Expression of prostaglandin E$_2$ prostanoid receptor EP2 and interleukin-1β in laryngeal carcinoma – preliminary study

Aim of the study: Expression of EP2 protein, the prostaglandin E$_2$ (PGE$_2$) receptor, produced by tumour microenvironment inflammatory cells as well as tumour cells, may promote cellular proliferation and growth in an autocrine and paracrine fashion. The phenomenon involving these proteins is regulated by interleukin 1β (IL-1β). Many researchers indicate a connection of EP2 and IL-1β in various types of neoplasms with higher tumour progression and poor prognosis. The aim of this study was to analyse the EP2 expression within laryngeal carcinoma tissue and IL-1β levels in peripheral blood mononuclear cell supernatants and to find relationships between clinicomorphological features.

Material and methods: A group of 50 patients with verified squamous cell laryngeal carcinoma was analysed in this study. The pathological evaluation included pTNM depth of invasion according to tumour front grading criteria. Immunohistochemical analysis for membranous staining of EP2 in tumour tissues was used. The IL-1β expression was determined by enzyme-linked immunosorbent assay (ELISA).

Results: Increased EP2 expression in carcinoma cells was confirmed for more advanced tumours (pT3-pT4 vs. pT1-pT2, $p < 0.0001$ and pN1-3 vs. pN0, $p = 0.02$). Tumours with the highest aggressiveness identified by deeper invasion of submucosa or cartilage were characterised by the highest expression of EP2 ($p < 0.0001$). In laryngeal carcinomas characterised by a lower differentiation the highest EP2 expression in tumour cells was noted ($p = 0.009$). A positive relationship between IL-1β expression and the presence of lymph node metastases was also confirmed ($p = 0.04$).

Conclusions: The study indicates the potential effect of EP2 receptor and IL-1β on tumour progression in laryngeal carcinoma.

Key words: laryngeal carcinoma, PGE$_2$ prostanoid receptor EP2, depth of invasion.

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Introduction

Interleukin-1β (IL-1β) is a member of the interleukin-1 family of cytokines. This cytokine is an important pro-inflammatory interleukin associated with chronic inflammation and inflammatory-related cancer development and progression [1]. Interleukin-1β induces the production of matrix metalloproteinases (MMPs) and collagenases in the mechanism of MEKK and MAPK kinase activation, which may promote an increased invasiveness of various tumours of epithelial origin as well as the presence of metastasis and recurrence of the cancer [1]. The regulation COX-2-dependent E-cadherin expression and the prostaglandin E$_2$ (PGE$_2$) metabolism are important components of signalling pathways leading to activation of IL-1β in neoplastic disease, as well as in head and neck carcinomas [2–4]. EP2 is one of the four subtypes (EP1-EP4) of receptors for PGE$_2$. The EP2 receptor is a representative of the G protein-coupled receptor (GPCR) family. Activation of EP2 is coupled to the Gs molecule and directs the synthesis of cytosolic cAMP [5]. Stimulation of EP2 receptor may cause an increase in angiogenesis process by promoting the expression of vascular endothelial growth factor (VEGF) [2]. EP2 signalling also upregulates COX-2 activation and PGE$_2$ production by the neoplastic and immunocompetent cells in tumour microenvironment. This is linked with myeloid-derived suppressor cells (MDSC) activity, and consequently a suppression of anti-tumour functions of T and NK lymphocytes, a reduction of dendritic cell maturation, as well as an increase in tumour angiogenesis [6]. Numerous publications indicate the presence of an association of both, EP2 and IL-1β in various types of neoplasms with tumour invasiveness, lymph node metastases, and poor prognosis [7].

The purpose of this study was to analyse the expression of prostanoid PGE$_2$ type EP2 receptor in the tumour tissue and the level of IL-1β in peripheral blood mononuclear cell cultures in order to find relationships between immunological parameters and clinicomorphological features to estimate tumour aggressiveness in squamous cell laryngeal carcinomas.

