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## The Molecular Prognosis on Bone Osteolysis in Epulides

### Molekularne prognozowanie osteolizy kości w nadziąślakach

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#### Abstract

**Background.** Epuli are the most frequent gingival tumours. The lesions are often associated with bone osteolysis which necessitates more extensive surgery or removal of tumour-adjacent teeth. Recurrence, related to non-radical tumour excision, is also common. No literature data have been found concerning molecular markers responsible for the processes of osteolysis and recurrence.

**Objectives.** The aim of the study is molecular prognosis of bone osteolysis in epuli patients.

**Material and Methods.** Molecular investigations by RT-PCR were carried out on 210 biopsies collected from 70 epulis lesions. Depending on the diagnosis of bone osteolysis/lesion recurrence, expression of genes, i.e., BCL-2, BAX, H3 histone, IFN $\gamma$  and its IFN $\gamma$ R1, IFN $\gamma$ R2 receptors was investigated.

**Results.** Recurrence and osteolysis were observed when IFN $\gamma$ R1/IFN $\gamma$ R2 quotient < 0.5. Apoptosis and proliferation genes expression did not show any significant changes.

**Conclusions.** When IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.5, recurrence and bone osteolysis can be predicted in epuli patients (Dent. Med. Probl. 2011, 48, 3, 319–324).

**Key words:** epulus, RT-PCR, IFN $\gamma$ R1, IFN $\gamma$ R2, IFN $\gamma$ .

#### Streszczenie

**Wprowadzenie.** Nadziąślaki należą do grupy najczęściej spotykanych guzów dziąsłowych jamy ustnej. Zmianie często towarzyszy osteoliza kości, która wymaga rozległego zabiegu chirurgicznego lub usunięcia guza razem z sąsiadującym zębem. Często dochodzi do nawrotu związanego z nieradykalnym wycięciem guza. W piśmiennictwie brakuje doniesień na temat molekularnych markerów odpowiedzialnych za osteolizę i nawrót w przebiegu nadziąślaków.

**Cel pracy.** Propozycja prognozowania osteolizy kości w przebiegu nadziąślaków.

**Materiał i metody.** Badania molekularne z użyciem RT-PCR były prowadzone na podstawie pobranych 210 badań histopatologicznych z 70 nadziąślaków. W zależności od stwierdzonej osteolizy kości/zmian nawrotowych oceniano ekspresję genów, np. BCL-2 BAX, histon H3, IFN $\gamma$  i jego receptorów IFN $\gamma$ R1, IFN $\gamma$ R2.

**Wyniki.** Nawrót i osteolizę obserwowano, gdy iloraz IFN $\gamma$ R1/IFN $\gamma$ R2 był mniejszy od 0,5. Nie stwierdzono istotnych zmian w ekspresji genów odpowiedzialnych za apoptozę i proliferację.

**Wnioski.** Gdy IFN $\gamma$ R1/IFN $\gamma$ R2 < 0,5, można przewidywać nawrót i osteolizę kości u osób z nadziąślakami (Dent. Med. Probl. 2011, 48, 3, 319–324).

**Słowa kluczowe:** nadziąślak, RT-PCR, IFN $\gamma$ R1, IFN $\gamma$ R2, IFN $\gamma$ .

Epulides (granulomas) are the most frequently observed gingival tumours. The etiopathogenesis of the hyperplasia is not yet clear, and classification inconsistent. Literature on the subject presents considerable discrepancies regarding the origin of the tumours. The factors determining their development, growth rate, and tendency to recur are still unknown although several options have been con-

sidered, i.e., type of injury or inflammatory process, hygiene, nutrition, alcohol, tobacco, pharmacotherapy, hormonal status, and immune efficiency.

Bone osteolysis observed in the course of the disease remains unaccounted for. However, as it has more frequently been found in giant cell epulis, giant cells might be the culprit. Giant cell epulis calls for particular oncological vigilance [1–4].

