

CXCR4-CXCL12 axis and distant metastatic outgrowth in head and neck malignancy

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Abstract

Background: To analyze whether distant metastatic outgrowth in different head and neck malignancies (HNM) underlies the CXCR4-CXCL12 axis as overriding molecular mechanism.

Methods: Clinic-pathological data of 1,250 HNM was included. HNM were collected due to different capability to exhibit distant metastasis comprising basal cell, squamous cell, and adenoid-cystic carcinoma as well as melanoma. MMP2/9, TIMP1/2, CXCR4, and CXCL12 immunohistochemistry was done in 190 randomly selected specimens.

Results: Immunohistochemistry visualized a significant increase in MMP2/9, TIMP1/2, CXCR4, and CXCL12 protein expression following the clinical occurrence of distant metastasis. CXCR4, CXCL12, and TIMP2-expression significantly increases with number of affected organs by distant metastasis. Cox regression demonstrated CXCR4-overexpression and advanced T-status being independent risk factors of distant metastasis associated death.

Conclusion: The CXCR4-CXCL12 axis is associated with the occurrence of distant metastases in different HNM. The increased risk of distant metastasis associated death was identified at primary tumour site and, therefore, potentially influences further treatment protocols.

Introduction

Head and neck malignomas (HNM) consist of a broad variety of malignant tumours with different histological subtypes and distinct clinical behavior. Risk factors range from ultraviolet irradiation in basal cell carcinoma (BCC) and malignant melanoma (MM), nicotine and alcohol abuse as well as HPV-infection in head and neck squamous cell carcinoma (HNSCC) to unknown predisposing factors in adenoid-cystic carcinomas (ACC) [1-4]. Subsequently, molecular mechanisms underlying the respective entity vary. However, in all HNM patient's prognosis is inherently associated with the TNM status. Thereby, the occurrence of loco-regional lymph node and particularly distant metastasis is associated with a significantly reduced survival [5, 6]. The current study analyzes whether different HNM share overriding molecular mechanisms that are associated with distant metastatic outgrowth. Therefore, different HNM were selected due to their clinical behavior to develop distant metastasis. BCC is a slowly progressive, locally destructive skin cancer that accounts for almost 80% of all non-melanoma skin cancers. The incidence of metastases in BCC is estimated to be about 0.0028-0.55 of all BCC cases [7]. HNSCC is the sixth common malignant neoplasm worldwide, accounting for more than 90% of head and neck cancers [8]. Overall, the loco-regional lymph node metastasis rate is estimated to be about 60% [9]. Distant metastases were diagnosed in 10-20% [9-12]. MM originates from melanocytes and accounts for 4% of skin malignancies. MM represents the most aggressive skin cancer and usually accompanies the initiation of loco-regional and distant metastasis with a dramatically reduced 5-year survival of <12% [13, 14]. ACC is a frequent malignant tumour of salivary glands, which is characterized by frequent local recurrences and almost exclusive distant metastasis [15]. ACC usually develops late-onset distant

metastases, years after resection of the primary tumour [16]. The current study focuses on the CXCR4-CXCL12 axis as overriding molecular mechanism in distant metastatic outgrowth that was suggested to be associated with poor survival in BCC, HNSCC, MM, and ACC [17-22].

Material And Methods

Patient selection

The current study includes a total of 1,250 HNM patients that were diagnosed in the ENT department of the University Hospital Rechts der Isar, Munich. Thirty-one patients with BCC, 1,057 patients with HNSCC, 146 patients with MM, and 16 patients with ACC were included. Tumour samples were histologically reviewed by at least two experienced pathologists. Dysplasia, carcinoma in situ, and other histologic subtypes were excluded. Clinical parameters and survival data were retrospectively collected: age, sex, TNM-Staging, grading, treatment modalities, recurrence, and death/loss to follow-up. Patients with lacking data, incomplete staging, and refused/not finished surgical and/or conservative treatment (radio-/chemotherapy) were excluded from survival analysis. The study was approved by the ethical committee, Technical University Munich (08/14/2019, 418/19S).

