of VEGFR-1, named soluble VEGFR-1 (sFlt-1), is an important causative agent in cancer.[6] A naturally occurring soluble form of VEGFR-2 has also been described, which could arise as a result of alternative splicing or through proteolytic processing.[7] In humans, an alternative splicing of VEGFR-3 generates two isoforms.[8,9]

Increasing clinicopathological evidence indicates that tumour lymphangiogenesis is associated with the metastatic spread of cancer. Moreover, studies in experimental models of cancer have demonstrated that the VEGF-C/VEGF-D/VEGFR-3 signalling system is a key regulator of tumour lymphangiogenesis,[10] especially in relation to progression or metastasis in leukaemia[11] and other cancers.[1] Under physiological conditions, VEGFR-3 is restricted to lymphatic and some fenestrated vascular endothelium in adult. However, it is upregulated in angiogenic blood vessels in tumours and wounds, and may contribute to tumour angiogenesis and solid tumour growth.[4,12] The observation that treatment of tumour xenograft models with neutralising VEGFR-3 monoclonal antibodies restricted both angiogenesis and tumour growth supports this hypothesis.[13] In addition, expression of VEGFR-3 by lymphatic endothelial cells was reported to be associated with lymph node metastasis in prostate cancer. Experimental tumours that overexpress VEGFR-3 ligands induce lymphatic vessel sprouting, enlargement and show enhanced metastasis to regional lymph nodes.[14] In experimental tumour models, overexpression of VEGFR-3 ligands by tumour cells induced intratumoural and peritumoural lymphangiogenesis and increased metastasis to the regional lymph nodes, whereas a soluble form of VEGFR-3, which inhibits VEGF-C/VEGF-D signalling, inhibited both lymphangiogenesis and metastasis.[1,15]

In order to evaluate whether VEGFR-3 (Flt-4) may affect tumour metastasis and prognosis in metastatic malignant melanoma patients, we retrospectively investigated if there is a relationship between the VEGFR-3 (expressions or level) and clinicopathological parameters of melanoma. Finally, we examined whether sVEGFR-3 has any value or relevance with respect to clinical response and survival.

2. Materials and methods

2.1. Material and antibodies

EIA micro titre plates were purchased from Co-star (Cat No. 2592A, Corning B.V., Life Sciences Koolhovenlaan, The Netherlands). Phosphate buffer saline (PBS), bovine serum albumin (BSA), H_2O_2 and tetramethylbenzidine substrate were purchased from Sigma–Aldrich, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France. All the following materials were purchased from R&D Systems, Abingdon, Oxford shire, UK. As capture antibody, we used a mouse monoclonal anti-human VEGFR-3 antibody (Mab349 Clone 54716). As detection antibody, we used a biotinylated mouse monoclonal anti-human VEGFR-3 antibody (BAM 3492, Clone 54733, Cat No. DY998**).** For standard curve construction, we used a recombinant human VEGFR-3 (Flt-4)/Fc Chimera (Cat No. 349-F4). For immunohistochemical study the Citric-acid-based antigen unmasking solution was purchased from Vector Laboratories (Burlingame, CA). The primary antibodies for VEGFR-3 detection, a rabbit polyclonal IgG (Flt-4, c-20, clone sc-321) and the corresponding isotypic control (a polyclonal rabbit IgG) were purchased from Santa Cruz Biotechnology, Inc. (Delaware Avenue, Santa Cruz, CA, USA). The secondary antibody, a Biotin-SP-Affinipure Goat Anti-Rabbit IgG (H+L), was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Streptavidin conjugated to horseradish-peroxidase **(**Streptavidin-HRP, Dako, Glostrup, Denmark).

2.2. Solution preparation

The washing buffer was prepared as follows: 0.9% sodium chloride containing 0.05% Tween 20 in PBS (pH 7.2–7.4). As a blocking solution, we used PBS containing 5% of BSA and 5% of sucrose at (pH 7.2–7.4). A solution of PBS containing 1% of BSA (pH 7.2–7.4) was used for samples and antibody dilution.