Epuli are characterized by tendency to recur, and especially in the case of non-radical surgery [5, 6]; recurrence rates vary between 5 and 70.6% of cases [5, 7–10]. It has seemed that an investigation into epuli subgroups with regard to prognosis on recurrence or bone osteolysis might yield quite interesting results; such analysis has not been carried out so far.

The present study was designed in attempt to use RT-PCR for recurrence/osteolysis markers detection; BCL-2, BAX, H3 histone, IFN $\gamma$  and its IFN $\gamma$ R1, IFN $\gamma$ R2 receptors expression was investigated for the purpose.

## Material and Methods

### Clinical Investigations

A total of 150 cases including 43 inflammatory, 34 fibrous, and 73 giant cell epulides diagnosed and treated in the Department of Craniomaxillofacial Surgery, Medical University of Silesia in Katowice from 1998 through 2006 were retrospectively analysed. Epulides were classified based on histopathological diagnosis. The patients were divided into four age groups, ie., Group I – up to 15 years; Group II – 16 to 44 years; Group III – 45 to 60 years; and Group IV – over the age of 60. The analysis was based on medical histories, X-rays, surgery reports, and follow-up records. Study group allocation was based on clinical records including preliminary diagnosis, age and gender. Medical history, often reviewed during follow-up care appointments showed hygiene and nutrition, stimulants (coffee, alcohol, tobacco), history of trauma, procedures and inflammatory conditions within the oral cavity, medication, hormonal status, presence of other local or general disease, and the course of the tumour (epulis) since its occurrence. Length of time elapsing from onset was noted, ie., up to 1 month, 1 to 3 months, 3 to 6 months, 6 to 12 months, and over 1 year. Dental examination report contained tumour location and topography, colour, diameter, and consistency, relationship to surrounding tissues; also, the presence of ulceration and other oral lesions. X-rays, surgery report, and the process of postoperative wound healing were also considered as well as the length of follow-up, and time to recurrence. Osteolysis and the presence of multifocal disease were also noted.

### Molecular Investigations

Molecular Investigations were carried out on intraoperative specimens collected from three sites per patient: the lesion, tissue margin (incision

line), and healthy tissue (opposite to lesion site). RT-PCR was used to determine expression profile of genes encoding IFN $\gamma$  receptor subunits, H3 histone gene (proliferation marker), and BCL-2, BAX (apoptosis markers).

The results were confronted with X-rays disclosing bone osteolysis (confirmed on histopathology), and with tumour recurrence.

Molecular analysis (RT-PCR) consisted of three stages: RNA extraction, amplification and quantification of amplification products, and, finally, evaluation of amplification products specificity.

### RNA Extraction

Total RNA was extracted from homogenized tissue samples by AGCP (Acid Guanidinium-Phenol-Chloroform) method – as described by Chomczynski and Sacchi (1987). RNA concentration was determined by absorbance at 260 nm using Gene Quant II spectrophotometer (Pharmacia). RNA degradation and purity was evaluated by electrophoresis on 2% agarose gel with ethidium bromide staining. The number of mRNA molecules in 1  $\mu$ g of total RNA was used to determine gene activity.

### Real-Time RT-PCR Assay

Gene *mRNA* levels were determined by Real-Time RT-PCR based on SYBR Green chemistry. RT was carried out in the same system immediately before PCR amplification.

RT-PCR reaction tubes contained 25  $\mu$ l QuantiTect SYBR Green RT-PCR Master Mix (Qiagen) (HotStart Taq DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, dNTP mix, SYBR Green I, ROX™ passive reference dye and 5mM MgCl<sub>2</sub>), 2.5  $\mu$ l of forward and reverse primers (10  $\mu$ M stock solution), 3  $\mu$ g of unknown RNA template, 0.5  $\mu$ l of OmniTect RT Mix (containing Omniscript Reverse Transcriptase, and Sensiscript Reverse Transcriptase) and deionized water to a total volume of 25  $\mu$ l. All RT-PCR reactions were conducted in 96-well microtiter plates. Each analysis was carried out in triplicate with the use of ABI PRISM 7000 Sequence Detector (Applied). The thermal profile was 48°C for 30 minutes for reverse transcription and 95°C for 15 minutes, 40 two-step cycles at 94°C for 15 seconds and 60°C for 30 seconds; followed by 72°C for 10 minutes for QPCR and finally a 30-minute dissociation protocol. RNA integrity was assessed using mRNA amplification of the house-keeping genes GAPDH and  $\beta$ -actin.

