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Infiltrates of M2-Like Tumour-Associated Macrophages Are Adverse Prognostic Factor in Patients with Human Papillomavirus-Negative but Not in Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma

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Keywords

Oropharyngeal cancer · Tumour-associated macrophages · M2 macrophages

Abstract

Introduction: Human papillomavirus with a high oncogenic potential (HR-HPV) is responsible for more than a half of squamous cell carcinomas of the oropharynx. The HR-HPV-dependent cases of this tumour have a better prognosis compared to the HR-HPV-negative cases, despite the usually more advanced disease at the time of diagnosis. In addition to genetic and epigenetic factors, the causes of this more favourable course of the disease are also seen in the participation of the tumour microenvironment, including the patient's immune system. Macrophages are one of the most important elements of the immunocompetent cells landscape that make up the tumour microenvironment. Traditionally, they are divided into 2 groups: inflammatory macrophages with the M1 phenotype and tumour-associated macrophages known as M2 phenotype macrophages. ***Objective:*** The aim of this study was to investigate the impact of the macrophage infiltrates intensity of the M1/M2 and M2 phenotype separately on the clinical outcome of patients with squamous cell carcinoma of the oropharynx (OPSCC), taking into account the HR-HPV status of tumours. ***Methods:*** The study involved 85 patients with OPSCC in which HR-HPV status in tumour tissue was determined using a double-check algorithm including the detection of viral DNA by RT-PCR method with subsequent confirmation of its biological activity by immunohistochemical demonstrating the P16^{INK4A} protein overexpression. In each of the groups formed on the basis of HR-HPV status, macrophages were discriminated using CD68 and CD163 proteins as markers of pan-macrophage and M2 phenotype. The intensity of infiltrates was quantified by means of computer-assisted analysis in digital images of whole slides (virtual slides) separately in tumour tissue and stroma. ***Results:*** In HPV-positive patients, significantly more intense infiltration of both M1/M2 and M2 macrophages was found in the tumour stroma compared to HPV-negative patients. The infiltrates from both types of macrophages in the tumour tissue were less intense and did not differ between these groups. Intensive infiltration of CD68^{+} macrophages in the tumour front was associ-

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ated with higher rate of nodal failures and a shorter nodal control in both HR-HPV groups. In the group of HR-HPV-negative patients, heavy infiltration of CD163$^+$ macrophages was associated with significantly shorter: loco-regional control (LRC), metastasis-free survival and overall survival (OS). These parameters and prognosis in patients with scanty CD163$^+$ infiltration were similar to favourable outcomes in HR-HPV-positive patients. The relative risk of local-regional recurrence, distant metastases and disease-related death in HR-HPV-negative patients with intense CD163$^+$ infiltrates was, respectively, 4.7, 5.4 and 5.7 compared to patients with scanty infiltrates. ***Conclusions:*** Tumours with a positive HR-HPV status demonstrate intense infiltrations of total pool M1/M2 and M2 macrophages. In the group of HPV-negative patients, intensive M1/M2 macrophage infiltrates correlate with higher risk of nodal failures, and intensive M2 infiltrates are an adverse prognostic factor for LRC, metastasis-free survival and OS.

Introduction

Papillomaviruses are small DNA viruses that can infect various host species, including reptiles, birds and mammals [1]. Human papillomavirus (HPV) infects mucosal and/or cutaneous epidermis of primates and are responsible for the development of a benign or malignant tumour. About 25 HPV genotypes of high oncogenic risk (HR-HPV) are described as causally associated with multiple human cancers, primarily cervical and oropharyngeal squamous cell carcinomas (OPSCC) [2].

Recent epidemiological studies have shown that HR-HPV is responsible for more than a half of squamous cell carcinomas of the oropharynx, and HR-HPV-positive OPSCC incidence is increasing at an epidemic rate [3, 4], suggesting that HPV-positive squamous cell cancer will likely comprise the majority of all head and neck cancers by 2020 [5]. The HR-HPV-dependent cases of this carcinoma are characterized by a distinct set of biological and clinical features that could be summarized by the conclusion that they carry a better prognosis compared to the HR-HPV-negative cases, despite the usually more advanced disease at the time of diagnosis [6–8]. In addition to genetic and epigenetic factors, the causes of this more favourable course of the disease are also seen in the participation of the tumour microenvironment, including the patient's immune system [9].

One of the elements of the tumour microenvironment are the immunocompetent cells forming a divergent network of interactions with each other, elements of the intercellular matrix and with cancer cells. It has been known for some time that some elements of the innate and adaptive immune response directed against cancer cells (cytotoxic response) are very similar to the response to viral infections. In this light, the specificity of the host's immunity to oncovirus-induced tumours may seem particularly interesting. In this case, synergistic immune response stimulated simultaneously with tumour and viral antigens could be expected. Indeed, infiltration of many types of cells associated with innate immune response such as dendritic, Langerhans cells (LC), natural killers, and natural killer T cells is observed in the microenvironment of HPV-positive tumours [10]. Recently, it has also become evident that these cells not only are involved in the innate immunity but also are an important element of the adaptive response. Dendritic and LC have been shown to be involved in the presentation of viral antigens and the activation of cytotoxic T lymphocytes (CD8$^+$) in cervical cancer [11, 12]. On average, more intense CD4$^+$ and CD8$^+$ lymphocyte infiltrates in the tumour stroma and longer overall survival (OS) were observed in patients with HPV-positive OPSCC compared to HPV-negative cases [13].

In fact, effective immune response in cases of HPV-positive tumours is not always clearly visible. An example of this type may be cervical cancer – a classic HR-HPV-dependent neoplasm with a relatively poor prognosis. Despite the presence of viral antigens in virtually all cases of this tumour and even the detection of anti-HPV antibodies in the peripheral blood of patients, the development of an effective immune response does not occur. Moreover, the expression of HPV E7 viral protein in infected cells has been shown to induce formation of a local, strong immunosuppression area where the functions of LC and CD8$^+$ T cells are significantly inhibited [14]. There is also strong evidence that regulatory T cells mediate this immunotolerance of cytotoxic lymphocytes [15].