2.3. Patients

Sixty patients with metastatic malignant melanoma (21 women and 39 men) with a median age of 45.5 years (range 22–70) were treated in the Medical Oncology Department of the Salpetriere Hospital in Paris, France by biochemotherapy regimen as described by Khayat et al.[16] Briefly, they received 100 mg/m^2 of cisplatinum (CDDP) over 4 h on day 1 followed by 18×10^6 IU/m^2/day of recombinant interleukin 2 (rIL-2) (Proleukin, Chiron, Amsterdam, The Netherlands) in a continuous intravenous infusion from days 3 to 6 and 17 to 21. The same induction cycle was repeated on day 28. Interferon-α (Roferon, Roche, Neuilly, France) was subcutaneously administered at a dose of 9×10^6 IU three times weekly throughout the treatment period. This study was conducted with the approval of the Local Ethics Committee; all patients gave informed consent and received the two induction cycles. Regarding tumour burden, patients were divided into two groups: a low tumour burden group ($n = 23$) with more than 5 and less than 10 metastatic sites and/or tumour size less than 6 cm, and a high tumour burden group ($n = 34$) with more than 10 metastatic sites and/or tumour size more than 6 cm; three unclassified patients were excluded.

2.4. Clinical response evaluation

Although results were evaluated before each course, tumour measurements that required scans were performed on day 66 (after the two induction cycles), confirmed 4 weeks later and then repeated every 2 months. Responses were assessed according to the World Health Organisation (WHO) criteria.[17]

Complete response (CR) was defined as the disappearance of all known disease on two separate measurements at least 4 weeks apart; partial response (PR) was defined as a reduction in the sum of the products of the largest perpendicular diameters of each lesion by at least 50%; stable disease (SD) was defined as a decrease of less than 50% or an increase of less than 25% with no new lesions; finally, progressive disease (PD) was defined as an increase >25% in measurable disease or appearance of new lesions. The response duration was measured for CRs from the onset of the CR to the date of disease progression, and for PR from the first day of treatment until the progression of disease. Duration of survival was measured from the first day of treatment until the date of death.

2.5. *Quantitative detection of VEGFR-3*

Sera and plasma from 30 healthy volunteers (15 females and 15 males ranging in age from 25 to 55 years) and melanoma patients were collected on a serum separator tube for serum or EDTA for plasma and kept frozen at −80 °C until assay.

2.6. *VEGFR-3 Sandwich ELISA*

The development of a sensitive and specific two-step sandwich enzyme-linked immunosorbent assay (ELISA) test for the detection of VEGFR-3 was achieved by using standard methods. Briefly, a 96-well micro titre plate was coated with 100 μl/well of the capture antibody diluted to a final concentration of 4 μg/ml in PBS (pH 7.2–7.4). After an overnight incubation at room temperature, the excess antibody was removed by 3 washes, and plates were blocked by adding 300 μl of blocking solution to each well at room temperature for 2 h. All subsequent incubation steps were carried out at room temperature using an orbital shaker with three washes between the incubation steps. Afterwards, 100 μl of the samples diluted at 1:10 in a dilution buffer was added to the plates in duplicate. Recombinant VEGFR-3 diluted serially with the same dilution buffer to generate a standard curve with different concentrations ranging from 20 to 0.1 ng/ml was used. One hundred micro litres of blocking solutions, dilution buffer or PBS were added in duplicate to the plate as negative controls. After 2 h of incubation, unbounded antigens were washed out as described earlier. One hundred micro litres of biotinylated mouse monoclonal anti-VEGFR-3 antibody (BAM 3492) diluted to a final concentration of 0.4 μg/ml in dilution buffer were added to each well and incubated at room temperature for 2 h. The plate was again washed, and 100 μl of streptavidin HRP diluted at 1:200 was added to each well. The plate was incubated for 20 min with shaking at room temperature and washed as described earlier. Finally, 100 μl of 1:1 mixture of H_2O_2 and tetramethylbenzidine substrate was added, and the enzymatic reaction was allowed to take place for 25–30 min in the dark at room temperature. The colorimetric reaction was stopped by adding 50 μl/well of 2 N H_2SO_4.