Table 1. Clinicopathological characteristics of laryngeal carcinoma cases

Characteristics		*n* (%)
Age (mean ± SD)	45–79 years (62.5 ±8.3)	
Overall survival (mean ± SD)	28–79 months (59.5 ±13.8)	
3- and 5-years survival	< 3 years	7 (14)
	3–5 years	12 (24)
	≥ 5 years	31 (62)
Gender	male	48 (96)
	female	2 (4)
Tumor size (pT)	pT1	6 (12)
	pT2	13 (26)
	pT3	16 (38)
	pT4	15 (30)
Nodal metastases (pN)	pN0	40 (80)
	pN1–3	10 (20)
Grade	G1	9 (18)
	G2	34 (68)
	G3	7 (14)
Depth of invasion (points)	1 – CIS or microinvasion	20 (40)
	2 – submucosa or muscle invasion	10 (20)
	3 – deep invasion (cartilage invasion)	20 (40)

Material and methods

A group of 50 (48 men, 2 women) patients with verified squamous cell laryngeal carcinoma was analysed in this study (aged 45–79 years; mean age 62.6 ±8.4 years). A control group consisted of a group of 30 healthy volunteers (aged 40–65 years; mean age 54.2 ±5.9 years). A pathologically confirmed diagnosis of *carcinoma planoepitheliale*, primary surgical resection without receiving prior immuno-, radio-, or chemotherapy, and the absence of distant metastasis were the criteria for patient participation in this study. The clinicomorphological features of cases are shown in Table 1.

Histological classification and morphological features

For histological classification of laryngeal carcinomas, archival paraffin-embedded tissue samples were utilised. All specimens were assessed according to the criteria conducted in accordance with the AJCC TNM classification of 2010 for laryngeal cancers [8]. Morphological estimation of depth of invasion was performed on H&E-stained sections in the most invasive, peripheral zones of the tumour, according to tumour front grading (TFG), one of the most reliable pathological methods for the analysis of neoplastic progress and determination of the dynamics of tumour growth, as well as a reasonably precise prognostic factor in laryngeal carcinoma [9]. The histological evaluation considered the depth of invasion. The factor was assessed in at least five different regions of the peripheral part of the

tumour (magnification 200×). The depth of invasion was graded according to a scale ranging from 1 to 3 as follows: 1 – CIS (carcinoma *in situ*) or microinvasion, 2 – nodular into submucosa or muscle invasion, and 3 – deep invasion (cartilage). The histological grade of differentiation, G, was measured according to the three-grade morphological system: G1 – well-differentiated tumour, G2 – moderately-differentiated tumour, and G3 – poorly-differentiated tumour.

Immunohistochemistry for PGE$_2$ prostanoid receptor EP2

Paraffin-embedded biopsy specimens were used for EP2 evaluation. Immunohistochemistry was performed on 2-μm-thick biopsy sections. Sections were collected onto Superfrost Plus slides (BDH). After deparaffinising through xylene, alcohol, and distilled water the sections were treated in a water bath for 40 minutes with a solution of citrate buffer, pH 6.0, and transferred to distilled water. Endogenous peroxidase activity was blocked through incubation with 0.3% hydrogen peroxide for 30 minutes. Slides were washed in TBS and incubated overnight at 4°C with the primary antibody. The primary antibodies Rabbit Anti-EP2 (Abcam, ab124419 dilution, 1 : 500) were used. Detection of membranous fraction of EP2 was performed with an appropriate EnVision/HRP System (Dako Cytomation). Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (Dako-Cytomation, Denmark). After washing, the sections were counter-stained with haematoxylin and coverslipped. For each antibody and for each sample a negative control were processed. Negative controls were carried out by incubation in the absence of the primary antibody, and they always yielded negative results. The intensity of immunohistochemical staining was assessed as a percentage of tumour cells with EP2 membranous (EP2-m) positive expression, and they were scored using a three-tier system: 1) < 25%; 2) 25–50%; and 3) > 50% positive cells. At least 10 high-power fields (magnification 40×) were assessed for each specimen. All slides were assessed in three independent sessions by two researchers.