Macrophages are one of the major populations of infiltrating leukocytes associated with solid tumours [16]. They can be recruited to the tumour site from surrounding tissues by the tumour itself through secretion of chemotactic molecules. In addition, monocytes circulating in the blood stream can infiltrate into the tumour microenvironment and mature into tumour-associated macrophages (TAM) [17].

Macrophages as immunocompetent cells derived from monocyte precursors undergo specific differentiation depending on the local tissue environment. They respond to environmental cues within tissues such as damaged cells, activated lymphocytes or microbial products, to differentiate into distinct functional phenotypes. The M1 macrophage phenotype is characterized by the production of high levels of proinflammatory cytokines, an ability to mediate resistance to pathogens, strong microbicidal properties and high production of reactive nitrogen and oxygen intermediates. In contrast, M2 macrophages are characterized by their involvement in parasite control, tissue remodelling, immune regulation, tumour promotion and efficient phagocytic activity. It has recently been demonstrated that in vitro, macrophages are capable of complete transformation from M1 to M2 and can reverse their polarization depending on the chemokine stimuli [18]. The change in polarization is rapid and is associated with the remodelling of signalling networks on the transcriptional and translational levels [19]. TAMs are known to promote tumour progression by inducing angiogenesis, lymphangiogenesis, stroma remodelling and immunosuppression. They also play a pivotal role in promoting tumour invasion and metastasis, and hence, they are associated with poor prognosis [20, 21]. Several studies have evaluated the prognostic or predictive significance of TAMs in different neoplasms with ambiguous results. Intense infiltrates of TAMs were linked to unfavourable prognosis in gastric or breast cancer [22, 23], whereas controversial results were shown for bladder, lung, and thyroid cancer [24–26]. Results obtained for oropharyngeal cancer are also contradictory especially in the context of HR-HPV status and macrophage phenotype. This study aimed to investigate the impact of the intensity of macrophage infiltrates of the M1/M2 and M2 phenotype on the clinical outcome of patients with squamous cell carcinoma of the oropharynx (OPSCC), taking into account the HR-HPV status of tumours. The work adopted a unique approach involving the use of computer-aided digital image analysis of the entire tissue sections.

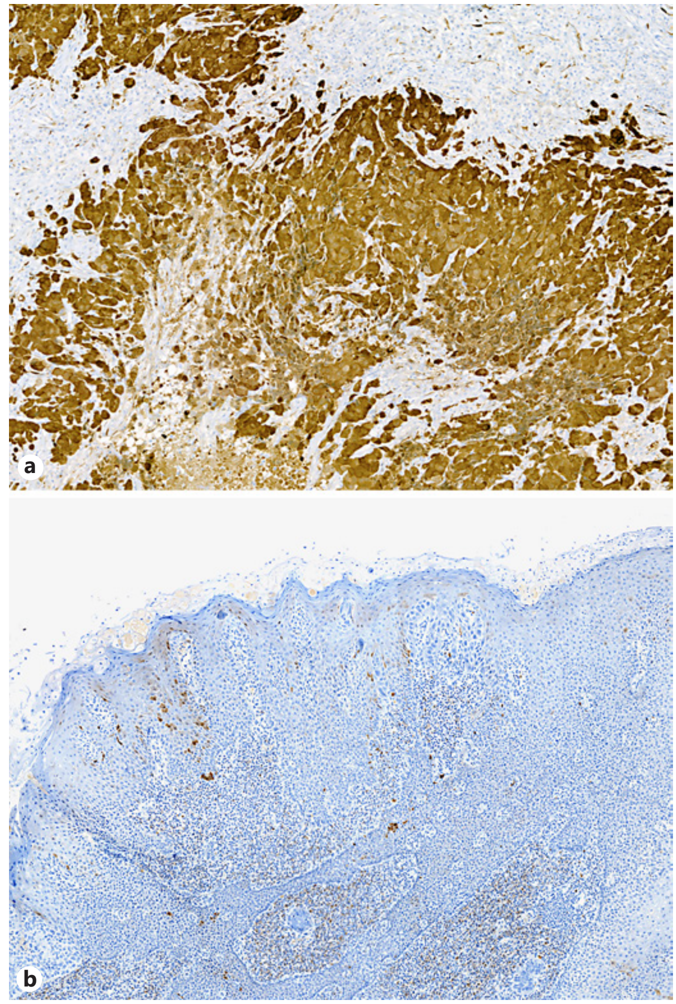

Fig. 1. Representative patterns of immunostaining for P16^{INK4A} protein. **a** Staining pattern characteristic for HR-HPV-positive squamous cell carcinoma of the oropharynx. **b** HR-HPV-negative OPSCC with evident lack of immunoreactivity in tumour cells. IHC staining with E6H4 monoclonal antibody (Roche). Original magnification 100×.

Materials and Methods

Study Groups

Eighty-five patients with diagnosed squamous cell carcinoma of the tonsil or other oropharyngeal locations (62 and 23 individuals, respectively) undergoing radical treatment with radiotherapy or radiochemotherapy were enrolled in the study. Formalin-fixed and paraffin-embedded tumour tissue samples were collected from all the patients as well as histopathological reports and clinical data concerning treatment and outcomes. Paraffin blocks were cut to 5-μm histological sections for further immunohistochemical staining and tumour DNA extraction.

HR-HPV Status Assessment

At the beginning to assess HR-HPV status, tumour tissue samples were examined for protein P16^{INK4A} expression that is considered a surrogate marker of high-risk HPV (HR-HPV) infection [27]. This was demonstrated by immunohistochemistry (IHC), using ready-to-use E6H4 monoclonal antibody and Benchmark Ultra automated staining system (Roche Diagnostics). According to the widely accepted criteria, only a strong homogenous chromogen concentration in over 70% of cancer cells was considered characteristic of HR-HPV infection (Fig. 1) [28].