Immunohistochemistry

Tumour samples were achieved from primary tumour sites at the time of diagnosis. Paraffin-embedded tumour (FFPE) samples from 31 BCC, 118 HNSCC, 32 MM, and nine ACC were randomly selected and analyzed via immunohistochemistry (IHC). FFPE tumor sections (2.5µm) were MMP2 (DCS Innovative Diagnostik-Systeme, Hamburg, Germany, 1:100), MMP9 (Biomol GmbH, Hamburg, Germany, 1:1000), TIMP1 (R&D, Wiesbaden, Germany, 1:500), TIMP2 (Biomol, 1:500), CXCR4 (R&D, 1:200), and CXCL12 (R&D, 1:1000) immuno-stained and visualized with the Bond Polymer Refine Detection Kit (Leica, Nussloch, Germany). Expression levels were classified using a scoring system analyzing the staining intensity (0=no staining, 1=low, 2=moderate, 3=strong staining intensity) and the relative proportion of stained cells (0, 1=<10%, 2=10-39%, 3=40-69%, 4=>70 of the tumor cells). A cumulative score (range 0-7 points) was assessed by adding both scores. A positive staining was defined by a cumulative score equal or greater than 3.

Statistical analysis

Differences between the groups were analyzed using the Chi square test and Fisher exact test for categorical, and the unpaired student's t-test for continuous variables. For the analysis of the independent groups, the one-way ANOVA tests were used. As main endpoint, the overall survival (OS) was assessed measuring the time from treatment to death of any cause. Survival rates and curves were calculated and illustrated by the Kaplan–Meier method and further analyzed by the log-rank test. Variables that revealed prognostic or effect modifying potential on the outcome were subsequently evaluated by the proportional Cox regression for forward selection. p-values <0.05 were considered statistically significant. Statistical analysis was done using SPSS (SPSS Inc., Chicago, IL).

Results

Demographic and tumour characteristics of analyzed HNC

The overall cohort demonstrated significant differences in the age and gender distribution. While HNSCC, MM, and ACC demonstrated a mean age of 60 years, BCC patients were significantly older (72 years; $p < 0.0001$). Our study demonstrated a significant predominance of male patients in BCC (2.5:1) and HNSCC (4:1). MM showed a more balanced gender distribution (1.3:1), while a female predominance was observed in ACC (1.6:1; $p < 0.0001$). The current study, performed in a tertiary ear nose throat department, revealed that the majority (88%) of ACC originated in major salivary glands (parotid and submandibular gland), BCC originated in head and neck cutis (100%). The majority of head and neck MM were diagnosed in the cutis (88%), while 10% originated in the sinonasal system, one percent in the oral cavity, and one percent in the nasopharynx, respectively. HNSCC demonstrated a pronounced occurrence in the oropharynx (39%), followed by the hypopharynx (21%), larynx (20%), and the oral cavity (15%) (Table 1).

In BCC, the vast majority of patients presented limited T-status (T1/2: 97%) and underwent primary tumour resection. R0 status was achieved in 97% of our patients. None of our BCC patients suffered from lymph node and/or distant metastases at the time of diagnosis or during the follow up period. HNSCC exhibited a balanced T-status (T1/2: 56%; T3/4: 44%). Head and neck surgery in HNSCC (66%) was sustained by adjuvant radio-(chemo-) therapy in 47% of our patients. R0-status was achieved in 83% of our HNSCC patients, while 10% remained insufficiently resected. In MM, 74% of our patients showed limited T-Status (T1/2). Twenty-six percent of our patients suffered from T3/4 MM that usually refers to a mucosal subtype with a missing T1/2-status in the UICC classification system. 64% of our patients underwent solitary tumour resection, while combined treatment regimens were applied in 36%. Tumour debulking was done in 8% of the MM cohort, resulting in a R2 status. Tumour surgery with adjuvant radio-therapy was done in 81% of the ACC patients. Despite radical parotid and submandibular gland surgery, tumour margins remained microscopically *non-in-sano* in 21% of our patients (Table 1).