Lymphocyte isolation and ELISA for Interleukin 1β measurement

For peripheral blood mononuclear cell (PBMC) isolation venous blood was obtained (10 ml) from each patient and transferred to test tubes containing heparin (10 U/ml). The control blood samples were obtained from 30 healthy volunteers without a history of malignancies or autoimmune disorders. PBMCs were isolated by Ficoll-Hypaque density gradient and resuspended in RPMI 1649 medium to obtain the concentration of 1×10^6 cells/ml. The recovered cells were checked and counted for viability with tryptan blue staining method. The isolated PBMC cultures were incubated for 21 hours at 37°C in a humidified atmosphere with 5% CO$_2$ (Cellstar Incubator) in 96-well plates in a final volume of 0.2 ml (per well). The supernatants of cultures were collected and the secretion pattern of IL-1β was measured with specific enzyme-linked immunosorbent assay ELISA Kit (R&D Systems, Inc.; Minneapolis, MN, USA) ac-

cording to the manufacturer's instructions. Absorbance was measured with an ELISA reader (Multiscan RC 351). The sensitivity of this assay was < 1 pg/ml. The investigations were performed with the approval of the Bioethical Commission of the Medical University of Lodz and the National Science Council, Poland (No. RNN/60/13/KE).

Statistical analysis of data

The statistical analyses were performed using the IBM SPSS STATISTICS 21 (Business Machines Corp., USA). Distributions of quantitative variables were described using means and standard deviations. Since levels of IL-1β expression did not show normal distribution (according to results of Shapiro-Wilk normality test) the non-parametric statistical tests: Mann-Whitney U test, Kruskal-Wallis test with post hoc multiple comparisons with Bonferroni correction, were used to identify the relationship between IL-1β expression and clinicopathological parameters. The χ^2 test was used to identify the relationship between EP2-m protein expression and clinicopathological features. Kaplan-Meier survival analysis was used to identify the relationship between EP2-m protein expression and disease-free survival. A p-value of less than 0.05 was considered as statistically significant.

Results

Analysis of EP2-m and Interleukin 1β expression in the studied groups

Our study confirmed positive expression of membranous fraction of EP2 prostanoid receptor, scored using a three-tier system, as follows: 1) < 25% positive cells in 38% (19/50), 2) 25–50% in 20% (10/50), and 3) > 50% in 42% (21/50) of tumours. The representative images of IHC EP2 membranous (EP2-m) positive staining are shown in Fig. 1. Analysis of the data showed a higher level of IL-1β in the supernatant of PBMCs obtained from patients with laryngeal carcinoma, as compared with the control group, but at the border of the of significance level ($p = 0.064$). The mean concentrations of this cytokine were 2.52 ±3.90 pg/ml and 1.87 ±2.55 pg/ml, for cases and controls, respectively.

The relationships between EP2-m and interleukin 1β expression and clinicomorphological features

Subsequently, the pattern of membranous fraction of EP2 receptor (EP2-m) in tumour tissue and the level of IL-1β in PBMC cultures in relation to clinicomorphological features was compiled. Our study disclosed that more intensive positive expression of EP2-m in laryngeal carcinomas was an indicator of more advanced lesions. Statistical analysis confirmed significant relationships of the studied protein with the local extension of the tumour pT ($p < 0.0001$). The presence of a higher content of EP2-m (> 50% positive cells) was more frequent for tumours with a higher pT status (pT3–pT4). Similarly, the statistical association between EP2-m expression and nodal extension of the neoplastic lesions ($p = 0.02$) was also noted. The greater number of positively stained cells for EP2-m (> 50% positive cells) was more characteristic for positive lymph nodes (pN1–pN3). Statistical dependences for depth of invasion estimated according to tumour front grading scale and grade were also noted. The presence of a higher content of positive EP2-m cells in tumour tissue was more frequent for tumours with more aggressive behaviour determined by submucosa or cartilage invasion ($p < 0.0001$). Similarly, an increased staining for EP2-m (> 50% positive cells) was more characteristic for less differentiated carcinomas ($p = 0.009$). Conversely, the lower content of EP2-m in neoplastic tissue (< 25% positive cells) was more frequent for tumours with pT1–pT2 status, negative lymph nodes (pN0), more differentiated tumours (G3), and low aggressive behaviour characterised by carcinoma in situ or microinvasion. The EP2-m expression in the studied laryngeal carcinoma group and statistical analysis results are shown in Table 2.