Subsequently, in all the examined cases, the presence and types of HR-HPV DNA in the tumour cells were confirmed using commercially available test, Sacace HPV Genotypes 14 Real-TM (Sacace Biotechnologies Srl, Italy), which is a real-time PCR Kit for

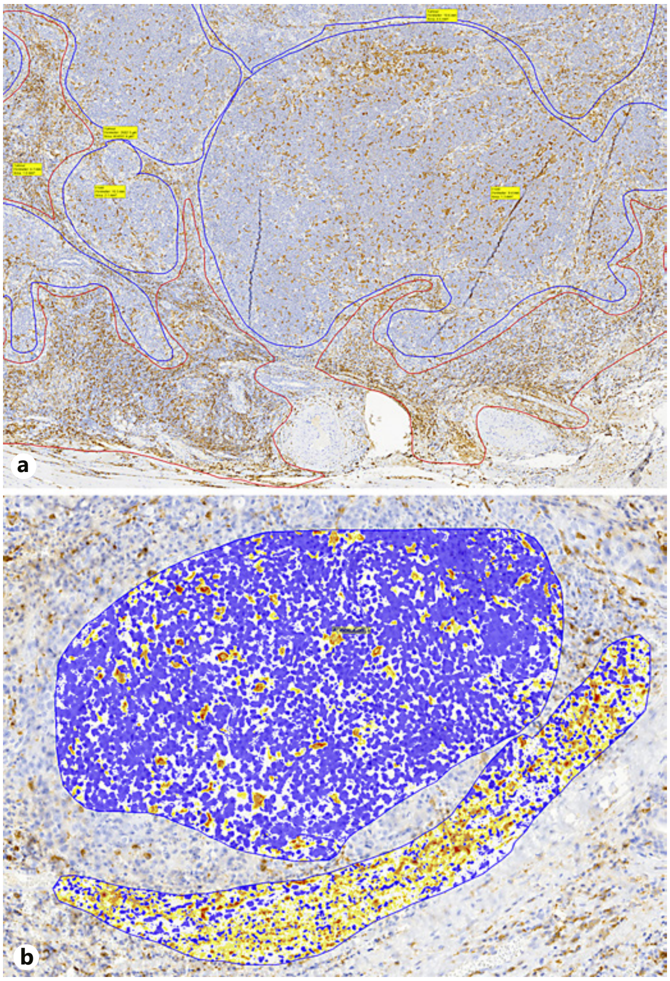

Fig. 2. Computer-aided quantitative assessment of macrophage infiltration intensities. **a** Definition of the ROI corresponding to tumour areas (blue contour) and stroma (red contour). **b** Automatic detection of positive immunostaining areas corresponding to macrophages (yellow to red colour). The ratio of the area occupied by macrophages (red) to the total area occupied by all cells (red + blue areas) constituting the area fraction parameter was treated as a measure of the infiltrates intensity. The image shows only a small fragment of the digital virtual slide – in fact, the analysis was conducted on the whole image representing a complete histological section.

quantitative detection and genotyping of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The first step of the procedure was to isolate the genomic DNA from all tumour tissue samples by using Mag Core Genomic DNA FFPE One-step Kit and MagCore Nucleic Acids Automatic Extractor HF16-Plus (RBC Bioscience Corp. Taiwan). Next, the reaction mixtures of 15 μL isolated DNA (25 ng/10 μL) and controls were prepared and cycled in ViiA7 Real-Time Cycler (Thermo Fisher Scientific/Life Technologies, USA) according to the manufacturer's recommendations. Only the cases with both strong P16^{INK4A} expression and HR-HPV DNA amplification were classified as truly HR-HPV positive. The detailed PCR protocol has been described earlier [29].

Immunohistochemical Staining of Macrophage Populations

In order to visualize macrophage infiltration within the tumour and at the tumour stroma, we used the CD68 pan-macrophage marker showing the expression in macrophage-like cells, predominantly in inflammatory M1 phenotype macrophages. A number of phenotypes were described within M2 macrophages. These phenotypes differed in terms of the expression of specific markers. To visualize them, simultaneous use of at least several antibodies would be necessary. Therefore, we decided to use immunohistochemical staining of the M2 macrophages with anti-CD163 antibody. CD163 is a marker present in almost all TAMs of M2 phenotype except M2d subtype [22].

The immunohistochemical stainings were performed from each block containing tumour tissues using monoclonal, mouse, anti-CD68 antibody – clone PG-M1 (catalogue no.: M 0876, Dako/Agilent, Denmark) and rabbit monoclonal anti-CD163 antibody – clone SP96 (catalogue no.: 503-3964, Zytomed Systems, Germany). Deparaffinization of the sections and tissue antigen retrieval were performed using PT-Link (catalogue no.: PT101, Dako/Agilent, Denmark) and EnVision Flex Target Retrieval Solution, High pH (catalogue no.: K8024, Dako/Agilent, Denmark) buffer. To avoid false-positive immunohistochemical reaction, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min.

EnVisionTM FLEX/HRP system was used to visualize the antigen-primary antibody reaction (catalogue no.: K8024, Dako/Agilent, Denmark), consisting of a mixture of goat anti-mouse and anti-rabbit antibodies linked to polymer containing immobilized horseradish peroxidase and chromogen 3,3′-diaminobenzidine (EnVisionTM FLEX DAB+ Chromogen, catalogue no.: K8024, Dako/Agilent, Denmark).

The sections were counterstained using aqueous solution of hematoxylin (Mayer's solution); they were dehydrated and closed using a permanent mounting medium (Dako Toluene-Free Mounting Medium, catalogue no.: CS705, Dako/Agilent, Denmark).