The optical densities of each well were quantified within 30 min at dual wavelengths of 450 corrected to 540 nm using a micro plate reader (eASYS UVM 340, Biochrom Ltd., Cambridge, UK). Standard curves were constructed using serial dilutions of recombinant human VEGFR-3 (Flt-4)/Fc Chimera after the removal of the average zero standard optical density. The concentrations of VEGFR-3 detected in the samples were calculated from the standard curve and multiplied by the dilution factor. Each serum sample was tested in duplicate, and results were presented in nanograms/millilitre (ng/ml).

2.7. *Immunohistochemistry*

The whole melanoma lesions were always included. Paraffin blocks were cut into 4-μm sections, deparaffinised and rehydrated. The sections were subjected to heat-induced epitope retrieval by placing them in a citric-acid-based antigen unmasking solution inside a plastic pressure cooker that was placed in a microwave oven. The sections were treated with 2 cycles of 15 min each. The endogenous peroxidase was blocked for 20 min in methanol containing 3% hydrogen peroxide. Sections were washed in phosphate-buffered saline (PBS), blocked in 10% serum and incubated for 1 h at 37 °C either with primary antibodies for VEGFR-3 (Flt-4, c-20, clone sc-321, dilution 1/100) or with a polyclonal rabbit IgG at equivalent dilutions. Sections were then washed in PBS and incubated with secondary antibody, a Biotin-SP-AffiniPure Goat Anti-Rabbit IgG (H+L), for 30 min at room temperature. Streptavidin-HRP was then applied for 30 min, and the sections were stained with diaminobenzidine. Positive and negative controls were systematically performed. A highly vascularised tissue (blood vessels) with constant overexpression of VEGFR-3 was used as a positive control. Replacing the primary antibodies by a polyclonal rabbit IgG at equivalent dilutions was used as negative controls. Haematoxylin eosin safran (HES) staining was performed to determine the tumour cells.

2.8. *Immunostaining analyses*

On each case, three slides were stained with VEGFR-3 antibody and then interpreted. The immunostaining was examined by light microscopy blindly and independently by 2 pathologists. The medium values of the three slides were taken for results. In case of interobserver discordance of more than 10%, slides were re-evaluated together to reach consensus. The VEGFR-3 reactivity in melanoma sections was scored according to the percentage of labelled tumour cells in comparison with a negative control and a known positive control, which in our cases it was a highly vascularised tissue (blood vessels) with constant overexpression of VEGFR-3. Staining was classified as 0 (negative <10% positive cells), 1 (10–70% positive cells) and 2 (>70% positive cells). Representative photomicrographs were taken with a Leica DMLB microscope and DC500 camera.

2.9. *Statistical analysis*

All statistical analyses were carried out using Stat View statistical software, version 5.0 (SAS Institute, Inc., Cary, NC). Unless specified, the data were expressed as median and range. As the data were not normally distributed; consequently the significance of differences between groups were calculated by applying non-parametric tests. The difference of VEGFR-3 concentration between healthy donors and melanoma patients and the difference in circulating VEGFR-3 level

according to age, sex, tumour burden, LDH and clinical response in melanoma patients were calculated using the Mann–Whitney U-test for unpaired mean. Cut-off values for circulating VEGFR-3 were determined using descriptive statistic and median was used. Correlations between the different parameters were calculated using the r' Spearman test. Two-sided $p < 0.05$ was considered to be statistically significant.