Distant metastatic profile

The distant metastatic profile differed significantly between the analyzed groups. At the time of diagnosis, distant metastases were diagnosed in 4% of HNSCC, 6% of ACC, and 10% of MM ($p = 0.01$). Metachronous metastases occurred in 14 of HNSCC, 19% of ACC, and 24% of MM resulting in an overall metastasis rate of 18% in HNSCC, 25% of ACC, and 34% of MM ($p = 0.0009$). Loco-regional and distant metastasis did not occur in BCC. Time from first diagnosis to the occurrence of metachronous metastases ranged from 13 to 37 months, without differences between the groups ($p = 0.6$). The mean follow up time was ≥ 60 months for all analyzed tumour entities. There were significant differences in the number and distribution of affected organs. While metastases in more than one organ occurred in 18% of the HNSCC and 25% of ACC, MM demonstrated multi-organ manifestation in 43% of our cases ($p < 0.0001$). The lungs represented the most affected organs in ACC (75%) and HNSCC (48%). The liver and bone were involved in 18% and 17% of all HNSCC patients, while the distant metastatic spread in

other organs occurred infrequently. In contrast, there was a balanced and extensive affection of different distant metastasis organs in MM ($p=0.04$; Table 2).

Impact of the CXCR4-CXCL12-axis in HNC distant metastases

There were significant differences in the expression patterns of analyzed proteins that are associated with the CXCR4-CXCL12 axis. Immunohistochemical (IHC) analysis visualized a stepwise increase of MMP2 and 9 expression from BCC, HNSCC, and MM to ACC ($p<0.0001$). TIMP1 was visualized in all tumour entities with an increased expression in MM and ACC ($p<0.0001$). There were striking differences in the expression of TIMP2, CXCR4, and CXCL12 with significant higher expression levels in MM and ACC (all: $p<0.0001$; Figure 1).

IHC scoring visualized significant differences in the TIMP2, CXCR4, and CXCL12 expression with respect to the number of organs affected by distant metastasis ($p=0.02$; $p=0.002$; $p=0.047$; Figure 2). Even MMP9 and TIMP1 showed a stepwise increase in protein expression with the lowest level in M0 and the highest level when three or more organ systems were affected. However, the trend failed to achieve statistical significance (Figure 2).

Analysis of the overall survival demonstrated a significantly better outcome of M0 individuals (mean: 254 months) when compared with their M1 counterparts (mean: 44 months) ($p<0.0001$; Figure 3). Forward selected, M0/1 adjusted, proportional Cox regression of survival modifying parameters (T, N, MMP2/9, TIMP1/2, CXCR4) identified CXCR4 positivity at primary tumor site (HR: 3.57; $p=0.017$) and increasing T status (HR: 1.823; $p=0.025$) being solitary survival modifying parameters in HNM.

CXCR4 expression levels at primary tumour site differed significantly due to the location of distant metastasis. Highest CXCR4 levels were detected in patients with skin and CNS metastases, moderate levels in patients with lung and liver involvement, and low CXCR4 levels in all other locations ($p=0.04$; Figure 4).

Discussion

Head and neck malignancies (HNM) represent a melting pot of different malignant lesions with distinct clinical behavior. Risk factors and molecular mechanisms underlying the respective entity differ significantly. However, malignant capacity is clinically characterized by an infiltrative and destroying growing pattern at primary tumour site, locoregional lymph node manifestation, and the exhibition of distant metastases. Particularly, the occurrence of distant metastases is inherently associated with reduced patients' survival and usually results in palliative treatment regimens. At the time of primary diagnosis, the reliable identification of patients with increased risk to develop distant metastasis is major clinical impact in order to modify treatment recommendations. The current study analyzes the CXCR4-CXCL12-axis as overriding molecular mechanism in the distant metastatic outgrowth of different HNM. HNM were selected due to a distinct clinical metastatic profile. Concordant with the present literature, our study did not observe loco-regional or distant metastases in BCC [7]. While loco-regional metastases were