Evaluation of Immunohistochemical Reactions

The initial technical assessment of immunohistochemical reactions was performed using a light microscope, Olympus (Tokyo, Japan) type BX43 with 4× and 10× magnification Plan-Apochromat objectives. All stained preparations were scanned to digital form in a Panoramic Flash III scanner (3DHistech, Hungary) at the optical resolution 0.24 μm/pixel. In all virtual slides, regions of interest (ROI) encompassing tumour tissue and tumour stroma (defined as tissue directly surrounding or adjacent to the tumour or cancer cells islets) were manually defined in the way that the cumulative ROIs area of each category accounted for at least 90% of the corresponding tumour or tumour stroma area (Fig. 2a). The intensity of macrophage infiltration was determined quantitatively in the defined earlier ROIs for tumour and tumour stroma separately using a computer image analysis system (QuantCenter/HistoQuant Plugin version 2.0.0.46136-3DHistech, Hungary) compatible with the virtual slide images generated by the scanner.

The intensity of macrophage infiltration due to their irregular shape and, consequently, difficulties in image segmentation were defined as the fraction of the area with immunostaining above the defined threshold (ratio of the area occupied by the cells with the positive reaction to the total area of the analysed tissue, expressed as a percentage; Fig. 2b). Subsequently data were analysed separately for each location and immunohistochemical marker.

Data Analysis

Statistical analysis of the results was performed using Statistica version 13 PL software (StatSoft Inc., USA). The comparisons of parameters in the analysed groups were carried out using the nonparametric Mann-Whitney U test for the variables in the ordinal and categorical scale or the Student *t* test for variables in the quantitative scale. Normal distribution of the analysed values was verified using the Shapiro-Wilk test. Univariate and multivariate survival analyses depending on the analysed factors were performed using the Gehan's-Wilcoxon test or Cox proportional hazards regression, whereas their cumulative proportion of surviving plots in regard to defined clinical endpoints (loco-regional control [LRC], metastases-free survival, OS) were prepared using the Kaplan-Meier method. The *p* value of statistical significance ≤0.05 and 95% CI was adopted for all tests.

Results

Informative results of molecular tests and immunostainings were obtained for all the participants. Forty-five tumour tissue samples were HR-HPV positive and 40 were HR-HPV negative. Study groups formed on the basis of HR-HPV status did not differ in terms of most of the analysed histo-clinical parameters except for alcohol consumption and the location of the predominant lesion at the time of diagnosis. In the HPV-positive group, the tumour was observed significantly more often in the tonsil, and the alcohol consumption in these patients was lower in comparison to the HR-HPV-negative ones (Table 1). Similarly, in the HPV-positive group, on average, much more intense infiltrates from $CD68^+$ and $CD163^+$ cells were found in the tumour stroma, while these groups did not differ in the intensity of infiltrates in the tumour tissue (Fig. 3). HPV-negative patients were characterized by greater variability of $CD68^+$ and $CD163^+$ infiltration intensity visible in immunohistochemical staining and expressed by higher standard deviation values in computer-aided measurements (Table 2, Fig. 4).

Interesting observations were found in the analysis of the intensity of $CD68^+$ and $CD163^+$ macrophage infiltrates depending on the occurrence of nodal failures. In the group of HPV-negative patients with nodal failure, statistically significantly more intense $CD68^+$ infiltrates were found than in the group without nodal failure ($CD68^+$ area fraction 67.3 vs. 48.9%; $p = 0.006$). Inverse relationship occurred in HPV-positive patients; however, this group included only 2 cases (53.9 vs. 80.9% $p = 0.005$).

Table 1. Clinico-pathological characteristics of the study groups defined on the basis of HR-HPV status

	HR-HPV positive (*n* = 45)	HR-HPV negative (*n* = 40)	*p* value[1]
Age, years, median (range)	62 (39–81)	61 (31–74)	ns
Gender			
Male	24	21	ns
Female	21	19	
Smoking status			
Never-smoker	19	7	ns
Present-smoker	20	26	
Former-smoker[2]	6	7	
Alcohol consumption			
Low/abstinent	22	8	0.006
Moderate	21	30	
Heavy	0	2	
Staging			
T			
T1	5	5	ns
T2	11	12	
T3	16	12	
T4	13	11	
N			
N0	6	10	ns
N1	9	8	
N2	22	16	
N3	8	6	
Histology			
SCC	45	40	ns
Predominant tumour site			
Tonsil	39	23	0.02
Other oropharynx	6	17	
Treatment regimen			
RT	6	18	ns
ChRT	23	9	
indChT + ChRT	10	7	
indChT + RT	6	6	
RT dose, Gy, median	68.5	69.0	ns

[1] Statistical significance of Mann–Whitney U test or Student *t* test. Tests with a *p* value <0.05 are considered as statistically significant; [2] Former-smoker, non-smoker for at least 5 years before the diagnosis. SCC, squamous cell carcinoma; RT, radiotherapy; ChRT, chemoradiotherapy; indChT, induction chemotherapy; ns, non-significant.

Similar relationships were not observed for $CD163^+$ infiltrates or analyses performed for all patients together without taking into account HR-HPV status (Fig. 5).

Survival analysis in relation to time to nodal recurrence conducted separately in the groups of HR-HPV-positive and HR-HPV-negative patients showed shorter nodal control times in patients demonstrating more in-

Table 2. Mean area fraction of CD163+ and CD68+ infiltrations in tumour and its stroma

Infiltration type	Mean and median of area fraction		p value[1]
	HR-HPV(+)	HR-HPV(–)	
CD163+ stroma	59.79	41.09	<0.0001
SD	19.86	20.01	
Mean	60.60	42.80	
CD163+ tumour	8.13	9.41	ns
SD	7.01	7.42	
Mean	6.00	8.10	
CD68+ stroma	79.19	54.85	<0.0001
SD	13.78	20.57	
Mean	80.40	54.10	
CD68+ tumour	14.48	13.19	ns
SD	9.70	14.46	
Mean	9.10	12.30	

[1] Statistical significance of Student *t* test. ns, non-significant.

