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Epidermal Growth Factor Is Increased in Conjunctival Malignant Melanoma

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Abstract. *Background/Aim: Conjunctival malignant melanoma (CMM) is a rare, but very aggressive tumor with a high metastasis rate. Not much is known about the CMM metastasis mechanisms. So far, epidermal growth factor (EGF) and its receptor (EGF-R) as well as macrophages and matrix metalloproteinase 9 (MMP-9) have been reported to lead to metastasis by epithelial-mesenchymal-transition and tumor migration in different solid tumors. Therefore, we evaluated whether EGF and EGF-R, CD68 and MMP-9 are altered in CMM samples in comparison to conjunctival nevi and healthy conjunctiva. Patients and Methods: EGF, EGF-R, the macrophage marker CD68 and MMP-9 expression were analyzed in human conjunctival melanoma (CMM, n=16), human conjunctival nevi (n=13) and disease-free human conjunctiva (controls, n=14) by immunohistology. Staining of each sample was evaluated using a standardized score ranging from negative (0) to triple positive (3). The groups were then compared by ANOVA, followed by Tukey's post-hoc test. Results: A statistically significant increase of EGF was seen in CMM samples in comparison to conjunctival nevi (p=0.03). In contrast, no statistically significant differences in EGF-R expression were noted between the three groups. A statistically significant increase of CD68 was only seen in conjunctival nevi compared to controls (p=0.04). MMP-9 expression was similar in all groups. Conclusion: In CMM, the study data demonstrated an up-regulation of EGF in comparison to conjunctival nevi. Hence, EGF might promote proliferation of CMM cells and induce the epithelial-mesenchymal transition. Therefore, our data suggest that an interplay between EGF and CMM might have a critical role in the developing CMM tumors and metastasis.*

Conjunctival malignant melanoma (CMM), with an estimated annual incidence of 0.04 per 100.000 people, is a rare but extremely malignant tumor (1-3). The 10 year mortality rate of 30% demonstrates its high malignancy (2). Progress in local tumor control and reduction in the rate of local recurrence in the last 20 to 30 years has not had any positive effect on CMM metastasis rate (4-10).

Several clinical and histopathological prognostic factors for CMM metastasis have been evaluated so far. Tumor thickness, ulcerative tumor invasion in the sclera and feeder vessels as well as lymph vascular invasion, microsatellites, mitotic figures and epithelioid cell types are clinical and histopathological risk factors for metastasis (5, 7-9, 11-15). Also, dermatoscopy may further clinically discriminate between nevi and CMM (16). Recently, Esmaeli *et al*. demonstrated that a higher TNM classification grading of conjunctival melanoma (following the 8th edition of the American Joint Committee on Cancer) exhibited a higher risk for metastasis. Also, this group noted that greater tumor thickness, ulceration or positive sentinel lymph node correlated with metastasis (17). Furthermore, Kenawy *et al*. detected a genetic deletion on chromosome 10 in conjunctival melanoma samples, which was correlated with metastasis, lymphatic invasion, increased tumor thickness and BRAF mutation (18). Larsen *et al*. discovered a higher metastasis risk for extrabulbar CMM as well as for CMM undergoing an incisional biopsy or an excisional biopsy

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Table I. *Summary of biodata of CMM samples.*

Sample	Eye	Age at excision	Gender	TNM classification	Adjuvant therapy	Metastasis	Location of metastasis	Recurrence
1	Right	57	Male	pT(is)N0M0	Strontium-90	No	-	No
2	Right	44	Male	pT(is)N0M0	Ruthenium-106	No	-	No
3	Left	77	Male	pT(is)N0M0	Strontium-90	No	-	Yes
4	Left	65	Male	pT(is)N0M0	External beam	No	-	Yes
5	Right	67	Male	pT(is)N0M0	Strontium-90	No	-	No
6	Right	58	Male	pT(is)N0M0	Strontium-90	No	-	No
7	Left	55	Male	pT(is)N0M0	Strontium-90	No	-	Yes
8	Left	28	Male	pT(is)N0M0	Ruthenium-106	No	-	No
9	Left	76	Male	pT(is)N1M1	Strontium-90	Yes	Cervical lymph nodes, pulmonal, pleural and hepatical distal metastasis	Yes
10	Right	31	Female	pT(is)N0M0	Strontium-90	No	-	Yes
11	Right	67	Female	pT(is)N0M0	Strontium-90	No	-	No
12	Right	81	Female	pT(is)N0M0	External beam	No	-	No
13	Right	65	Male	pT(is)N0M0	External beam	No	-	No
14	Right	73	Female	pT(is)N0M0	Mitomycin C eye drops, cryocoagulation	No	-	No
15	Right	65	Male	pT(is)N0M0	External beam	No	-	No
16	Left	87	Female	pT(is)N1M0	Mitomycin C eye drops, cryocoagulation	Yes	Lymph nodes glandula parotis	Yes