The level of IL-1β in PBMC cultures from patients with laryngeal carcinoma in relation to clinicomorphological parameters was also compiled. The present study demonstrated that increased expression of IL-1β was an indicator of the presence of nodal metastases (pN1–pN3) in the patient group ($p = 0.042$). Unfortunately, no correlation between the level of this cytokine and the aggressiveness
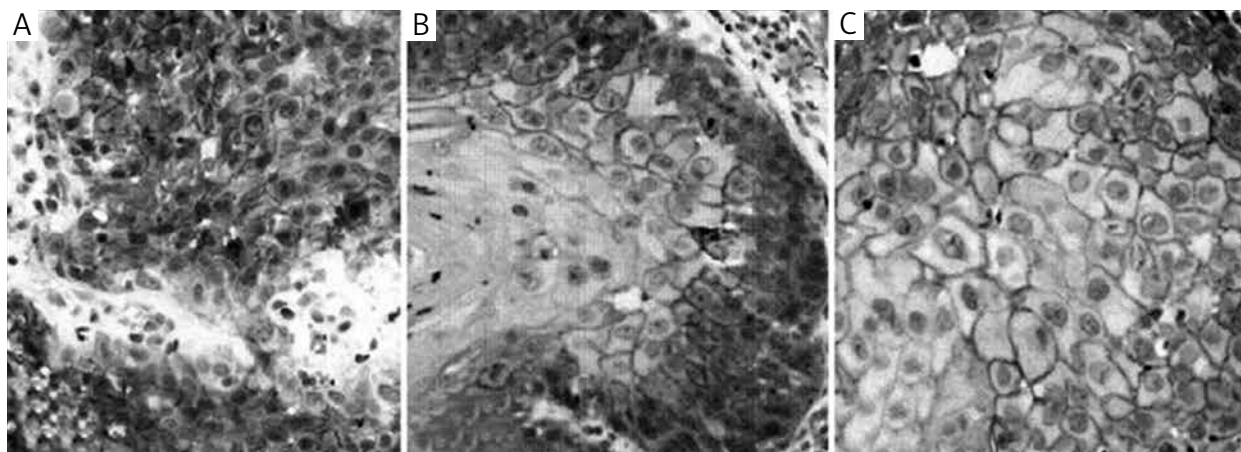

Fig. 1. The representative images of IHC EP2 membranous (EP2-m) scored using a three-tier system: **A)** 1: < 25% (magnification 100×); **B)** 2: 25–50% (magnification 200×); **C)** 3: > 50% (magnification 200×) positive cells

Table 2. Clinicopathological features of laryngeal carcinomas and EP2-m expression

Characteristics		EP2-m			p
		< 25%	25–50%	> 50%	
Tumor size (pT status)	pT1	100% (6/6)	0	0	
	pT2	84.6% (11/13)	15.4% (2/13)	0	
	pT3	12.5% (2/16)	31.3% (5/16)	56.3% (9/16)	< 0.0001
	pT4	0	20.0% (3/15)	80.0% (12/15)	
Nodal metastases (pN)	pN0	47.5% (19/40)	17.5% (7/40)	35% (14/40)	0.02
	pN1–3	0	30.0% (3/10)	70.0% (7/10)	
Grade	G1	88.9% (8/9)	11% (1/9)	0	
	G2	29.4% (10/34)	20.6% (7/34)	50.0% (17/34)	0.009
	G3	14.3% (1/7)	28.6% (2/7)	57.1% (4/7)	
Depth of invasion	1–2 points	90.0% (18/20)	10.0% (2/20)	0.0% (0/20)	
	3 points	0.0% (0/10)	30.0% (3/10)	70.0% (7/10)	0.0001
	4 points	5.0% (1/20)	25.0% (5/20)	70.0% (14/20)	
EP2-m total expression		38% (19/50)	20% (10/50)	42% (21/50)	100% (50/50)