Table 3. Median time of nodal control in relation to HR-HPV and CD68+ status in tumour stroma

HPV status	Median of nodal control, months (percentage of 3-year surviving)		p value[1]
	CD68+ high	CD68+ low	
HPV(–), *n* (%)	9.1 (28.6)	16.4 (55.7)	0.049
HPV(+), *n* (%)	13.5 (45.0)	17.0 (54.5)	0.062

[1] Statistical significance of Gehan's-Wilcoxon test. CD68+ high, CD68+ area fraction > median; CD68+ low, CD68+ area fraction ≤ median.

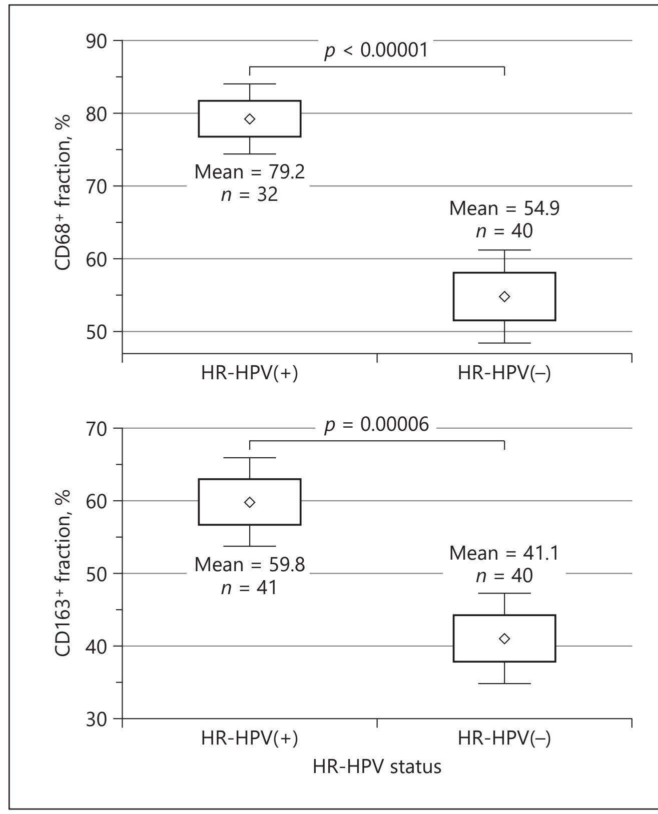

Fig. 3. Comparison of CD68+ and CD163+ infiltration intensities expressed in the form of mean area fractions (middle point) in groups formed depending on the HR-HPV status. Box value – SE, whiskers – 1.96× SE.

tense infiltration of CD68+ macrophages (median time to recurrence = 9.1 months in patients with infiltrates above the CD68+ median versus 16.4 months in patients with infiltrates less or equal to the median). In the HR-HPV-negative group, a similar trend was observed, but it did not reach the assumed statistical significance (Table 3). The analysis of Kaplan-Meier curves of nodal control time revealed that a higher level of nodal failure was primarily responsible for reducing the time of nodal control in the group of HPV-negative patients with intense CD68+ infiltrations (Fig. 6).

The most interesting finding of this work is a demonstration of the relationship between the intensity of CD163+ macrophage infiltrates in the tumour stroma and the time of local control, metastases-free survival and OS. In the group of HR-HPV-negative patients, infiltrates of low intensity from CD163+ (M2) macrophages were associated with favourable prognosis in regard to LRC, distant metastasis-free survival and OS – comparable to patients with positive HR-HPV status (Fig. 7). Intensive CD163+ infiltrates were associated with a significant reduction in LRC (5.5 vs. 26.7 months), metastasis-free survival (9.8 vs. 30.5 months) and OS (10.1 vs. 27.2 months) compared to HPV-negative patients with scanty infiltrates (Table 4).

In the Cox proportional hazard model, the occurrence of intense CD163+ infiltrates in HPV-negative patients carried a 4.7-fold increase in the risk of loco-regional recurrence, a 5.4-fold increase in the risk of distant metastases and a 5.7-fold increase in the risk of dying from the disease compared to HPV-negative patients with scanty infiltrates of CD163+ macrophages (Table 5).