without adjuvant radiotherapy (19). Therefore, many clinical and histopathological risk factors for CMM metastasis are known and can be linked with epithelial-mesenchymal-transition and tumor cell migration, but not much is known regarding molecular mechanisms of CMM metastasis. So far, upregulation of mucin 4 protein (MUC 4) in CMM has been linked with CMM migration (20). Additionally, investigations in CMM noted a UV light-induced mutational signature (21). Also, Heindl and colleagues observed the development of lymphatic vessels in primary CMM and especially in CMM with metastasis (22, 23).

Hanahan and Weinberg have postulated that local tumor invasion capacity as well as epithelial-mesenchymal-transition are critical steps for migration and metastasis in solid tumors (24, 25). In different solid tumors, especially in cutaneous melanoma, epidermal growth factor (EGF) and EGF-receptor (EGF-R), macrophages and matrix metalloproteinase 9 (MMP-9) trigger metastasis, through *e.g.*, local tumor migration and epithelial-mesenchymal-transition. Li *et al.* noted activation of metastasis inducing pathways by EGF, EGF-R and MMP-9 in two cutaneous cell lines (26). Furthermore, Treskova *et al.* investigated EGF plasma levels and correlated higher levels with lymph node metastasis in cutaneous melanoma (27). Also, Bracher *et al.* showed a significant upregulation of EGF in human primary cutaneous melanoma tissue of metastatic patients (28). Foks *et al.* revealed a correlation between macrophages and more advanced cutaneous melanoma including higher microvessel density and poor prognosis (29). In a study by Licarete *et al.*, the effect of tumor-associated macrophages was suppressed in a cutaneous melanoma cell line and hereby reduced the activity of these cells (30). Until now, upregulation of EGF, EGF-R, macrophages and MMP-9 has not been investigated in CMM. Therefore, expression patterns and localization of these factors in CMM might provide new information to facilitate our understanding of the pathogenesis of CMM metastasis.

The objective of this study was to analyze whether EGF, EGF-receptor, CD68 (a macrophage marker) and MMP-9 are up-regulated in human CMM samples in comparison to conjunctival nevi and healthy conjunctiva. These data might elucidate tumor cell migration to vessels for metastasis and might therefore indicate how metastasis develops in CMM.

Patients and methods

Sample collection. For sample collection, approval was obtained from the Ruhr-University Bochum ethics committee (register no 4850-13; Bochum, Germany); the study was conducted following the guidelines of the Helsinki declaration.

Analysis was conducted on CMM (n=16 for EGF and EGF-R; n=14 for CD68 and MMP-9; Table I), conjunctival nevi (n=13), and healthy conjunctival epithelial samples without any pigmentation (controls, n=14). CMM and conjunctival nevus tissue was obtained by excision for diagnostic reasons. Healthy conjunctival tissue was

Table II. *Primary and secondary antibodies used for immunohistology.*

Primary antibody	Company	Dilution factor	Secondary antibody	Company	Dilution factor	Reference
EGF goat anti-human	Santa Cruz	1:100	Donkey anti-goat Alexa 488	Invitrogen	1:500	(60)
EGF-R rabbit anti-human	Santa Cruz	1:100	Donkey anti-rabbit Alexa 555	Invitrogen	1:700	(31)
MMP-9 rabbit anti-human	Millipore	1:400				(33)
CD68 mouse anti-human	DAKO	1:100	Goat anti-mouse Alexa 488	Invitrogen	1:500	(32)

obtained from patients undergoing strabismus surgery. For all CMM samples, clinical biodata was collected (Table I).