Probabilities and curves of time to progression (defined as the time between date of entering to the treatment and tumour progression) and overall survival (OS) (defined as the time between date of entering to the treatment until last follow-up or death) were generated using the Kaplan–Meier method[18] in combination with the log-rank test.

3. Results

3.1. Clinical response

All patients received the two induction cycles and could be fully evaluated. Overall response rate was 45% with 1 of 60 (1.7%) patients achieving a long-term complete response for more than 60 months and 26 of 60 (43.3%) achieving a partial response with a median duration of 12 (3–20+) months. Fifty five percent were non-responding including 13 of 60 patients (21.6%) with stable disease and 20 of 60 (33.4%) with progressive disease. Response rate was equivalent in visceral and non-visceral metastasis. No significant difference in age and sex was observed between responder and non-responder patients.

3.2. Assay optimisation of the sandwich ELISA tests for VEGFR-3

The best dilution for the capture antibody was 4 μg/ml and 0.4 μg/ml for the detection antibody. This combination resulted in the most sensitive results with a signal to noise ratio of less than 5. The mean optical density (OD) of the background was 0.098 ± 0.022 (7 different experiments range (0.054–0.15). The minimum detection limit estimated by a serial dilution was less than 0.1 ng/ml of recombinant human VEGFR-3. The intra-assay coefficient of variation was 5.72%, and the inter-assay coefficient of variation was 7.5% demonstrating the reproducibility and reliability of the assay.

3.3. Detection of VEGFR-3 by ELISA test

The best results in the linearity of the dilution were obtained with the 1/10 dilution. The study of the VEGFR-3 level in plasma and sera was performed on samples of 7 healthy donors and 7 patients. The median VEGFR-3 level was slightly higher in sera (34.12 ng/ml) as compared to plasma levels (32.53 ng/ml) without any significant difference ($p = 0.75$); so we decided to continue all our study using only sera samples. As shown in Table 1, and Fig. 1, serum VEGFR-3 level was detectable and variable in healthy donors, and median level was 22.92 ng/ml (range 0.2–33.2 ng/ml). In melanoma, 96% of melanoma patients showed a high VEGFR-3 level (above the median concentration observed in healthy donors). The median VEGFR-3 level was 39.05 ng/ml (range 18.5–49.52 ng/ml). A highly significant difference was found in the median VEGFR-3 level in melanoma patients ($p < 0.00001$) as compared to healthy donors.

3.4. Immunohistochemical analysis of the VEGFR-3 expression

As shown in Fig. 2A, no staining was observed with the negative control. The presence of malignant melanoma tumour was confirmed using haematoxylin eosin safran (HES) staining (Fig. 2B), As shown by immunoperoxidase staining (Fig. 2C), more than 70% of melanoma cells were positive with anti-Flat-4 polyclonal antibody as compared to our negative control (Fig. 2A). Furthermore, the staining was very high in membrane and cytoplasmic localisation (Fig. 2D). The specificity of the staining was confirmed using a positive control, which was a highly vascularised tissue (blood vessels) with constant overexpression of VEGFR-3. On the other hand, the intensity of VEGFR-3 expression was significantly correlated with VEGFR-3 levels measured by ELISA ($p < 0.01$).

3.5. Relationship with clinicopathological parameters

The sVEGFR-3 level according to patients characteristics is listed in Table 1. No relationship was observed between sVEGFR-3 level and age ($rs = -0.04$, $p = 0.89$), gender ($rs = -0.10$; $p = 0.58$) and LDH levels ($rs = -0.1$, $p = 0.75$). Using pre-treatment samples, we found a positive correlation between tumour burden and sVEGFR-3 concentration ($r' = 0.53$; $p = 0.01$); but not to tumour localisation ($p = 0.19$). It was significantly higher ($p = 0.03$) in the high tumour burden group as compared to the low tumour burden group.