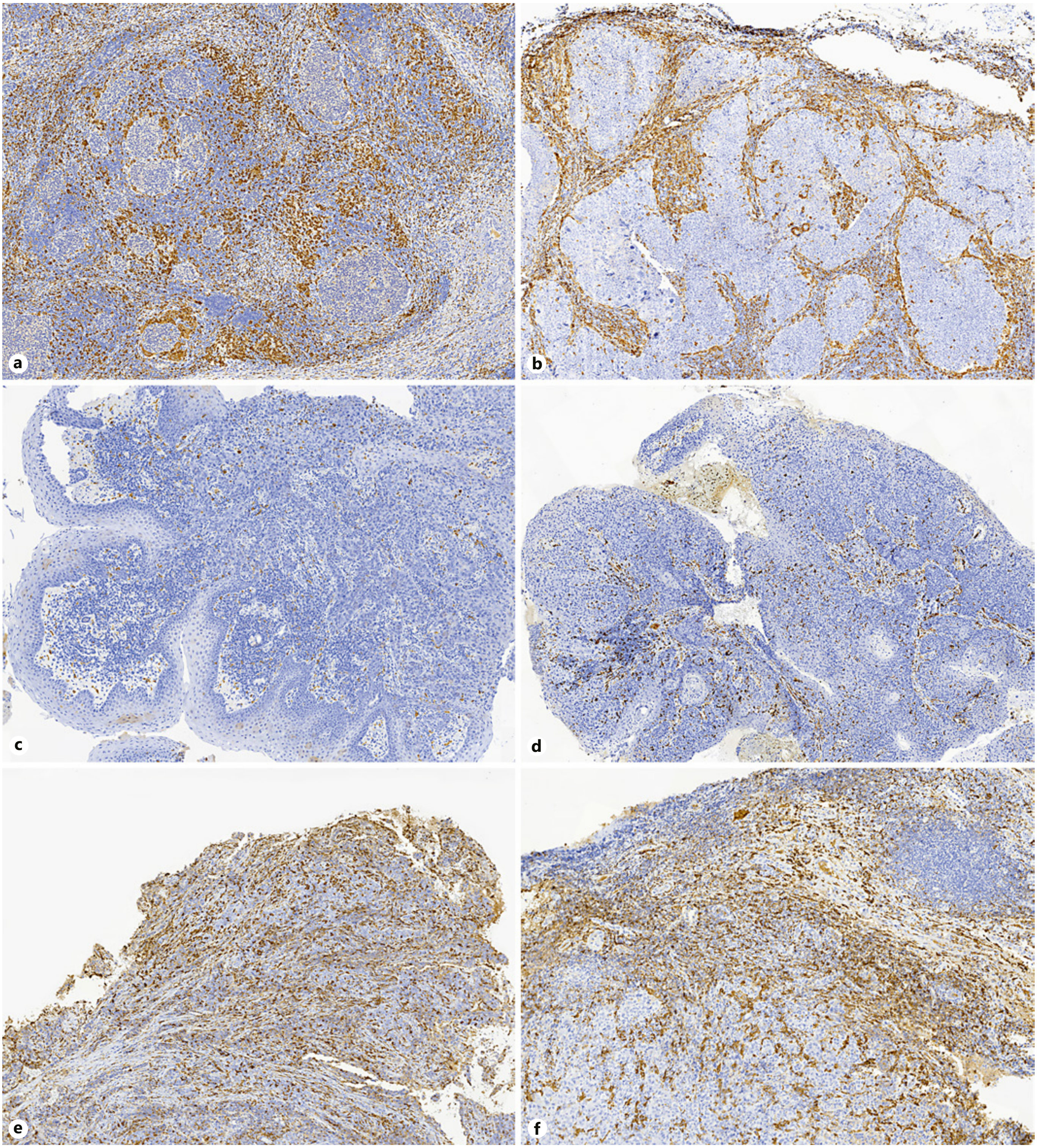

Fig. 4. Microphotographs demonstrating a representative immunostaining of CD163^{+}/M2 macrophages with anti-CD163 monoclonal antibody (clone SP96, Zytomed Systems, Germany). **a**, **b** Intense infiltration of tumour stroma in HPV-positive samples. **c**, **d** Typical scanty CD163^{+} infiltrates in HPV-negative tumours associated with favourable prognosis. **e**, **f** HR-HPV-negative tumour tissue samples with CD163^{+} infiltrates accompanied by poor clinical outcome. Original magnification 100×.

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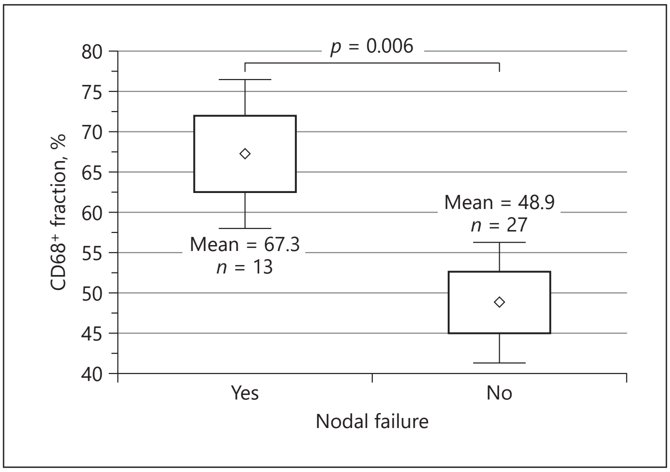

Fig. 5. Statistically significant differences in the intensity of M1/M2 ($CD68^+$) infiltration between patients with and without nodal failures. *n*, number of subjects in groups.

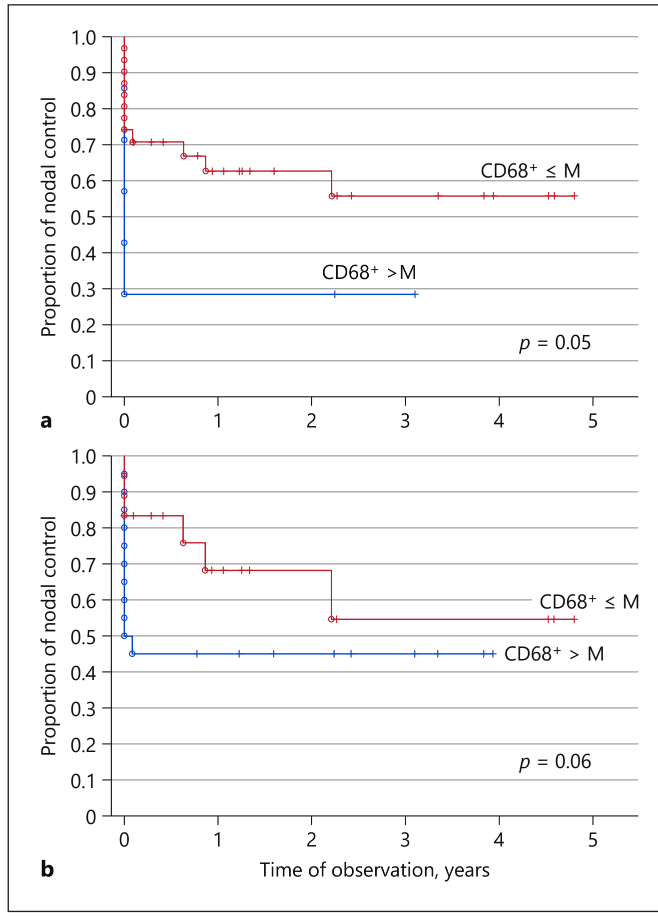

Fig. 6. Kaplan-Meier approximation of nodal control times in groups of patients with $CD68^+$ infiltrates higher than median ($CD68^+ > M$) and equal or less than median ($CD68^+ \leq M$). **a** HR-HPV-negative group; (**b**) HR-HPV-positive group (a tendency was observed).