Immunohistology. Immunohistology was performed as previously described (20). Conjunctival biopsies were fixed in 4% (w/v) formalin, dehydrated in an ethanol series and then embedded in paraffin. Serial sections (4 μm thick) were de-paraffinized and rehydrated. High-temperature antigen-retrieval was performed by applying 0.05% sodium-citrate buffer (pH 6) for 20 min. Staining was performed for all samples to enable association of immune histochemical staining with structures within the samples.

Sections were processed for immunofluorescence microscopy using antibodies against EGF (31), EGF-R (31), CD68 (32) and MMP-9 (33) (Table II). Sections were blocked for 60 minutes at room temperature. After two washing steps with PBS for 5 minutes, the primary antibody was incubated overnight. After four further wash out steps with PBS, appropriate secondary antibodies were applied for 60 minutes. DAPI co-staining was applied to visualize cell nuclei. Negative controls were performed by using only secondary antibodies.

Digital images were taken from two sections per sample with six images per section using an Axio Image M1 Microscope (Zeiss, Oberkochen, Germany). Immunohistological analysis was performed following a previously established score for CMM, analog to the Remmele score for breast cancer (34, 35). Each sample was divided in sectors of similar area and separately scored following an established scoring system ranging from 0 to 3 (0=no signal/not a single cell was stained; 1=minor signal/cells were stained sporadically; 2=moderate to high signal/couple of cells were stained within a cell layer; 3=high signal/couple of cells were stained within different cell layers). The scoring system was used for EGF, EGF-R, CD68 and MMP-9. Data was used for further statistical analysis.

Statistical analysis. Data are presented as mean±SEM. The three groups were compared by ANOVA followed by Tukey's post-hoc test using Statistica software (V13.0, Statsoft, Dell, Tulsa, OK, USA). *p*-Values below 0.05 were considered statistically significant with $*p<0.05$, $**p<0.01$ and $***p<0.001$.

Results

Bio data. For EGF analysis, 16 CMM samples (10 primary tumors and 6 recurrent tumors, two of which developed CMM metastasis) as well as 13 conjunctival nevi samples and 14 healthy conjunctival samples (controls) were used. The average age of CMM patients was 62±17 years with 5 females and 11 males. The conjunctival nevi patients had an average age of 41±17 years and this group had 8 female and 5 male patients. The healthy conjunctiva group consisted of 14 patients with an average age of 14±22 years; 7 were females and 7 males. Detailed biodata (gender, age at diagnosis, eye, TNM level, recurrence after, excision metastasis after excision, therapy) for all 16 CMM samples are displayed in Table I. For CD68 and MMP-9 analysis, only CMM sample numbers 1 to 14 were used, due to limited tissue.

EGF upregulation around epithelial cell membranes in CMM. EGF staining was localized at the epithelial cell membranes of CMM, conjunctival nevi, and controls (Figure 1A). Staining investigations and scoring demonstrated increased EGF expression in CMM (mean score of 0.56±0.12) in comparison to conjunctival nevi (0.18±0.05, $p=0.03$). No statistical significance was seen between CMM and controls (mean score 0.23±0.09, $p=0.055$) and between conjunctival nevi and controls ($p=0.94$; Figure 1B).

Sub-analysis of two CMM with history of metastasis even showed a mean EGF score of 1.12. These samples displayed an even higher score than all other CMM samples. Statistical analysis was not done between CMM with and without metastasis history due to the low number of CMM with metastasis history.

Sub-analysis of two CMM recurrences showed a mean EGF score of 0.39. These samples displayed a lower score then all other CMM samples. Statistical analysis was not done between CMM with recurrence history and primary CMM due to the low number of CMM recurrences.

EGF-R staging intensity and localization was comparable in all samples. EGF-R staining was localized at the epithelial cell membranes of CMM, conjunctival nevi, and controls (Figure 1A). Staining investigations demonstrated no statistical difference of EGF-R score between CMM (mean score of 0.99±0.15) and conjunctival nevi (mean score of 1.08±0.12, $p=0.92$), as well as between CMM and controls (mean score of 0.75±0.18, $p=0.53$). No statistically