3.6. Relationship with the clinical response to biochemotherapy

The follow-up of sVEGFR-3 level (Fig. 3) showed that before treatment, the median level of sVEGFR-3 detected in the 33 non-responding patients (40.7 ng/ml range 22.57–49.8 ng/ml) was significantly higher ($p = 0.013$) as compared to the median level of sVEGFR-3 detected in the serum of the 27 responding ones (34.54 ng/ml range 18.5–44.64 ng/ml). After treatment, no change in the median sVEGFR-3 was observed ($p = 0.42$).

3.7. Relationship with time to progression and overall survival

Kaplan–Meier analysis showed that the 28 patients with a low sVEGFR-3 concentration had a higher median disease free survival than the 32 patients with high sVEGFR-3 (16.2 versus 10.8 months) (Fig. 4a). Log-rank section test was significant ($X^2 = 3.85$, $p = 0.022$). Regarding overall survival (Fig. 4b), patients with low sVEGFR-3 concentration had a higher median overall survival than patients with high VEGFR-3 (16.8 versus 12 months), but the log-rank section test was not significant ($X^2 = 2.38$; $p = 0.12$).

4. Discussion

The mechanisms governing lymphatic spread of tumour cells in metastatic melanoma are still poorly understood.[19,20]

Table 1 – Pre-treatment soluble VEGFR-3 levels according to patient's characteristics. Statistical analysis was performed using the Mann–Whitney U-test.

	No. of patients	VEGFR-3 (ng/ml) mean ± SE median (min–max)	*p*-Value
Healthy controls	30	24.28 ± 7.57 26.35 (0.2–33.18)	<0.00001
Overall patients	60	37.84 ± 9.97 38.89 (18.47–49.52)	
Age ⩽45 years	28	38.71 ± 12.14 38.95 (18.47–49.52)	0.722
Age >45 years	32	37.2 ± 6.76 38.65(20.77–48.97)	
Gender Male	39	38.57 ± 11.65 39.16 (20.77–49.52)	0.766
Gender Female	21	36.56 ± 6.01 38.71 (18.47–44.8)	
Low tumour burden	23	34.62 ± 7.19 36.37 (18.47–44.8)	0.034
High tumour burden	34	38.27 ± 3.56 39.35 (37.53–49.52)	
LDH level ⩽ 450 (IU/L)	33	36.34 ± 6.19 37.53 (34.59–39.45)	0.226
LDH level > 450 (IU/L)	27	37.99 ± 42.05 39.65 (35.98–49.52)	

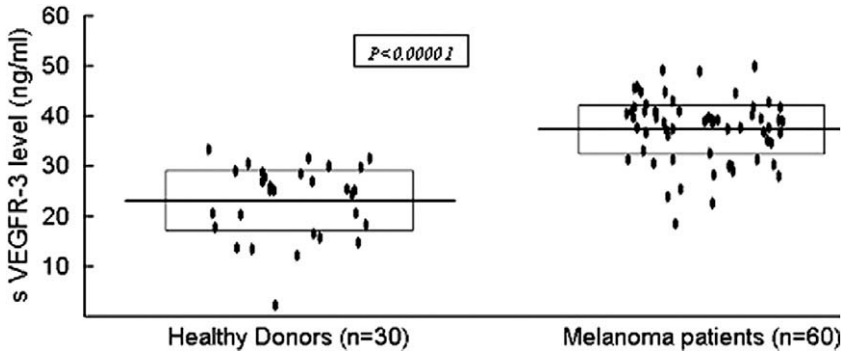

Fig. 1 – Pre-treatment serum VEGFR-3 levels in healthy donors and metastatic melanoma patients. Each point represents an individual determination. Statistical analysis was performed using the Mann–Whitney U-test. The means ± SD of the individual samples is shown by the solid and dashed lines, respectively.