diagnosed in 60% of the HNSCC and MM at the time of diagnosis, lymph node metastases were not seen in ACC. The occurrence of distant metastases differed significantly between the groups. At the time of diagnosis, distant metastases were diagnosed in 4% of HNSCC, 6% of ACC, and 10% of MM ($p=0.01$). Metachronous metastases occurred in 14 of HNSCC, 19% of ACC, and 24% of MM resulting in an overall metastasis rate of 18% in HNSCC, 25% of ACC, and 34% of MM ($p=0.0009$). The aggressive clinical phenotype in MM was highlighted by a high proportion of multiple organ manifestation. While HNSCC and ACC demonstrated multiple organ manifestation in 18% and 25% of the analyzed cases, multilocularity was observed in 43% of our MM patients. The lungs were predominantly affected in HNSCC and ACC. MM showed an extensive metastatic spread with systemic disease manifestation in many organs. Several studies demonstrated the association of the CXCR4-CXCL12 axis and clinicopathological features of malignancy [23-25]. In our study, the protein expression of CXCR4, CXCL12, TIMP1, and TIMP2 was significantly higher in MM and ACC when compared with HNSCC. BCC, that did not develop loco-regional or distant metastasis, showed the lowest expressions of the analyzed proteins. Recently, MMPs, TIMPs, CXCR4, and CXCL12 were associated with distant metastatic capacity [26-29]. Moreover, a CXCL12 dependent increase of MMP2 and MMP9 secretion by activating ERK-1/2 signaling pathway was demonstrated [30, 31]. Interestingly, our study demonstrated that patients with distant metastases in three or more organs showed significantly higher TIMP1, CXCR4, and CXCL12 protein expression in tumor samples at initial diagnosis than patients with distant metastases in 1-2 organs, or particularly than patients without distant metastases. Although the lung was the most affected metastatic organ in HNSCC and ACC, the CXCR4 expression in primary tumors did not show the organ-specificity.

A pooled model from meta-analysis showed a significant shorter OS in HNM patients with CXCR4 over-expression (HR=2.02, 95% CI, 1.37-2.97) [32]. Accordingly, forward selected, M0/1 adjusted, proportional Cox regression of survival modifying parameters (T, N, R, MMP2/9, TIMP1/2 and CXCR4) identified CXCR4 positivity being a solitary survival modifying parameter in HNM. In a large study of 233 HNSCC patients with inoperable tumors undergoing primary RT/RT-CT CXCR4 expression was also associated with increased risk of distant metastasis [33]. Recently, a multicenter study revealed that intracellular CXCL12 expression was associated with lower loco-regional control after postoperative RT-CT in a cohort of 221 HNSCC patients [34]. Consistent with our findings, McConnell et al. demonstrated that patients with AJCC stage II melanomas had 3-fold risk of disease recurrence in individuals with high total CXCR4 expression (>50%). Nevertheless, CXCL12 over-expression in the adjacent epidermis of all melanoma stages with tumour progression was associated with increased time to metastasis [35]. Due to the autocrine and paracrine CXCL12 secretion, the contradictory phenomenon remains to be further studied. In primary nasal-surface BCC tumor, upregulation of CXCR4 in normal skin tissues compared to normal skin tissues revealed that potential capacity of CXCR4 in progression and invasion of nasal-surface BCC [22]. Bazal et al. demonstrated that BCC patients with deep tissue invasion showed significant high CXCR4 staining rate (75%) than patients with papillary dermis invasion (14.3%) [36]. In our study Cox regression showed that CXCR4 expression and T1/2 vs. T3/4 can be solitary predictors for overall survival.

Conclusion

The CXCR4-CXCL12 axis plays a pivotal role in the distant metastatic capacity of different HNM. The increased risk of distant metastasis associated death can be identified at primary tumour site and, therefore, potentially influences further treatment protocols. Functional studies have to investigate the interaction of CXCL12 and CXCR4 in HNM.

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Declarations

Ethics approval and consent to participate: This article does not contain any studies with human participants or animals performed by any of the authors. The study was approved by the local ethical committee.