Sequence-specific PCR primers for the detection of:

IFN $\gamma$ F – (5'TGAAGTCATCCAAGTGATGGCT-GAACTGTGCG3'),

IFN $\gamma$ R – (5'CAGGCAGGACAACCATTACTGG-GATGCTC3'),

IFNGR1F – (5'ATACCGAAGACAATCCAGGAA-AAGTGGAACA3'),

IFNGR1 R – (5'GCGATGCTGCCAGGTTTCAG-CTGGTTACTA 3')

IFNGR2F – (5' CAAGGACAGCTCACCAAAG-GATGACG 3'),

IFNGR2R – (5' CAGCTCCGATGGCTTGATCT-CTTCCA 3')

RNA detection was designed using computer software Primer Express Version 1.0 ABI PRISM (Applied).

PCR amplimers were separated on 8% polyacrylamide gel with silver staining. Plasmid pBR 322/Hae III was the size marker. Specificity of PCR amplimers was additionally confirmed by establishing their melting temperatures in a dissociation curves assay after every RT-PCR amplification and by enzymatic sequencing carried out with the use of ABI PRISM 310 DNA Sequencer (Applied).

The values were expressed as SEM and standard deviation. Quantitative data were compared by variance ANOVA (Tukey test).  $P < 0.05$  was considered statistically significant. All calculations were performed with Statistica Version 6.0 software.

Based on literature data, an own map was designed/developed with representations of IFN $\gamma$ /IFN $\gamma$ R1/IFN $\gamma$ R2 stimulation of genes participating in the following processes: apoptosis: BAX, BCL-2, proliferation: H3 histone.

## Results

The pathology was diagnosed in 89 women and 61 men and included 43 inflammatory, 34 fibrous, and 73 giant cell epulides.

The incidence of fibrous epulides was significantly higher among patients aged 15–44 years (29.4%) and among those over the age of 60 (35.5%). Inflammatory epulides were most frequently diagnosed in patients aged 15 to 44 years and 45 to 60 years (41.9% and 44.2%, respectively). The occurrence of giant cell epulis was comparable in all age groups ( $p = 0.0003$ ) (Tab. 1).

Fibrous and giant cell epulides were significantly more frequent in posterior mandible (premolars and molars) whereas inflammatory epulides were predominantly located in anterior maxilla ( $p = 0.01$ ).

Recurrence was observed in 4 cases of fibrous, 4 cases of inflammatory and 3 cases of giant cell epulis. Multifocal disease was diagnosed in 3 patients with inflammatory epulides.

Osteolysis revealed on pantomograms was significantly more common in patients with giant cell epulis than those suffering from inflammatory or fibrous lesions ( $p = 0.02$ ). The shortest time from onset was given by patients with inflammatory epulides (up to 1 month and to 3 months). The longest by those with fibrous epulides (over 1 year). Halfway period of disease development was significantly more common among patients with giant cell epulides (3 to 6 months). The bone osteolysis were in 2 cases of fibrous epulis, 28 cases of giant cell epulis and 14 inflammatory epulis.

The patients with bone osteolysis had higher levels of IFN $\gamma$ R1/IFN $\gamma$ R2 expression in healthy tissue ( $p = 0.004$ ) (Tab. 2). In recurrence cases low-level IFN $\gamma$ R2 expression was found in healthy tissue and tissue margin ( $p = 0.05$ ;  $p = 0.02$ ) (Tab. 3). When IFN $\gamma$ R1 < IFN $\gamma$ R2, IFN $\gamma$ R1 > IFN $\gamma$ R2, and, similarly, at IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.9, 0.9 < IFN $\gamma$ R1/IFN $\gamma$ R2 < 1.0, and IFN $\gamma$ R1/IFN $\gamma$ R2 > 1.0, no statistically significant correlations were found between the expression of genes under consideration and bone osteolysis/lesion recurrence.