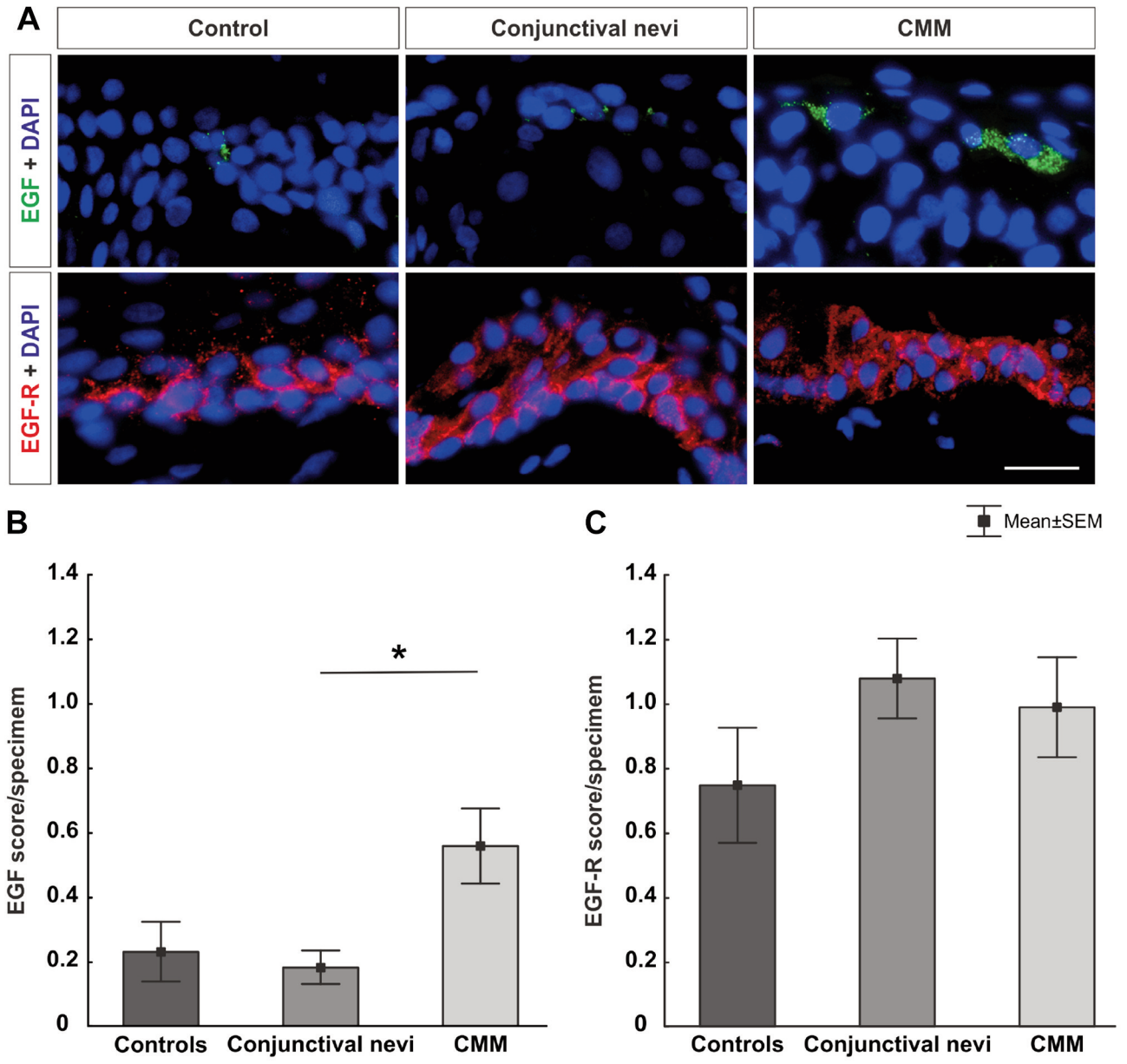

Figure 1. *Immunofluorescence analysis of EGF and EGF-R-expression. A) Immunofluorescence staining of EGF (green) and EGF-R (red) was performed on disease-free conjunctiva (control, n=14), conjunctival nevi (n=13), and conjunctival melanoma (CMM, n=16). Cell nuclei were visualized with DAPI (blue). B) Significantly increased EGF expression was noted in melanoma compared to nevi (p=0.03), but not compared to controls (p=0.055). C) In regard to EGF-R, comparable scores were observed in the three investigated groups. Values are mean±SEM. Scale bar: 20 μm. *p<0.05.*

significant difference was noted between conjunctival nevi and control tissue (p=0.33, Figure 1C).