Consent for publication: All authors gave consent for publication.

Competing interests: Authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence their work.

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Authors' contributions: Conception: YL, LB, KF, PK, AKr, HB, AK; experiments: YL, LB KF, AK; data acquisition: YL, LB, KF, PK, AKr, HB, AK; interpretation: YL, LB, KF, PK, AKr, HB, AK; analysis: YL, LB KF, AK; manuscript preparation; YL, KF, AK; manuscript correction: YL, LB, KF, PK, AKr, HB, AK

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Tables

Table 1: Clinical characteristics

	BCC	HNSCC	MM	ACC	p-value
n	31	1057	146	16	
Age (years)					<0.0001
Median [25%; 75%]	74 [64; 81]	60 [53; 67]	62 [49; 70]	62 [50; 71]	
Mean ± SD	72±13	60±11	60±16	60±12	
Sex, n (%)					<0.0001
Male	22 (71)	841 (80)	83 (57)	6 (38)	
Female	9 (29)	216 (20)	63 (43)	10 (62)	
Location					
Sinunasal		35 (3)	15 (10)	0	
Nasopharynx		20 (2)	1	0	
Oropharynx		415 (39)	0	0	
Hypopharynx		220 (21)	0	0	
Larynx		208 (20)	0	2 (12)	
Oral cavity		154 (15)	1	0	
Head and neck skin	31 (100)	0	129 (88)	0	
Major salivary glands		0	0	14 (88)	
CUP		5	0	0	
T stage, n (%)					<0.0001
Tx	0	8	0	0	
T1	22 (71)	276 (26)	80 (55)	2 (11)	
T2	8 (26)	313 (30)	17 (12)	10 (63)	
T3	1 (3)	221 (21)	29 (20)	3 (20)	
T4	0	239 (23)	20 (14)	1 (6)	
N stage, n (%)					<0.0001
N0	31 (100)	417 (40)	59 (40)	16 (100)	
N+	0	640 (60)	87 (60)	0	
M stage, n (%)					0.01
M0	31 (100)	1015 (96)	132 (90)	15 (94)	

M1	0	42 (4)	14 (10)	1 (6)
Grading, n (%)				
G1		45 (4)		
G2		506 (48)		
G3		469 (45)		
G4		13 (1)		
Gx		24 (2)		
R stage, n (%)				
				0.28
R0	30 (97)	590 (83)	11 (92)	10 (71)
R1	1 (3)	58 (8)	0	3 (21)
R2	0	14 (2)	1 (8)	0
Rx	0	50 (7)		1 (7)
Treatment, n (%)				
				<0.0001
OP only	30 (97)	187 (19)	93 (64)	1 (6)
OP + RTX	0	300 (28)	16 (11)	13 (81)
OP + RCTX	0	203 (19)	12 (8)	0
OP + CTx	0	0	15 (10)	0
Prim. RCTX	0	329 (31)	1	0
Prim. RTX	1 (3)	36 (3)	1	2 (13)
Immunotherapy	0	0	9 (6)	0

Table 2: Distant metastatic profile

	HNSCC	MM	ACC	p-value
M stage, n (%)				
M1 synchronous	42 (4)	14 (10)	1 (6)	0.01
M1 metachronous	143 (14)	35 (24)	3 (19)	0.0009
Time frame, Mean \pm SD				
Diagnosis to metachronous M1	18 \pm 14	37 \pm 33	13 \pm 5	0.6
Location, n (%)				
Multi-organ disease	34 (18)	21 (43)	1 (25)	<0.0001
Liver	34 (18)	15 (31)	1 (25)	0.04
Lung	88 (48)	18 (37)	3 (75)	
Mediastinum	12 (7)	1 (2)	0	
Bone	32 (17)	7 (14)	0	
Skin	16 (9)	5 (10)	1	
Central nervous system	7 (4)	16 (33)	0	
Peripheral lymph nodes	12 (7)	20 (41)	0	
Other	24 (13)	1 (2)	0	