VEGF-C and VEGFR-3 have been associated with lymphatic metastasis mainly via tumour lymphangiogenesis in animal models and human tumours.[2,21] Moreover, autocrine/paracrine activation of cancer cells via VEGFs and their receptors has been suggested to play a role in tumour aggressiveness.[22–25] Our study was therefore designed to evaluate the expression of VEGFR-3 in tumours, the detection of its soluble form in sera, plasma and to analyse the relationship with clinicopathological parameters, response and survival in metastatic malignant melanoma patients.

It has been shown that VEGFR-3 is expressed not only in lymphatic endothelial cells but also in tumour cells, and has been seen in the cytoplasm, along the nuclear and cellular membranes underlying its potential role in tumour growth.[13,26,27] In our study, we demonstrated that metastatic melanoma tumour cells overexpress VEGFR-3 more than do the normal cells. In addition, more than 70% of melanoma cells were positive for VEGFR-3 and were localised in the membrane and cytoplasm; this high expression was not surprising since all our melanoma patients were metastatic. This is consistent with the study of Clarijs and colleagues,[28] in which they demonstrated that in melanoma, VEGFR-3 expression gradually increased with tumour stages. Furthermore, in NSCLC patients Saintigny and colleagues[29] showed that VEGFR-3 was expressed at a higher level in metastatic lymph node than in the primary tumour from the same patient.

Despite a vast literature on VEGFR-3 expression in tissue (quantitative PCR and immunohistology),[26,27,30–33] few data on serum and plasma exist for this factor, which is the major axis specific for lymphangiogenesis. In this study, we used an ELISA test for the measurement of VEGFR-3 protein levels in normal and clinically relevant samples from metastatic malignant melanoma patients. We found a significantly ($p < 0.00001$) elevated level of sVEGFR-3 concentration in 96% of melanoma patients, while it was detectable at a 2-fold lower level in healthy donors; this result corroborates those observed by Debrah and colleagues[34] in lymphatic filariasis patients and by Bolander and colleagues[34,35] in patients with

