Ectopic Myoglobin Expression Is Associated with a Favourable Outcome in Head and Neck Squamous Cell Carcinoma Patients

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Abstract. *Background/Aim:* Ectopic myoglobin (MB) expression, mediated by alternative and hypoxia-inducible transcription, has recently been demonstrated in several epithelial tumours. This study aimed to examine the expression of MB in hormone-independent head and neck squamous cell carcinomas (HNSCCs). *Patients and Methods:* Using immunohistochemistry, ectopic MB expression was analyzed on tissue microarrays (TMAs) of 524 patients with localized and locally advanced primary and recurrent HNSCC who had undergone surgical treatment with curative intent. Associations of MB expression with survival and clinicopathological parameters were analyzed. *Results:* MB expression was found in 45.8% of HNSCC patients being significantly lower in normal adjacent tissue (NAT)

compared to primary and recurrent tumours ($p < 0.001$) and significantly associated with a favourable overall survival (OS) in HNSCC [$p = 0.037$, hazard ratio (HR) = 0.72, 95% confidence interval (CI) = 0.53-0.98]. Furthermore, MB expression negatively correlated with human papillomavirus (HPV) status ($p = 0.013$). *Conclusion:* MB is differentially expressed in HNSCC and correlates with a better OS.

Myoglobin (MB) is a cytoplasmatic monomeric oxygen-binding hemoprotein known to be localized mainly in cardiac myocytes and mitochondrial-rich skeletal muscles. In contrast to its relative haemoglobin, MB is only found in the blood stream after muscle injury. The functional paradigm of MB is the storage and intracellular transport of oxygen (O_2) (1, 2) to the mitochondria for oxidative phosphorylation within the myocyte. However, MB also maintains homeostasis of the nitric oxide free radical ($NO\bullet$) in muscle through scavenging or producing $NO\bullet$ molecules (3, 4) and appropriating excessive reactive oxygen species (ROS) (5). In addition, MB is able to bind fatty acids with physiological binding constants *in vitro* (6). On the other hand, MB-knockout mice are viable and exhibit normal exercise capacity but their survival depends on the activation of compensating mechanisms, *i.e.* increasing capillary densities and haematocrit (7, 8).

In the last years, the existence of non-muscle MB has been increasingly acknowledged, demonstrating MB, *e.g.*, in the liver, brain or gills of carps (9) and in humans,

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particularly in various epithelial tumours and cancer cell lines (10-14). So far, the biological function and pathophysiological role of ectopically expressed MB is poorly understood. Recent studies have shown that MB expression in breast tumours is associated with a better prognosis and a more differentiated cell phenotype (11). Similarly, a trend towards a better prognosis in patients with high-grade tumours was observed in prostate cancer (12). In both tumour entities, MB presence correlates with the expression of fatty acid synthase (FASN), a key enzyme of the *de novo* synthesis of fatty acids and homeostasis, which may relate to the reported fatty acid-binding properties of MB. For both breast and prostate tumours, it was further demonstrated that MB is transcribed from an alternative and hypoxia-inducible promoter different from the one used in muscle (12, 15, 16), thereby supporting the hypothesis of a non-canonical role of MB in tumours. Additional data also suggest a role of steroid hormones in regulating MB in these tumours (11, 12, 17).

The aim of the present study was to analyze ectopic MB expression in a hormone-independent tumour entity. Therefore, the MB expression and its association with clinicopathological parameters, including follow-up data, tumour hypoxia, human papillomavirus (HPV) and FASN, were investigated in a large cohort of head and neck squamous cell carcinoma (HNSCC) patients.

Patients and Methods

Ethics statement. Studies were approved by the Institutional Review Board of the University Hospital of Bonn, Germany, which waived the need for written informed consent from the participants.

Patients. The patient cohort comprised of 524 patients with histologically confirmed HNSCC. Patients had undergone surgery with curative intent at the University Hospital of Bonn between 1997 and 2015. Tumour origin was distributed as follows: hypopharynx (n=55), oropharynx (n=172), oral cavity (n=139), larynx (n=134), lymph nodes (n=7) and 17 "other locations", such as facial skin and lips. Clinicopathological parameters and follow-up data were available (Table I). Median follow-up time was 27 months (mean=34) ranging from 0 to 145 months.