Sub-analysis of two CMM with metastasis history even showed a mean value EGF-R score of 1.1 and did not show a striking difference to other CMM samples. Sub-analysis of two CMM recurrences revealed a mean EGF-R score of 0.89. These samples displayed a lower score then all other CMM samples. Statistical analysis was not done between CMM with recurrence history and primary CMM due to the low number of CMM recurrences. CMM recurrence samples had a mean EGF, EGF-R, CD68 and MMP-9 score of 0.39, 0.89, 0.55 and 1.0, respectively.

$CD68^+$ macrophage expression did not demonstrate any differences between CMM and conjunctival nevi. $CD68^+$ macrophage staining was localized at the epithelial cell

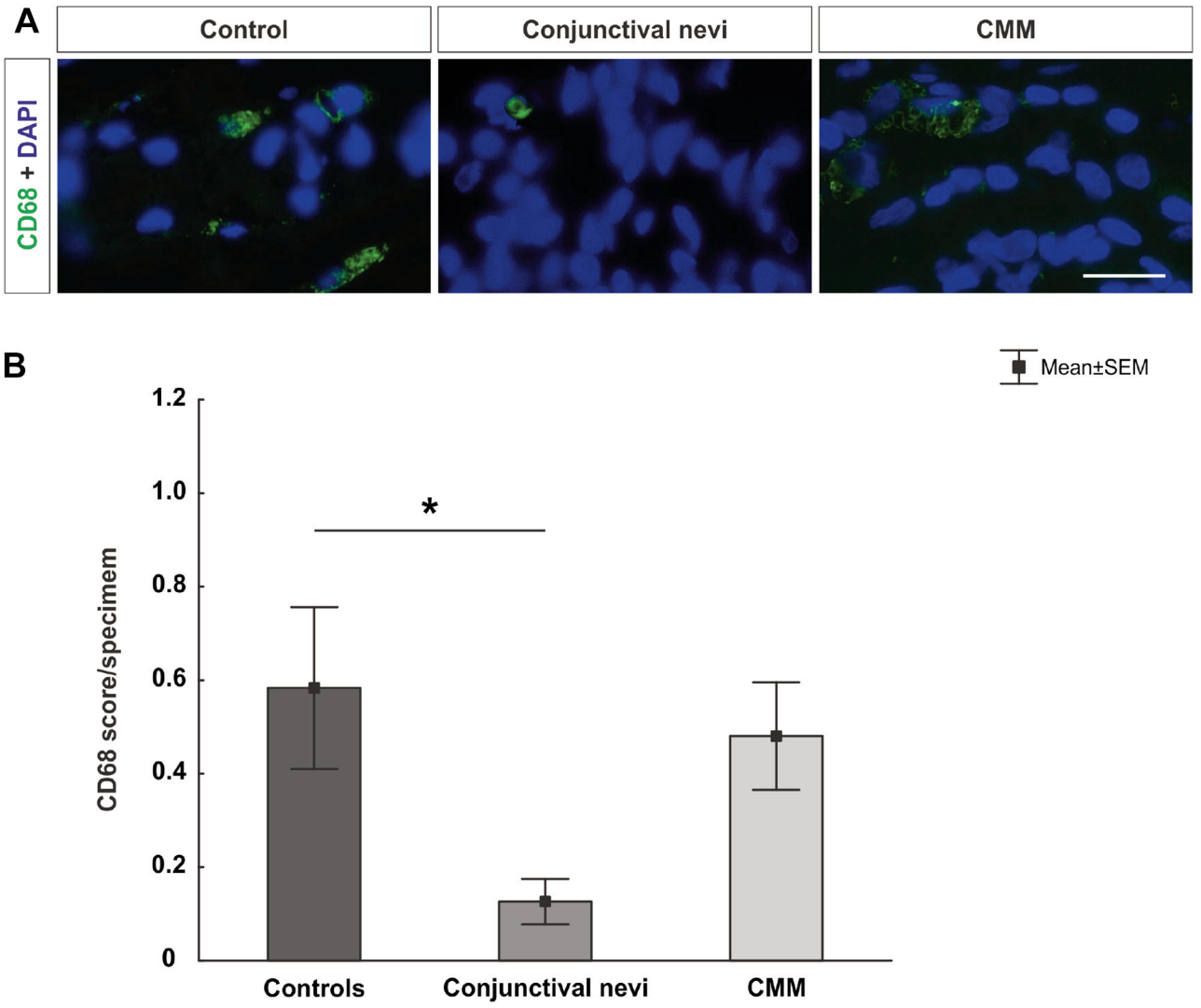

Figure 2. *Immunofluorescence analysis of macrophage incidence. A) Immunofluorescence staining of CD68 (green), a highly glycosylated lysosomal membrane protein found in macrophages, was performed on healthy conjunctiva (control, n=14), conjunctival nevi (n=13), and conjunctival melanoma (CMM, n=14). Cell nuclei were stained with DAPI (blue). B) There was no statistical difference for CD68$^+$ cell scores in CMM compared to conjunctival nevi. Healthy conjunctiva presented more macrophages in comparison to conjunctival nevi (p=0.04). Values are mean±SEM. Scale bar: 20 μm. *p<0.05.*

membranes of CMM, conjunctival nevi, as was well as controls (Figure 2A). Staining investigations demonstrated no statistical difference of CD68$^+$ cell score between CMM (mean score of 0.48±0.11) and conjunctival nevi (mean score of 0.13±0.05, p=0.12), as well as between CMM and controls (mean score of 0.58±0.17, p=0.83). A statistically significant difference was only seen between conjunctival nevi and control tissue (p=0.04, Figure 2B). Sub-analysis of two CMM with metastasis history even showed a mean CD68 score of 0.49 and did not display a striking difference to other CMM samples.

Sub-analysis of two CMM recurrences showed a mean CD68 score of 0.55. These samples displayed a higher score than all other CMM samples. Statistical analysis was not carried out between CMM with recurrence history and primary CMM due to the low number of CMM recurrences.

MMP-9 intensity and localization were similar for CMM, nevi, and controls. MMP-9 staining was localized at the epithelial cell membranes of CMM, conjunctival nevi, and controls (Figure 3A). MMP-9 staining investigations demonstrated no statistical difference between CMM (mean score of 0.97±0.46) and conjunctival nevi (mean score of 1.07±0.52, p=0.85), as well as between CMM and controls (mean score of 0.89±0.43, p=0.91; Figure 3B). Also, no