Discussion/Conclusion

The prognostic significance of various macrophage subpopulations in many types of cancer has not been clearly established. According to some reports, they can be positive and, according to others, negative prognostic factors. Prominent macrophage infiltrates are considered to be an adverse prognostic factor in gastric or breast cancer [22, 23], whereas controversial results were shown for bladder, lung and thyroid cancer [24–26].

In the present work, the intensity of infiltration of total pool macrophages (M1/M2, $CD68^+$) and M2 ($CD163^+$) phenotype macrophages in a group of 85 patients with squamous cell carcinoma of the oropharynx with known tumour HR-HPV status was assessed simultaneously. To assess HPV status, a double-detection algorithm was used based on viral DNA detection and $P16^{INK4A}$ protein overexpression as a determinant of viral biological activity. As we know this is the first publication covering such a homogeneous group of patients with a comparable number of HPV-positive and HPV-negative cases and one of the few in which the HPV double-detection algorithm was used. Most of the publications in which the intensity of macrophage infiltration within head and neck tumours was analysed concerned locations in the oral cavity [30] or other locations outside the oropharynx [31], where HR-HPV status was unknown. Due to the low frequency of HR-HPV infections in the 2.5–4% range in these anatomical sites, it can be assumed that the results presented in them relate mainly to the HPV-negative population [32–34].

In a group of 43 patients with oral squamous cell carcinoma, He et al. [35] assessed the intensity of $CD68^+$ and $CD163^+$ infiltrates in the tumour without distinguishing the specific location. The intensity of infiltration of both types of macrophages was positively associated with lymph node involvement and tumour size, while the intensity of $CD163^+$ infiltrate correlated with the reduced OS [35].

In another study, in a group of 127 patients with OPSCC, the intensity of CD68 and CD163 macrophage infiltrates was analysed in the tumour environment and in the tumour cells themselves. The authors showed a relationship between lymph node involvement and the intensity of the infiltration of both types of cells. In addition, they were an unfavourable prognostic factor in relation to the OS of patients [36]. Similarly, in 2 other studies conducted on groups of 108 and 73 patients, respectively, intense infiltrates of CD68+ and CD163+ were associated with an unfavourable outcome and correlated with nodal involvement. In the present study, intensive CD68+ infiltrates were associated with a higher incidence of nodal failures and a shorter nodal control in both HPV-positive and HPV-negative patients. The latter parameter may be affected by a high percentage of nodal failures. At the same time, the prognostic significance of CD68+ infiltration in relation to other clinical endpoints could not be demonstrated. This finding is in line with the conclusions of the meta-analysis presented by Troiano et al. [30], taking into account the results of 17 studies conducted on 1,528 patients with oral cancer, which excluded the prognostic significance of CD68+ infiltrates in this group of cancers and confirmed the usefulness of M2-like macrophage infiltrates assessment in the tumour stroma as an adverse prognostic factor. The results presented by us also indicate the usefulness of the CD163+ infiltration assessment in the tumour stroma as a negative prognostic factor in regard to the LRC, distant metastases-free survival and OS in the group of HPV-negative patients. These are in good concordance with the results of the meta-analysis, which as mentioned above includes mainly patients with HPV-negative tumours. We failed to demonstrate similar relationships for HR-HPV-positive patients and for localization within the tumour itself. However, significantly more intense infiltration of both the total macrophage pool and M2-like macrophages was found in HR-HPV-positive tumours compared to HPV-negative ones. Similar observations were described in the work of Seminerio regarding the total macrophage pool [37] and by Ryu et al. [38] in relation to M2-like macrophages. In the first study, the authors also showed the significance of CD68+ macrophage infiltrates in tumour tissue as an independent factor of poor prognosis in regard to relapse-free survival and OS. Unfortunately, unlike our work, the study was conducted on a heterogeneous group of patients with tumours of different locations – mainly outside the oropharynx. Patients with transcriptionally active HR-HPV accounted for only 11% (6 cases) in that study. The impact of M2 macrophages on recurrence rate in chemoradiotherapy was investigated by Balermpas et al. [39]. The study group included 106 patients with locally advanced squamous cell carcinoma of various head and neck regions, of which 42% in the oropharynx treated with radical chemoradiotherapy. The authors showed a high prognostic value of CD163+ infiltrates in the tumour front in regard to progression-free survival, local failure-free survival and distant metasta-