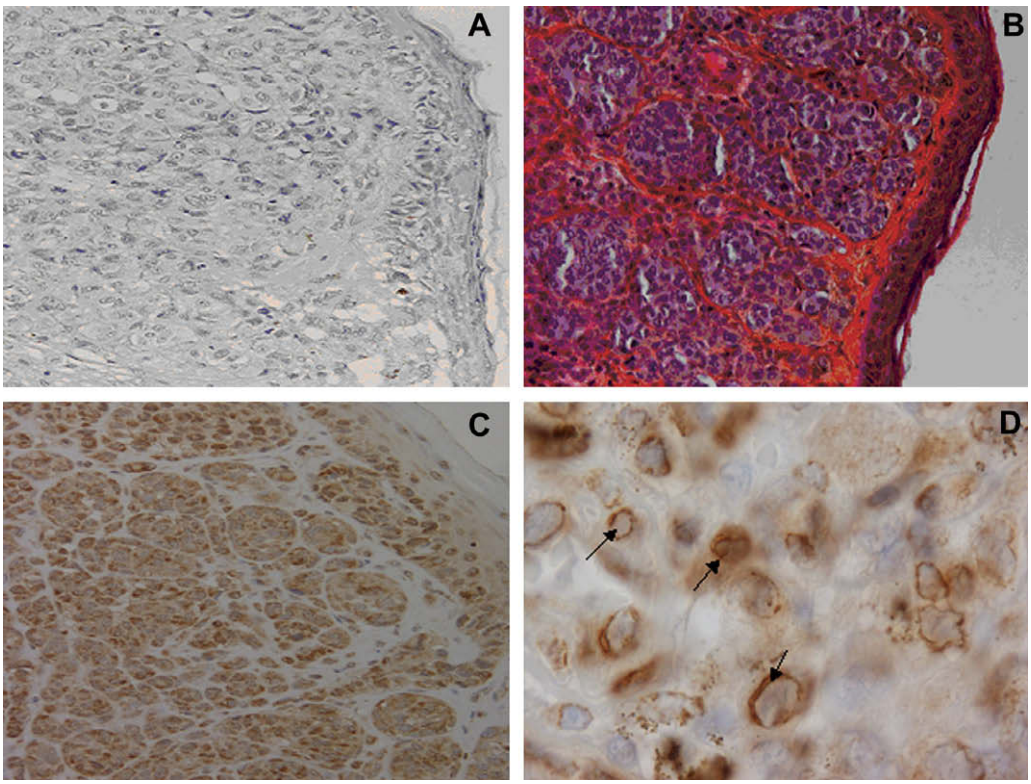

Fig. 2 – Immunohistochemical analysis of VEGFR-3 expression in human malignant melanoma tissue. (A) Negative control (magnification ×40). (B) HES staining showing malignant melanoma (magnification ×20), (C) immunoperoxidase labelling with anti-Flat-4 pAb showing positive staining of melanoma tumour cells (magnification ×20), (D) immunoperoxidase staining showing membrane and cytoplasmic localisation (magnification: ×100).

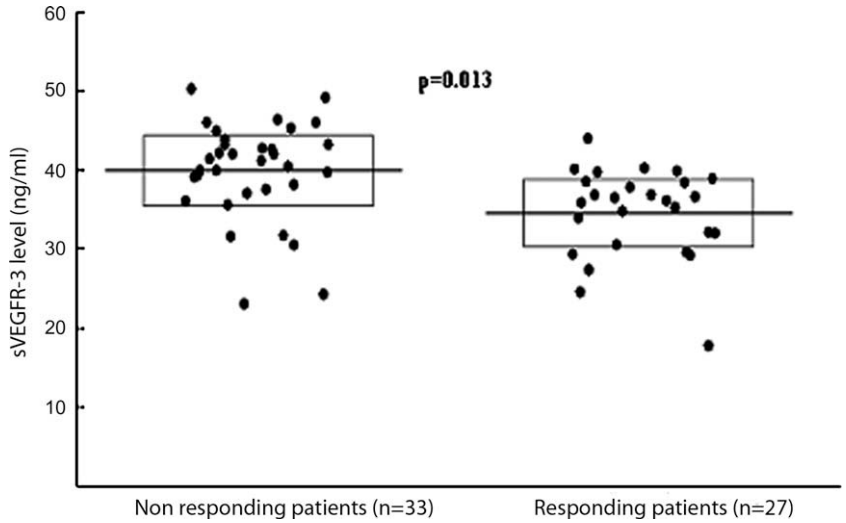

Fig. 3 – Comparison of pre-treatment serum VEGFR-3 levels between non-responding and responding patients. Statistical analysis was performed using the Mann–Whitney U-test. Each point represents an individual determination. The means ± SD of the individual samples is shown by the solid and dashed lines, respectively.

localised malignant melanoma. Unfortunately, no data exist as yet with regard to sVEGFR-3 level and its biological significance for tumours. However, the physiological significance of the 2-fold elevation in melanoma patient is not clear at the moment and still needs to be explored in which way VEGFR-3 can contribute to tumour lymphangiogenesis, angiogenesis, tumour progression or metastasis.

To address the biological and clinical significance of pre-treatment sVEGFR-3 levels in advanced melanoma, we compared sVEGFR-3 level with the clinicopathological parameters