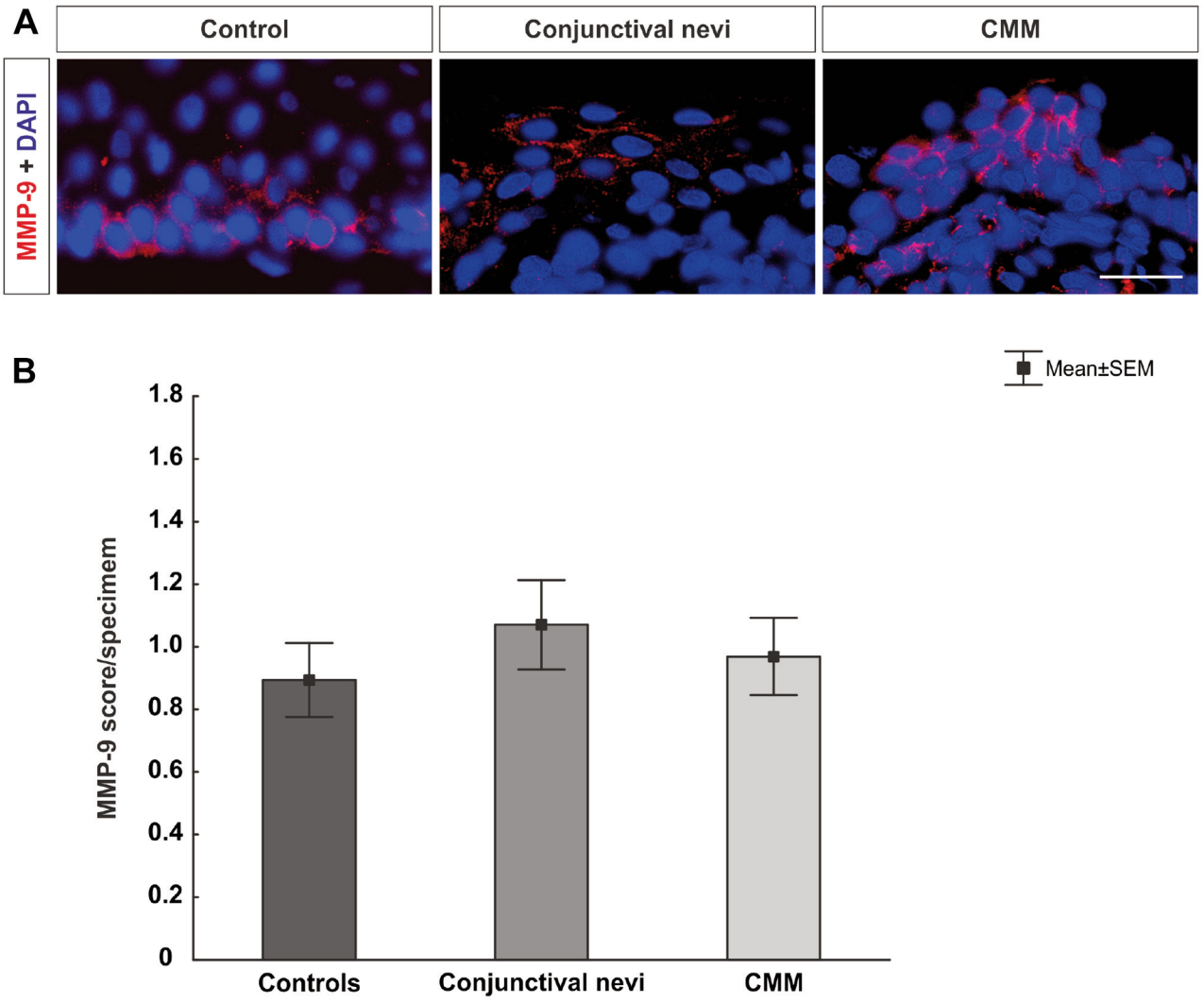

Figure 3. *Evaluation of MMP-9 expression. A) Immunofluorescence staining of MMP-9 (red) was performed on healthy conjunctiva (control, n=14), conjunctival nevi (n=13) and conjunctival melanoma (CMM, n=14). Cell nuclei were visualized with DAPI (blue). B) No significant differences were noted regarding MMP-9 expression in CMM, conjunctival nevi and healthy conjunctiva. Values are mean±SEM. Scale bar: 20 μm.*

statistically significant differences were noted between conjunctival nevi and controls (p=0.61). Sub-analysis of two CMM with metastasis history even showed a mean MMP-9 score of 1.07 and did not reveal a difference to other CMM samples. Sub-analysis of two CMM recurrences presented a mean MMP-9 score of 1.00. These samples displayed the highest score of all CMM samples. Statistical analysis was not done between CMM with recurrence history and primary CMM, again due to the low number of CMM recurrences.

Discussion

In our study, EGF, EGF-R, CD68 and MMP-9 were located on the epithelial cell membrane of CMM nevi and controls. We detected a statistically significantly increased EGF score in CMM samples compared to conjunctival nevi. In contrast, values for EGF-R were comparable in all groups. No significant differences were seen regarding $CD68^+$ macrophages in CMM samples compared to conjunctival nevi and controls. Also, a similar expression of MMP-9 was noted in all these tissues.

So far, there have not been any investigations regarding EGF and its receptor in CMM. Meng *et al*. observed that EGF is significantly upregulated in human breast cancer tissue and associated with metastatic progression and enhanced cancer cell migration and invasion (36). In addition, Xu *et al*. discovered that epithelial-mesenchymal-transition is EGF-dependent in hepatocellular carcinoma cells and mouse