Recurrence and bone osteolysis rates were significantly higher when IFN $\gamma$ R1/IFN $\gamma$ R2 quotient < 0.5, with differences between IFN $\gamma$ R2 expression (healthy tissue) and IFN $\gamma$ R1/IFN $\gamma$ R2 (tissue margin and healthy tissue). Recurrence was characterized by higher values of IFN $\gamma$ R1/IFN $\gamma$ R2 quotient, and lower IFN $\gamma$ R2 median.

When IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.5, significant differences were observed as to tissue margin IFN $\gamma$ R1 ( $p = 0.007$ ) and IFN $\gamma$ R2 ( $p = 0.02$ ) expression levels in osteolysis. This was associated with lower median values of the genes. Tumour sections of all recurrence patients showed IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.5.

## Discussion

Surgery is the method of choice in the treatment of epuli; the extent of surgical intervention is disputable. Some authors recommend sparing procedures [11, 12], others advocate radical management with teeth and teeth germs removal, alveolar process resection, tumour bed, and periosteum removal [3, 13]. Considering the possibility of more aggressive growth, more extensive surgery has been used in each recurrence [14, 15]. In epuli, recurrence tends to be quite common due to non-radical excision of primary tumours [5, 6]. Recurrence rates vary between 5 and 70.6% of cases [5, 7–10]. Bone osteolysis also requires rather extensive intervention; thus, decisions are made to remove the alveolar process or teeth adjacent to the tumour. The question remains whether this should be recommended; however, such strategy might

**Table 1.** Epulis subclasses – distribution of analysed factors**Tabela 1.** Podklasy nadziąsłaków – rozkład analizowanych czynników

Factor	Study parameters	Fibrous epulis (%)	Giant cell epulis (%)	Inflammatory epulis (%)	Univariate analysis CHI <sup>2</sup>
Age	≤ 15	3 (8.8)	20 (27.4)	1 (2.3)	p = 0.0003
	15–44	10 (29.4)	13 (17.8)	18 (41.9)	
	45–60	9 (26.5)	21 (28.8)	19 (44.2)	
	> 60	12 (35.3)	19 (26.0)	5 (11.6)	
Gender	female	20 (58.8)	39 (53.4)	30 (69.8)	ns. (p = 0.22)
	male	14 (41.2)	34 (46.6)	13 (30.2)	
Trauma	no	15 (44.1)	36 (49.3)	20 (46.5)	ns. (p = 0.87)
	yes	19 (55.9)	37 (50.7)	23 (53.5)	
Hygiene	poor	19 (55.9)	33 (45.2)	22 (51.2)	ns. (p = 0.57)
	good	15 (44.1)	40 (54.8)	21 (48.8)	
Stimulants (coffee, alcohol, tobacco)	no	24 (70.6)	48 (65.8)	38 (88.4)	p = 0.02
	yes	10 (29.4)	25 (34.3)	5 (11.6)	
Inflammation	no	16 (47.1)	46 (63.0)	31 (72.1)	ns. (p = 0.08)
	yes	18 (52.9)	27 (37.0)	12 (27.9)	
Medication	no	18 (52.9)	56 (76.7)	34 (79.1)	p = 0.02
	yes	16 (47.1)	17 (23.3)	9 (20.9)	
Presence of other local or general disease	no	18 (52.9)	57 (78.1)	34 (79.1)	p = 0.01
	yes	16 (47.1)	16 (21.9)	9 (20.9)	
Ulceration	no	34 (100.0)	72 (98.6)	43 (100.0)	ns. (p = 0.99)
	yes	0	1 (1.4)	0	
Bleeding	no	28 (82.4)	60 (82.2)	40 (93.0)	ns. (p = 0.20)
	yes	6 (17.7)	13 (17.8)	3 (7.0)	
Pain	no	34 (100.0)	73 (100.0)	41 (95.4)	ns. (p = 0.08)
	yes	0	0	2 (4.6)	
Period	up to 1 month	2 (5.9)	14 (20.0)	10 (24.4)	p = 0.02
	1–3 months	11 (32.4)	13 (18.6)	15 (36.6)	
	3–6 months	4 (11.8)	20 (28.6)	4 (9.8)	
	6–12 months	6 (17.7)	12 (17.1)	5 (12.2)	
	over 1 year	11 (32.4)	11 (15.7)	7 (17.1)	
Location	anterior maxilla	5 (15.2)	16 (22.9)	17 (42.5)	p = 0.01
	posterior maxilla	4 (12.1)	8 (11.4)	10 (25.0)	
	anterior mandible	10 (30.3)	15 (21.4)	6 (15.0)	
	posterior mandible	14 (42.4)	31 (44.3)	7 (17.5)	
Osteolysis	no	32 (94.1)	45 (61.6)	29 (67.4)	p = 0.002
	yes	2 (5.9)	28 (38.4)	14 (32.6)	
Recurrence	no	30 (88.2)	70 (95.9)	39 (90.7)	ns. (p = 0.30)
	yes	4 (11.8)	3 (4.1)	4 (9.3)	
Multifocal disease	no	34 (100.0)	73 (100.0)	40 (93.0)	–
	yes	0	0	3 (7.0)	
Treatment	removal	24	12	12	ns. (p = 0.40)
	electrosurgery	9	45	29	
	tooth extraction	4	12	3	
	alveolar process re-section	1	16	2	
	recurrence	4	3	4	