Immunohistochemistry. Construction of tissue microarrays (TMAs) has been described in previous studies (18, 19). Each patient was represented by up to four cores with 0.6 mm diameter. TMAs were stained in the immunohistochemistry laboratory of the Institute of Pathology, Bonn. HPV status was assessed by p16 immunohistochemical staining as a surrogate marker. Immunohistochemical staining of MB, carbonic anhydrase IX (CAIX), p16 and FASN was conducted using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA). The following antibodies and dilutions were used: myoglobin, clone EP3081Y, (1:200; Abcam, Cambridge, UK); fatty acid synthase, clone S2, (1:750; Abgent, San Diego, CA, USA); CAIX, rabbit polyclonal (1:300; Abcam.); p16, mouse monoclonal, clone E6H4™ (CINtec® Histology

Kit; Ventana Medical Systems). Slides were counterstained with haematoxylin, dehydrated and mounted. Evaluation of the slides was performed by two pathologists (GK, VS). MB, FASN and CAIX immunoreactivity was scored using the immunoreactive score (IRS), which is the product of categorized staining intensity (0-3) and immuno-positive tumour area (0-4) and, hence, ranges from 0-12. For statistical analysis, MB immunoreactivity was semiquantitatively categorized as follows: (IRS=0) negative; (IRS=1-3) weak; (IRS=4-7) moderate and (IRS=8-12) strong. p16 immunoreactivity was assessed as positive or negative.

Statistics. Statistical analyses were performed with SPSS, Version 21 (SPSS Inc., Chicago, IL, USA). Student's *t*-tests were used to evaluate the statistical significance between MB expression and clinicopathological parameters. Spearman's Rho was used for bivariate correlation analysis. ANOVA was conducted to test differences between tumour locations. Univariate survival analyses were conducted according to univariate Cox proportional hazards and Kaplan-Meier analyses. *p*-Values refer to Wald test and log-rank test, respectively.

Results

MB expression in HNSCC. MB was predominantly localized in the cytosol. However, in single cases, a strong membrane staining and/or nuclear localization was observed. Representative immunohistochemical stainings of cytoplasmic and membrane-bound MB in HNSCC are shown in Figure 1. No association between MB levels in primary tumours and emerging local tumour recurrence or primary tumours and the corresponding recurrence was seen. MB expression in normal adjacent tissue (NAT) was significantly lower compared to tumours or recurrent cancers ($p<0.001$ each). In the HNSCC cohort, 247 of 456 assessable primary tumour samples (54.2%) showed no MB expression, 137 (30.0%) displayed a weak expression, 47 (10.3%) were moderately positive and 25 (5.5%) had a strong MB staining. Recurrent tumours were MB-negative in 33 of 57 cases (57.9%), 14 cases (24.6%) were weakly positive, 6 cases (10.5%) displayed a moderate staining and 4 cases (7.0%) were strongly stained.

Association of MB with clinicopathological parameters and overall survival (OS). A correlation between MB expression and age ($p<0.001$), sex ($p=0.016$) and tumour localization ($p<0.001$) was found (Table I). Younger patients showed the highest MB expression as expression levels decreased with age. A high percentage of women showed MB expression with on average higher MB levels compared to men. With respect to the tumour localization, the highest MB expression could be detected in the oral cavity and the oropharynx, whereas the larynx showed the lowest MB expression. No significant correlation could be drawn between MB expression and (i) the TNM staging, (ii) different noxae, (iii) FASN ($p=0.077$) expression or (iv) the hypoxia marker

Table I. Association of myoglobin (MB) expression with clinicopathological parameters in head and neck squamous cell carcinoma (HNSCC) patients. MB expression was dichotomized into MB- (negative) vs. MB+ (weak, moderate, strong).