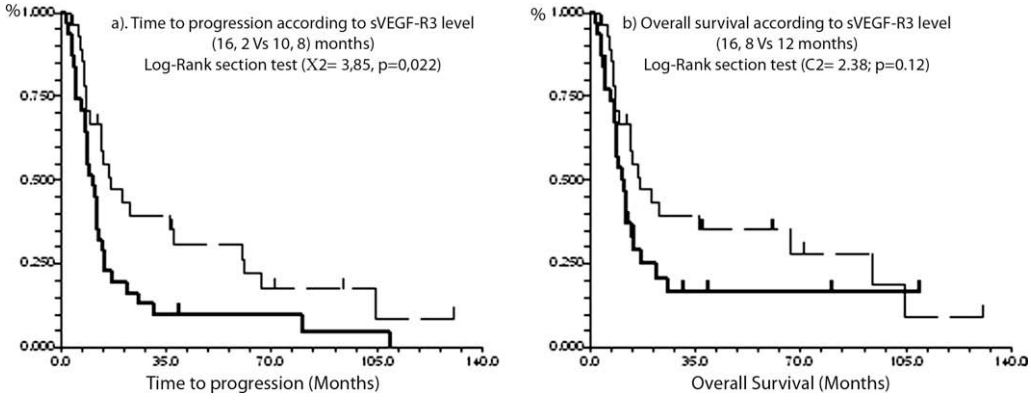

Fig. 4 – Time to progression (a) and overall survival (b) in melanoma patients according to sVEGFR-3 classification. Based on cut-off values calculated using descriptive statistics, patients were divided into 2 subgroups low sVEGFR-3 (dotted line *n* = –28) and high sVEGFR-3 (bolded line; *n* = 32).

related to melanoma. Gender, age or LDH was not associated with pre-treatment sVEGFR-3 levels. Although flt-4, like its ligand, has been proposed as a lymphatic marker, we were unable to demonstrate a significant association between the level of pre-treatment sVEGFR-3 and lymph node status or the number of involved nodes. In contrast, we found that pre-treatment sVEGFR-3 concentration was significantly correlated with the tumour burden ($r' = 0.53$; $p = 0.01$) but not with tumour localisation ($p = 0.18$). More interestingly, the pre-treatment soluble VEGFR-3 levels measured by ELISA significantly correlated with intratumoural VEGFR-3 positive intensity ($p < 0.01$). However, the observed correlation must be seen with the limitation that only a very limited number of sections have been used for VEGFR-3 expression which may not be representative. The physiological significance of these results remains unclear at the present time, but it should be further noted that the expression of VEGFR-3 could be attributed to blood, lymphatic capillaries, endothelial cells[36] and also to a subset of tumour cells. This imply a possible role in an autocrine/paracrine loop promoting tumour cell survival and proliferation[22–25,33] probably by different underlying mechanisms. It is also not known in which way VEGFR-3 contributes to tumour angiogenesis, lymphangiogenesis, tumour progression and metastasis[4,37] However, these results are the first data from tissue, plasma and sera, and further investigations are necessary to confirm our observation.

In a recent study regarding circulating protein biomarkers of anti-angiogenic treatment, DePrimo and colleagues[38,39] showed that the decrease of the novel soluble variant of VEGFR-3 could be a marker of sunitinib activity in patients with metastatic renal cell carcinoma. In our study, after the treatment, no significant decrease in the median sVEGFR-3 level was observed. In contrast, elevated pre-treatment concentrations of sVEGFR-3 were found to exert a significantly unfavourable impact on clinical response ($p = 0.013$) and disease free survival ($X^2 = 3.85$, $p = 0, 02$). Similar to our results, George and colleagues[40] showed that a high baseline sVEGFR-3 level was related to non-response to the treatment and lower progression free survival in RCC patients refractory to bevacizumab when they were receiving sunitinib; nevertheless, in that report, after sunitinb therapy, a higher reduction in sVEGFR-3 levels was observed in responding patients accompanied by concomitant improvement in the conditions of the patients.

In conclusion, the expression of VEGFR-3 on melanoma cells and the presence of high circulating VEGFR-3 in metastatic malignant melanoma patients may prospectively identify high-risk patients with a worse prognosis and shorter survival and may be promising targets for new therapeutic strategies in melanoma disease.

Additional studies are required to clarify the mechanisms of function of VEGFR-3 and to investigate the possible role of VEGF-C/VEGFR-3 axes in lymphangiogenesis in this disease.

Conflict of interest statement

None declared.

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