Table 3. Clinicopathological features of laryngeal carcinomas and IL-1β expression

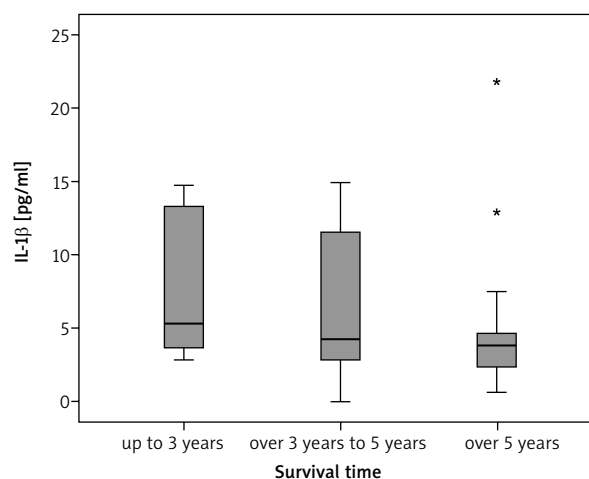| Characteristics		IL-1β (pg/ml)	p
Tumor size (pT status)	pT1	2.18 ±1.90	
	pT2	3.10 ±2.60	
	pT3	3.92 ±2.70	> 0.05
	pT4	3.95 ±3.12	
Nodal metastases (pN status)	pN0	3.85 ±2.32	0.042
	pN1–3	6.39 ±3.40	
Degree of differentiation (Grade)	G1	2.67 ±2.45	
	G2	3.85 ±2.34	> 0.05
	G3	5.30 ±3.90	
Depth of invasion	1 points	2.64 ±1.90	
	2 points	3.92 ±3.80	> 0.05
	3 points	4.23 ±2.99	
Survival	< 3 years	5.30 ±1.90	
	3–5 years	4.23 ±1.40	> 0.05
	≥ 5 years	3.80 ±1.50	

Results are given as mean ± standard deviation

Fig. 2. The levels of IL-1β in the group studied depending on the 3-year and 5-year survival

of studied tumours according to pTNM classification and depth of invasion was confirmed. However, a clear tendency towards higher values for IL-1β in laryngeal carcinomas characterised by higher local status (pT3–pT4), less degree of differentiations (G3), and higher aggressive behaviour characterised by submucosa or cartilage invasion was noted. The IL-1β expression in PBMC cultures in the studied laryngeal carcinoma group and statistical analysis results are shown in Table 3.

The relationships between EP2-m and IL-1β expression and patient survival

The relationship between membranous fraction of EP2 receptor (EP2-m) in tumour tissue and IL-1β level in supernatants of purified PBMCs and survival of patients with laryngeal carcinoma was analysed. Statistical analysis did not disclose significant differentiation of IL-1β concentration in relation to patients three- and five-year survival. However, a tendency towards higher content of IL-1β in PBMC cultures in patients who lived for less than three years af-

ter treatment was noted. The IL-1β level in PBMC supernatants in the studied group in relation to three- and five-year survival is shown in Fig. 2. A significant differentiation of EP2-m expression in tumour tissue in relation to overall survival was observed (p = 0.001). A higher content of positive stained tumour cells in patients who lived for less time after treatment was observed. The expression of EP2-m in laryngeal carcinoma cases in relation to overall survival is shown in Fig. 3.