Figures

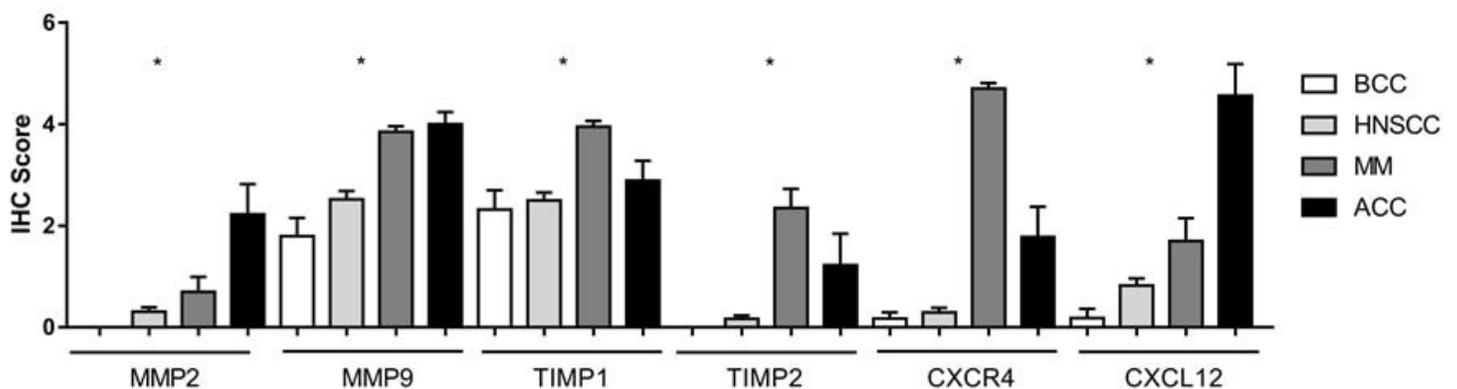


Figure 1

Immunohistochemical markers in HNC primary tumors Immunohistochemistry of primary tumor specimens visualizes a stepwise increase of all analyzed proteins following the clinical occurrence of distant metastasis.