Variable	Patients (n=524)	MB-	MB+	p-Value
Gender				0.016*
Female	138 (26.3%)	65 (47.1%)	73 (52.9%)	
Male	386 (73.7%)	229 (59.3%)	157 (40.7%)	
Age				<0.001 [‡]
Median Age (Range) [Years]	63 (26-90)			
0-50	68 (13.0%)	29 (42.6%)	39 (57.4%)	
51-60	149 (28.4%)	73 (49.0%)	76 (51.0%)	
>60	302 (57.6%)	188 (62.3%)	114 (37.7%)	
Unknown	5 (1.0%)			
Localization				<0.001 [‡]
Oral cavity/tongue/lips	139 (26.5%)	61 (43.9%)	78 (56.1%)	
Oropharynx and tonsils	172 (32.8%)	81 (47.1%)	91 (52.9%)	
Hypopharynx	55 (10.5%)	29 (52.7%)	26 (47.3%)	
Larynx	134 (25.6%)	91 (67.9%)	43 (32.1%)	
Others (nasopharynx, facial skin)	17 (3.2%)	12 (70.6%)	5 (29.4%)	
Unknown	7 (1.3%)			
Tumour				0.93*
Primary tumour	456 (87.0%)	253 (55.5%)	203 (44.5%)	
Locoregional recurrence	57 (10.9%)	34 (59.6%)	23 (40.4%)	
Unknown	11 (2.1%)			
p16 [#]				0.011*
p16-positive	63 (22.6%)	39 (61.9%)	24 (38.1%)	
p16-negative	216 (77.4%)	96 (44.4%)	120 (55.6%)	
245				
Tumour category				0.80 [‡]
T ₀	3 (0.6%)	1 (33.3%)	2 (66.7%)	
T ₁	34 (6.5%)	17 (50.0%)	17 (50.0%)	
T ₂	94 (17.9%)	50 (53.2%)	44 (46.8%)	
T ₃	50 (9.5%)	32 (64.0%)	18 (36.0%)	
T ₄	34 (6.5%)	18 (52.9%)	16 (47.1%)	
N/A	309 (59.0%)			
Nodal category				0.52 [‡]
N0	187 (35.7%)	100 (53.5%)	87 (46.5%)	
N1	63 (12.0%)	37 (58.7%)	26 (41.3%)	
N2	136 (26.0%)	74 (54.4%)	62 (45.6%)	
N3	7 (1.3%)	4 (57.1%)	3 (42.9%)	
N/A	131 (25.0%)			
Smoking				0.54 [‡]
Non-smokers	37 (7.1%)	19 (51.4%)	18 (48.6%)	
Smokers (current and former)	229 (43.7%)	119 (52.0%)	110 (48.0%)	
Unknown smoking status	258 (49.2%)	156 (60.5%)	102 (39.5%)	
Alcohol				0.36 [‡]
Alcoholic (current and former)	97 (18.5%)	54 (55.7%)	43 (44.3%)	
No alcohol	76 (14.5%)	37 (48.7%)	39 (51.3%)	
Moderate alcohol	82 (15.6%)	42 (51.2%)	40 (48.8%)	
Unknown alcohol consumption	269 (51.3%)			

[#]Larynx excluded; [‡]Spearman's Rho; *Student's *t*-test; [†]ANOVA.

CAIX ($p=0.72$). In a univariate Cox proportional hazard analysis, MB-positive HNSCC patients showed a significantly lower risk for death compared to MB-negative cases ($p=0.037$, hazard ratio (HR)=0.72, 95% confidence interval (CI)=0.53-0.98). This finding could be confirmed in Kaplan-Meier analysis ($p=0.035$, Figure 2). Patients with

MB-negative tumours had a mean survival time of 59.9 months (95% CI=53.9-65.9), whereas the MB-positive cases had a mean survival time of 68.7 months (95% CI=60.4-69.2). In multivariate survival analysis, including age, TNM stage, p16 and gender, MB failed to demonstrate an independent prognostic value.

Figure 1. Immunohistochemical staining of myoblobin (MB) in head and neck squamous cell carcinoma (HNSCC) samples. (A) Negative and (B) weak, basally accentuated MB expression in cancer specimens. Note the strong staining in the encased muscle fibres (arrows). (C) Squamous cell carcinoma with pronounced membranous staining. (D-F) Squamous cell carcinoma with the typical mosaic-like, diffuse expression pattern combining membranous, cytoplasmic and nuclear staining with a pronounciation in the basal layer.

P16 expression was detected in 64 of 379 (16.9%) primary HNSCC tumour samples and shown to have a strong correlation with localization ($p=0.008$). A total of 45/106 (42.5%) cases from the oropharynx, 8/31 (25.8%) cases from the hypopharynx and 10/79 (12.7%) patients from the oral cavity were p16-positive (Table I). Except for one, all larynx samples were p16-negative (1/96, 1%). For a sub-analysis the larynx samples were further excluded from the cohort. In univariate Cox proportional hazard analysis, p16-positive HNSCC patients ($n=62$) showed a significantly lower risk for death compared to p16-negative cases ($n=208$, $p=0.005$, $HR=0.41$, 95% $CI=0.22-0.77$). This finding was confirmed in Kaplan-Meier analysis ($p=0.004$).

In summary, MB expression, in our study, showed a significant negative correlation with p16 positivity ($p=0.013$), when excluding the larynx samples. The prognostic value of MB expression remained almost unchanged in univariate Cox proportional hazard analysis ($p=0.035$, $HR=0.70$, 95% $CI=0.50-0.98$) and Kaplan-Meier analysis ($p=0.033$). However, in multivariate survival analysis, including age, TNM stage, p16 and gender, MB still failed as an independent prognostic factor but remained significant when adjusted for p16.

Discussion

Apart from the primary localization of MB in cardiac myocytes and skeletal muscle (2, 20), MB also endogenously occurs in different tumours, *i.e.* breast, non-small cell lung, colon, ovary cancers, as well as medulloblastomas (10, 14, 21).