Discussion

The role of IL-1β in inflammation-related cancerogenesis and tumour progression is widely discussed [10–12]. Increased expression of IL-1β leads to the activation of other inflammatory cytokines such as IL-6, IL-8, IL-17, TNF, and IFN-γ produced by T cells and enhances the activity of DC cells and the Th17 lymphocyte subpopulation [13]. Interleukin-1β also induces p38 kinase pathway and, through increased expression of metalloproteases MMP2 and MMP9, plays an important role in cancer metastatic abil-

ity [11]. Researchers also point to the role of certain molecules called inflammasomes (caspase-1 activation complexes), such as NOD-like receptor P3 (NLRP3) and RIG-like receptor-I (RIG-I) in IL-1β secretion [10, 14]. Interleukin-1β enhances COX-2 activity and alters PGE2 metabolism. The precise mechanism of IL-1β activity in cancer and its relationship with tumour development and aggressiveness is still poorly characterised. Nevertheless, in most studies a higher expression of IL-1β and close connection of this cytokine secretion with invasive tumour phenotype and prognosis in various cancers were confirmed [1, 10–17].

The presence of PGE$_2$ in a tumour microenvironment and concomitant overexpression of COX-2 associated with PGE$_2$ activity can promote the growth of head and neck carcinoma cells in an autocrine and paracrine fashion by acting on prostanoid receptors [18]. Stimulation of EP2 receptor, which leads to PGE$_2$ production, may affect tumour behaviour and invasiveness through multiple mechanisms [5, 19]. One such mechanism is the regulation of tumour promoting and angiogenic cytokines such as IL-1β and IL-6 [5, 19]. Another mechanism is connected with the stimulation of G protein, which directs the synthesis of cAMP. The first pathway activated by cAMP involves protein kinase A (PKA) stimulation, which causes a phosphorylation of glycogen synthase kinase-3α (GSK-3α), which enhances expression of beta-catenin as well as the activation of transcription factors, such as Tcf/Lef and lymphoid enhancer-binding factor (CREB) that leads to VEGF and COX-2 gene expression. The second pathway is associated with cAMP-Epac-Rap (aAMP-exchange proteins directly activated by cAMP-Rap GTP-binding protein) activation, which regulates a variety of different cell-specific processes, such as cell motility, and genes expression, such as S100A8, in cancer [20]. Prostaglandin E$_2$ signalling via EP2 receptors by the aAMP-dependent induction of amphiregulin, which can lead to hyperplasia, is also discussed [20]. Moreover, stimulation of EP2 receptor suppresses T and NK cell function and dendritic cell maturation, and it may have an effect on the activity of myeloid-derived suppressor cells (MDSC) [5, 19]. The literature survey also indicates a connection of EP2 receptor stimulation with upregulation of Snail through EGFR/Akt/mTOR pathway [21].

In recent studies, an increasing amount of evidence indicates that EP2 receptor signalling and IL-1β secretion play a crucial role in cancer behaviour and prognosis [11–13, 22–27]. In this study, both proteins were significantly related to cancer invasiveness. The higher EP2 expression in tumour tissue and increased IL-1β level in PBMC cultures were observed in tumours characterised by increased local and nodal extension, lower degree of differentiation, and deeper invasion. These observations are consistent with the results regarding other types of cancer; for instance, Kuo *et al.* [26] reported that overexpression of EP2 receptor in neoplastic tissue exerts tumour cell activity and that it may be significant in the aggressive behaviour of oesophageal squamous cell carcinoma. The authors revealed a positive association between the prostanoid receptor and pT, pN, and overall survival prognosis parameters. Similar results for EP2 immunohistochemical expression relating to such clinical parameters as larger primary tumour size,
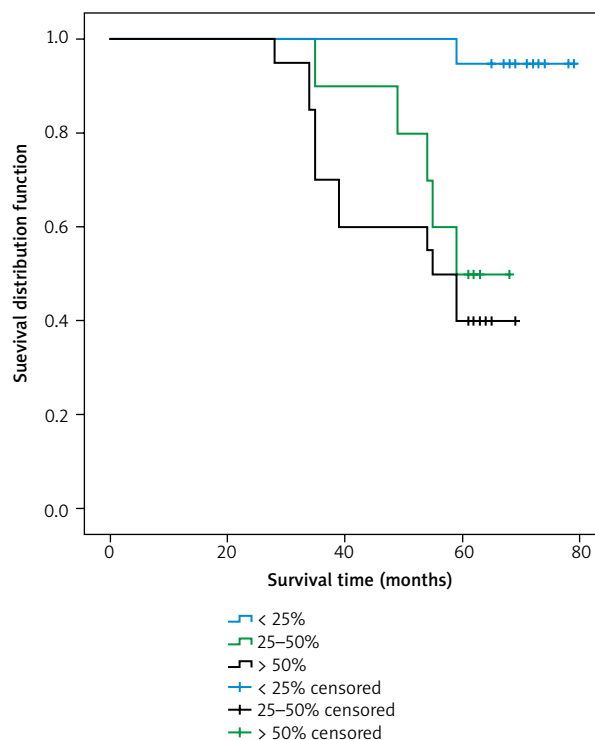