xenographs (37). In another study, Cheng *et al*. noted that ovarian tumor cell lines downregulate E-cadherin after EGF incubation, including a higher motility and invasiveness of the tumor cells. These effects could be inhibited by siRNA or inhibitors for EGF-R (38). Ma and Niederkorn injected different uveal cell lines into a nude mouse model and observed higher grades of metastasis to the liver depending on the grade of EGF-R in each cell line (39). Furthermore, several research groups have demonstrated that BRAF inhibitor resistance is linked with the upregulation of EGF and EGF-R in cutaneous melanoma cells and that this inhibitory effect is associated with EGF and EGF-R downregulation (40-46). Therefore, an inhibition of BRAF is probably valuable, when EGF-EGF-R activity is also reduced in tumors, *e.g.,* in colon carcinoma or CMM. All these findings are in accordance with the EGF upregulation detected in our study. Therefore, EGF upregulation might play a role in CMM malignancy. Nevertheless, no increase in EGF-R expression was seen in our CMM analysis. Recognizing the upregulation of EGF in our study, an upregulation of EGF-R was expected in CMM but was not seen in our samples.

So far, different research groups demonstrated the important role of macrophages in cutaneous melanoma metastasis (47-49). Storr *et al*. observed a correlation between higher macrophage levels and increased ulceration rates, tumor thickness, mitosis rates, as well as infiltration rates of lymph and blood vessels in cutaneous melanoma (49). Also, Jensen *et al*. reported a slightly lower survival rate in cases with a higher number of macrophages in the primary tumor tissue in this skin malignancy but did not differentiate between tissue with and without metastasis history (47). In addition, EGF expression is induced by macrophages in different solid tumors and may explain the EGF upregulation in CMM (50-53). Furthermore, in breast cancer, cell migration and invasion are triggered by macrophages and EFG (54). Our data are not in line with the previous results from cutaneous melanoma; our results showed a higher, but statistically not significant, level of macrophages in CMM in comparison to conjunctival nevi. These results are therefore not a hint for tumor associated macrophages as a mechanism for metastasis. Furthermore, the highest number of macrophages was seen in controls, which might be due to the younger patients in this group.

Kim *et al*. previously investigated MMP-9 levels in CMM in comparison to conjunctival nevi without seeing any differences. A sub-analysis of CMM samples with a clinical diameter >1.5 mm has demonstrated a higher level of MMP-9. The authors concluded that MMP-9 might play a role in advanced CMM (55). In addition, Candrea *et al*. analyzed cutaneous malignant melanomas thicker than 2 mm and noted a higher amount of active and inactive MMP-9 in these samples compared to benign nevi. The same study revealed a correlation between expression of inactive MMP-9 and lymphatic metastasis (56). Van den Oord *et al*. also detected an upregulation of MMP-9 in cutaneous malignant melanoma. Surprisingly, thinner lesions had higher amounts of MMP-9 than thicker lesions (57). An accumulation of macrophages in lung metastasis tissue of gastric cancer patients was also noted in a current study (58). In addition, in a mouse model for this disease, macrophages secreted MMP-9 and induced an epithelial-mesenchymal transition (58). Increased nasopharyngeal cancer cell migration was described after MMP-9 downregulation (59). In contrast to these important effects of MMP-9 in solid tumors, we did not detect an MMP-9 upregulation in CMM samples.

The study presented here has some limitations. The patient's history has been collected to the best of our knowledge. However, previous studies and clinical experience has shown that especially in cases of metastasis, a loss of patients has been seen and follow-up information is limited. Further, due to the rarity of the disease, there are limited cases in this study.

Taken together, we previously noted a possible function of MUC4 in CMM metastasis by epithelial-mesenchymal transition and tumor migration (20). Our new data revealed an upregulation of EGF in CMM. EGF may promote proliferation of tumor cells and induce an epithelial-mesenchymal transition. Therefore, EGF might play an important role in converting benign conjunctival lesions into CMM and for developing metastasis.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization: V.K.; Methodology: S.R., V.K., S.C.J.; Software: S.R., S.C.J.; Validation: C.W., M.S, S.G., M.R., A.T., H.B.D.; Formal Analysis: V.K., S.R., S.C.J.; Investigation: V.K., S.R., S.C.J, C.W., M.S,; Resources: H.B.D, S.C.J.; Data Curation: V.K., S.R., S.C.J; Writing – Original Draft Preparation: V.K.; Writing – Review & Editing: V.K., S.R., S.C.J., C.W., M.S, S.G., M.R., A.T., H.B.D.; Visualization: V.K., S.R., S.C.J; Supervision: V.K., S.C.J.; Project Administration: V.K, S.C.J.; Funding Acquisition: V.K., S.C.J.; S.G..

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