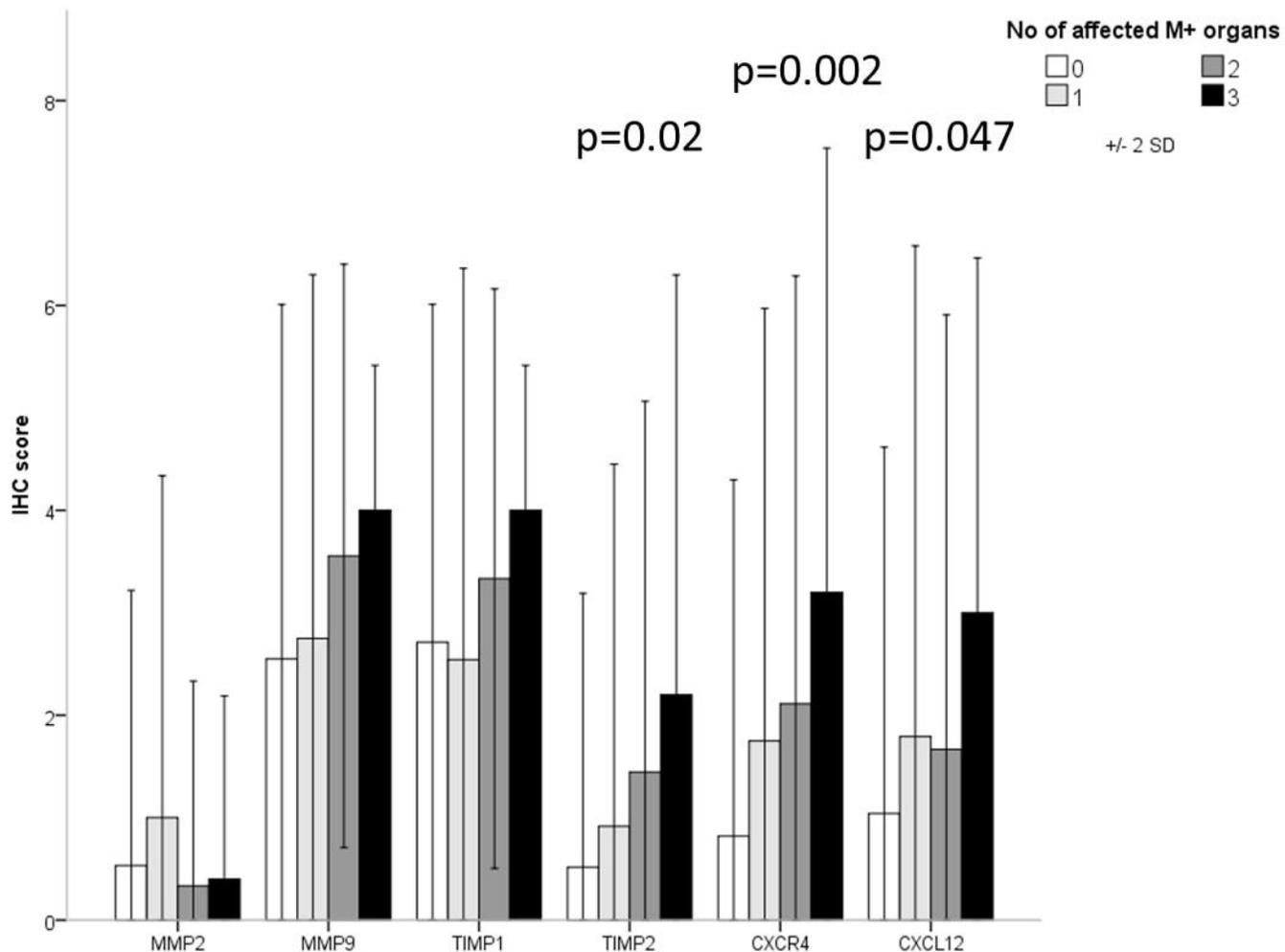


Figure 2

Immunohistochemical markers due to the number of affected organs Immunohistochemistry of primary tumor specimens visualizes a significant increase in TIMP2, CXCR4, and CXCL12 expression with the number of distant metastasis affected organs.