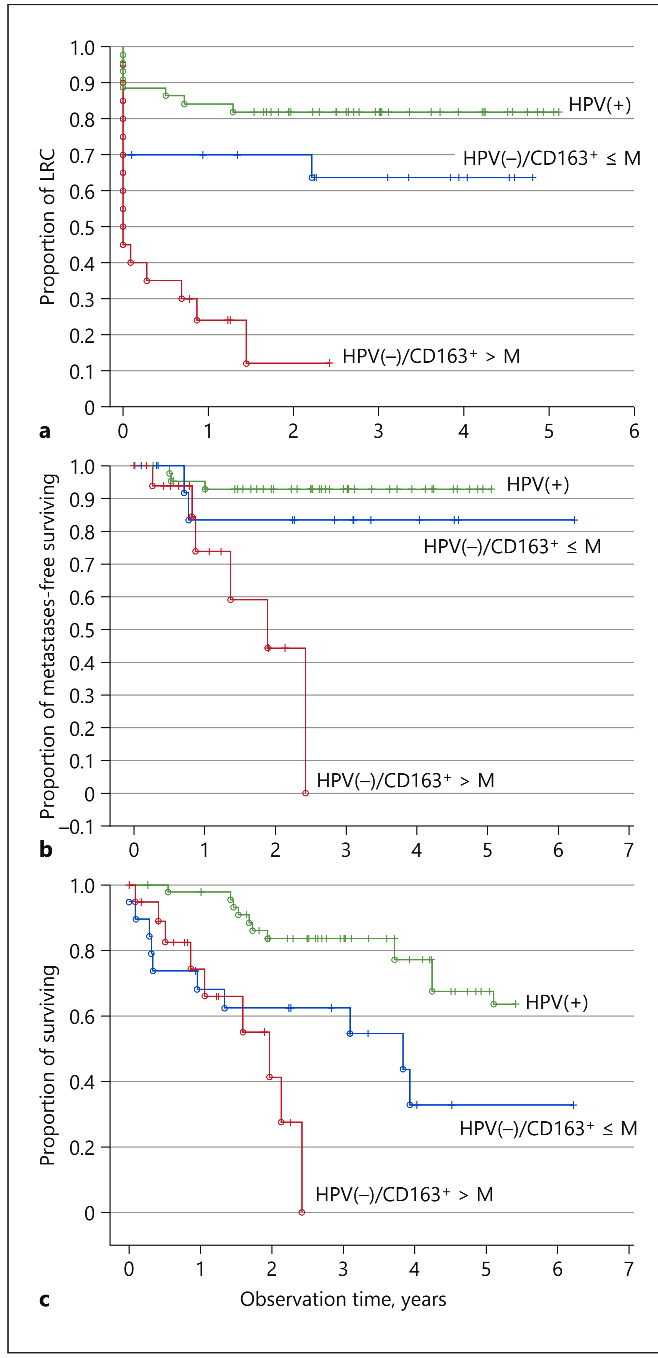

Fig. 7. Kaplan–Meier approximations of LRC (**a**), metastases-free survival (**b**) and OS (**c**) in 2 groups of patients: HR-HPV-positive regardless of CD163+ status (green curve), HR-HPV-negative patients with scanty CD163+ tumour stroma infiltrates (blue curve) and HPV-negative subjects with intense CD163+ (greater than median in HPV-negative group) infiltrates (red curve). Differences between HPV(+) and HPV(–)/CD163+ > M were highly statistically significant (p values 0.00002, 0.018, 0.0015, respectively), whereas differences between HPV(+) and HPV(–)/CD163+ ≤ M did not reach statistical significance. HPV, human papillomavirus; LRC, loco-regional control.

Table 4. Median time of LRC, META and OS in relation to CD163+ status in tumour stroma

Clinical endpoint	Median of survival, months (percentage of 3-year surviving)			*p* value[1]
	HPV(+)	HPV(–)		
		CD163+ high	CD163+ low	
LRC, *n* (%)	32.0 (81.8)	5.4 (12.0)	26.7 (63.6)	0.00002
META, *n* (%)	31.6 (83.3)	9.8 (0.0)	30.5 (92.7)	0.018
OS, *n* (%)	33.1 (67.5)	10.1 (0.0)	27.2 (27.5)	0.0015

[1] Statistical significance of Cox proportional hazard test. LRC, loco-regional control; META, metastases-free survival; OS, overall survival; HPV, human papillomavirus; CD163+ high, CD163+ fraction > median; CD163+ low, CD163+ fraction ≤ median.

Table 5. Relative risk in Cox proportional hazard model in group of HPV-negative patients with intense M2 (CD163+) macrophage infiltrates compared to patients with scanty infiltrates

	RR	95% CI	*p* value[1]
LRC	4.67	1.41–8.92	0.007
META	5.37	0.98–29.28	0.052
OS	5.71	1.70–19.14	0.005

[1] Statistical significance of Cox proportional hazard test. RR, relative risk; LRC, loco-regional control; META, distant metastases occurrence; OS, overall survival; HPV, human papillomavirus.

ses-free survival only in the HPV-negative group (without P16^{INK4A} overexpression) – similarly to the present study in which prominent M2-like macrophage infiltrates were associated with shorter LRC, distant metastases-free survival and OS time [39]. Our and the above-cited studies are the only ones that showed a relationship between CD163+ infiltrates and distant metastases. This phenomenon can be explained by many published data, suggesting that TAMs help tumour cell egress by increasing the density of leaky blood vessels that in turn may also provide pro-tumourigenic factors such as CXCL8 (also known as IL-8) and CXCL2, which increase the invasiveness of cancer cells. TAMs also directly help invasion of the surrounding tissue and intravasation of tumour cells. Intravital imaging revealed that cancer cells frequently invade surrounding tissues together with TAMs [17], and epidermal-to-mesenchymal transition of tumour cells facilitates intravasation [36]. Somewhat contradictory data compared to previously cited authors were published by Ou et al. [40], who failed to demonstrate the prognostic significance of macrophage infiltration in HPV-negative patients. However, they were characterized by a higher ratio of M2-like to the total pool macrophages. In HPV-positive patients, however, a correlation was found between total pool macrophage infiltrates in tumour and longer progression-free survival [40].

In summary, it should be emphasized that the clinical significance of individual macrophage phenotypes appears to be different depending on the HR-HPV status of the tumour and the spatial location of the infiltrate. Data obtained in this study suggest that intensive M2 macrophage infiltration in the tumour front is a strong negative prognostic factor in HPV-negative patients with oropharyngeal cancer.

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Statement of Ethics

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All subjects have given their written informed consent. For this type of study, institute's committee approval is not required.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.S. conception, arrangement and coordination of the research, selection of methods, verification of pathology reports and slides, assessment and interpretation of IHC results, collection, comparing, analysis and interpretation of the results, writing and editing manuscript including all pictures and microphotographs. A.B. selection of the subject groups, collecting, analysis and interpretation of clinical data, co-authoring of the manuscript. A.K. assessment of tissue samples' usefulness for molecular tests, performing laboratory work in parts relevant to preparing, running and interpretation of real-time PCR tests, performing of IHC, editing of the manuscript. T.R. arrangement and coordination of the research, selection of the subject groups, collecting, analysis and interpretation of clinical data, co-authoring of the manuscript.

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