Fig. 3. The expression of EP2-m in the group studied depending on overall disease-free survival

positive nodal status, higher grade of malignancy, and increased metastases incidence were also reported in another study of prostate cancer [23]. Miyata *et al.* [23] noted a histological correlation between EP2 receptor expression and tumour progression, angiogenesis, and lymphangiogenesis in prostate cancer. In another study, Cheng *et al.* [21] also reported a higher invasiveness of hepatocellular carcinoma with overexpression of EP2 receptor.

Many researchers emphasise that inflammasomes can regulate proinflammatory cytokines such as IL-1β and IL-18, which are implicated in the relationship between tumour development and progression; for instance, in various types of tumours the direct linkage between the inflammasome-mediated inflammation and the connected blockade of IL-1β and IL-18 leading to inhibition of tumour growth has been presented. On the other hand, inflammasome activation has potent antitumourigenic effects due to elimination of malignant precursor cells through pyroptotic cell death and through increasing the efficacy of certain chemotherapies [28]. Despite different mechanisms of IL-1β activation in neoplastic disease, the data of many studies demonstrate that this regulatory interleukin may be a promising molecular biomarker of tumour aggressiveness and a target for proper therapy [29]. For instance, Chen *et al.* [29] revealed that both at mRNA and protein levels of IL-1β were significantly higher in cancer specimens compared to non-malignant tissues in oesophageal cancer. The authors observed that blocking of IL-1β signalling in the tumour cell line was connected with enhanced tumour growth, invasion ability, and chemoradiotherapy resistance. The underlying mechanisms of

the more aggressive tumour behaviour were related to increased activation of NF-κB nuclear factor and subsequent epithelial-mesenchymal transition. Moreover, the immunochemistry findings indicate that positive staining of IL-1β in tumour tissue correlated significantly with higher clinical stage, lower response rate to chemoradiotherapy, and higher incidence of recurrence after curative treatment [29]. The objective of the Kamatani *et al.* [30] study was to evaluate cytokines in saliva from patients with oral squamous cell carcinoma as compared to those with pre- and post-surgery treatment. As a result, IL-1β proved to be useful for detection of early tumour stage. These observations explain the fact that the assessment of salivary IL-1β level is indicated as s biomarker for cancer detection as well as a prognostic marker in various tumours, including head and neck carcinoma [29–31].

It should be emphasised that discrepancies among various researchers' results exist due to differences in tumour biology caused by variation of tumour type, histological differentiation status, and proliferative index. Also, different materials used in the research, e.g. cell culture or laboratory animal material, and the diversity of tissues (fresh tumour samples, paraffin-embedded samples, neoplastic cell lines) may have had an impact on the results. Moreover, the relatively small size of the study group may have influenced the resulting data. In conclusion, despite the limitations discussed, the presented results illustrate the importance of EP2 receptor signalling and IL-1β secretion in inducing a malignant phenotype of laryngeal carcinoma, providing possible targets for diagnosis and opening new perspectives for cancer treatment.

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