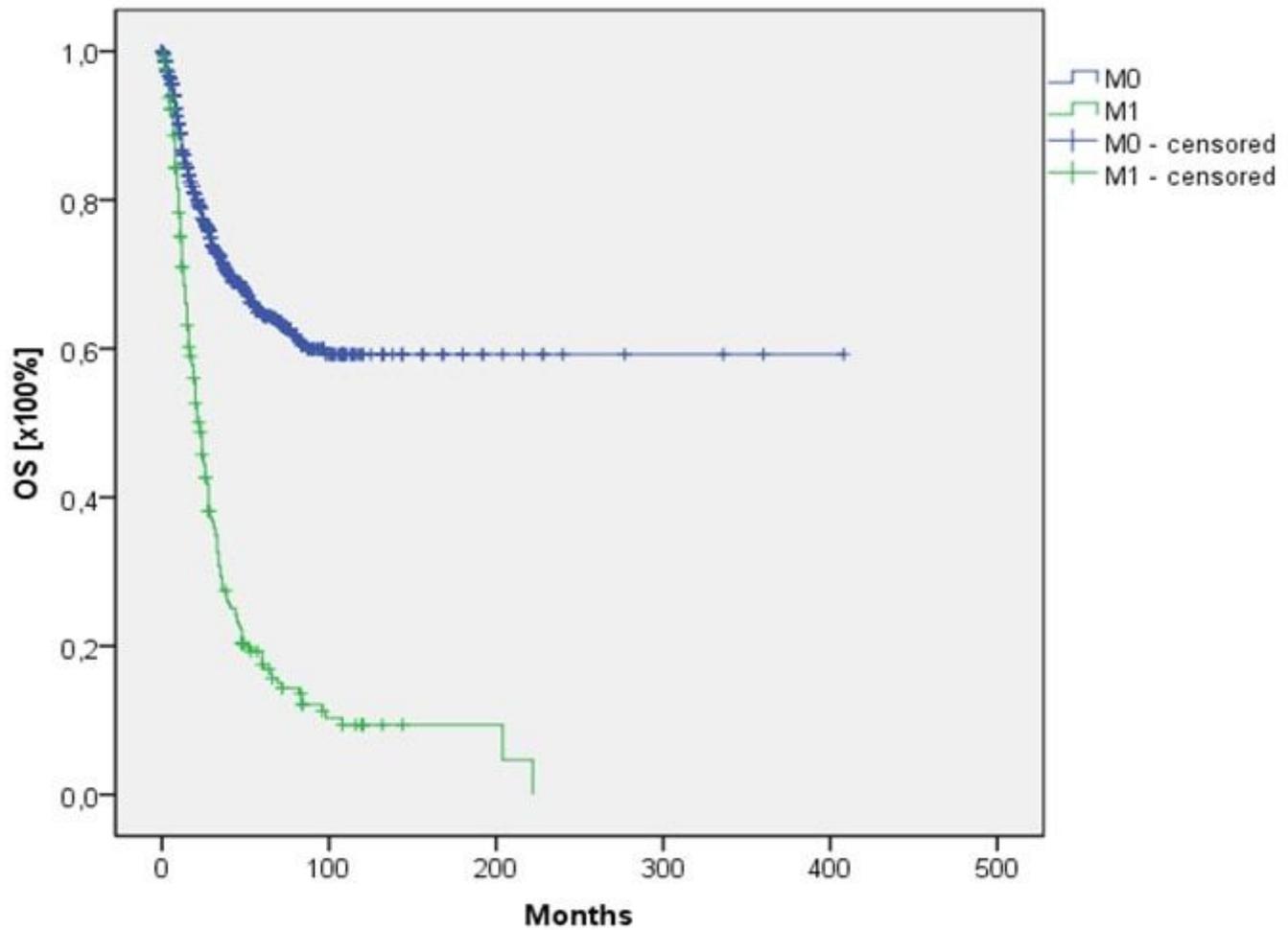


Figure 3

Overall survival in metastasizing HNM Overall survival (OS) was significantly better in patients without distant metastasis when compared with their M1 counterparts.

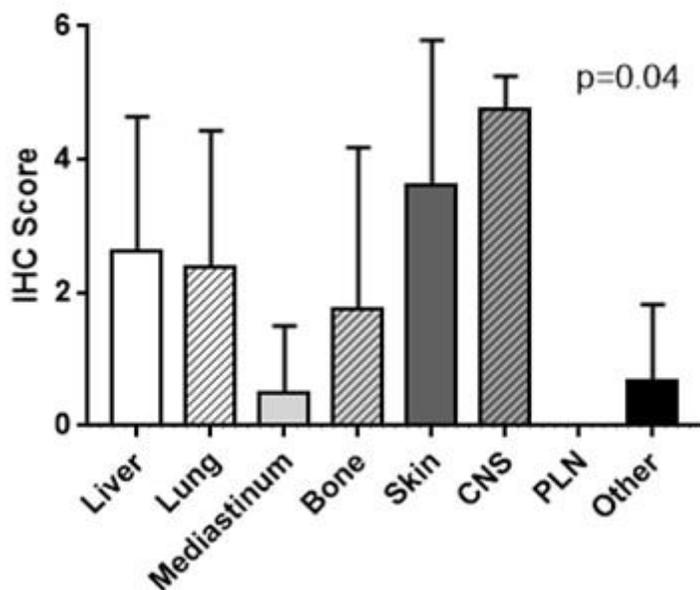


Figure 4

Immunohistochemical markers in different distant metastasis organs CXCR4 expression at primary tumour site differed significantly due to the distant metastasis location. Highest CXCR4 levels were detected in skin and CNS metastases, moderate levels in lung and liver involvement, and low CXCR4 levels in all other locations.