For breast cancer, we have previously demonstrated that MB-positive tumours have a significantly better prognosis in conjunction with the presence of oestrogen signalling (11). Furthermore, high-grade prostate cancer patients have also shown a trend towards a better prognosis when compared to MB-negative tumours (12). This poses questions about the effect and cause of this observation, since the biological function of ectopically expressed MB remains unclear.

Our study is the first to investigate MB expression in a large HNSCC patient cohort. Meanwhile it is known that MB expression can arise from the tumour-associated alternative MB transcript variants, which are hypoxia-inducible and differ from the canonical muscle-associated mRNA in their 5' untranslated regions (12, 15, 16). Based on immunohistochemical and cell culture results, it seems that MB protein levels in hormone-dependent epithelial tumours, such as breast and prostate cancer, exceed those of hormone-independent entities (11, 12). This could further support the hypothesis that MB expression in prostate and breast cancer is partly regulated by the androgen or oestrogen receptor signalling pathway (11, 12, 16, 17). Since HNSCC are hormone-independent tumours and MB expression in these

Figure 2. Kaplan-Meier analysis (log-rank test). In 497 head and neck squamous cell carcinoma (HNSCC) patients, a significant association between myoglobin (MB) expression and extended survival time was observed. MB expression was dichotomized into MB- (negative, $n=277$) vs. MB+ (weak, moderate and strong ($n=220$)) patients.

tumours lacks a correlation with the hypoxia marker CAIX, we speculate that MB expression in HNSCC is dependent either on the canonical, muscle-specific activation or on a so far undiscovered transcriptional regulation. This hypothesis would need to be investigated in further studies. Moreover, we assume that the potential beneficial effect of MB expression in cancer is independent from its primary respiratory functions, since the amount of MB protein expression would be too low to confer meaningful O_2 storage or buffering capacities (11). However, MB might play a scavenger role to support reduction of harmful ROS (5, 22). In HNSCC, apart from mitochondrial processes, ROS are mainly produced by cigarette smoke; 86% of patients in our cohort have a documented smoking history. A ROS-driven up-regulation of MB expression was already described in MCF7 breast cancer cells (10). Furthermore, localization of MB in close proximity to the cell membrane has not yet been described. We, therefore, hypothesize that MB might protect the cell membrane against ROS as described for membrane-bound globin X (23).

It should be noted that MB abundance is inversely associated with age, which also could explain the better prognosis. Such an age-correlated MB expression has not been observed in previous patient cohorts (12). In contrast, a positive correlation of MB expression with age was observed in breast cancer, possibly due to altered hormone levels in pre-

or post-menopausal patients (11). With respect to HNSCC, MB appears to have a minor influence on the aggressiveness of the tumour. This assumption seems to be confirmed by the observation that MB levels in the primary tumour have no effect on metastasis, despite a trend of approximately 40% of recurrent tumours being MB-positive. Perhaps, a larger HNSCC cohort might reveal an association between increased disease recurrence and loss of MB function. The association of MB expression with gender likely stems from the distribution of tumour localizations. In this cohort, the proportion of laryngeal carcinoma, which mostly exhibits no MB expression, amounts to 12% in women as opposed to 30% in men. In contrast, 45% of women, but only 20% of men, were shown to have oral cavity squamous carcinomas that express high MB protein levels.

Furthermore, recent research has shown that patients with HPV-positive oropharyngeal tumours have a better prognosis (24-26). A favourable outcome for HPV-positive HNSCC patients could also be confirmed in this study. In addition, HPV infection is strongly associated with the onset of squamous cell carcinoma in the oral cavity and oropharynx and less in the larynx (reviewed in 27, 28). Cancers of the oral cavity and oropharynx were also the tumours with the highest MB levels, whereas cancers of the larynx were mostly MB-negative. However, due to the negative correlation between MB expression and p16 positivity, the better prognosis for MB-positive HNSCC patients cannot be explained by HPV infection. It rather seems that HPV infections repress MB expression. It is known that the tumour suppressor gene *p53* is almost never mutated in HPV-related malignancies (29). Another study demonstrated that cells with mutated or silenced *p53* have increased ROS levels and that relatively low levels of *p53* are sufficient for the up-regulation of several genes with antioxidant products (30). HPV-negative HNSCC samples with a, therefore, increased proportion of mutated *p53* might have an elevated MB protein expression that is mediated by higher ROS levels. However, the regulation of MB expression in tumours appears to be complex and, at least, partly different between hormone-dependent and -independent tumours, a finding that clearly necessitates further studies.

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