**Table 2.** Statistical analysis based upon the number of mRNA copies of genes under consideration (median) depending on IFN $\gamma$ R1/IFN $\gamma$ R2, bone osteolysis, biopsy collection site and histopathology result. The all patients with recurrences had IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.5 in lesion

**Tabela 2.** Analiza statystyczna na podstawie liczby kopii transkryptów mRNA zależnych od IFN $\gamma$ R1/IFN $\gamma$ R2, osteolizy kości, wycinku i wyniku badań histopatologicznych. Wszyscy pacjenci z nawrotami mieli IFN $\gamma$ R1/IFN $\gamma$ R2 < 0,5

	Lesion		Margin		Control	
Parameter	Osteolysis	No osteolysis	Osteolysis	No osteolysis	Osteolysis	No osteolysis
Number	44	106	44	106	44	106
BCL-2	ns. (p = 0.47)		ns. (p = 0.19)		–	
BAX	ns. (p = 0.79)		ns. (p = 0.38)		–	
BCL-2/BAX	ns. (p = 0.53)		ns. (p = 0.15)		–	
Histon H3	ns. (p = 0.32)		ns. (p = 0.31)		–	
IFN $\gamma$ R1						
Median	270	498	62	473	628	754
Lower Quartile	50	82	14	235		278
Upper Quartile	24098	1186	111	889		1843
U Mann-Whitney test	ns. (p = 0,72)		p = 0,007		–	
IFN $\gamma$ R2						
Median	1061	2592	722	3296	2965	3429
Lower Quartile	653	850	106	1295		1312
Upper Quartile	69814	9340	1338	7275		10654
U Mann-Whitney test	ns. (p = 0.53)		p = 0,02		–	
IFN $\gamma$ R1/ IFN $\gamma$ R2	ns. (p = 0.28)		ns. (p = 0.34)		–	
IFN $\gamma$	ns. (p = 0.93)		ns. (p = 0.19)			

**Table 3.** Statistical analysis based upon the number of mRNA copies of genes under consideration (median) depending on IFN $\gamma$ R1/IFN $\gamma$ R2, bone osteolysis, biopsy collection site and histopathology result. The all patients with recurrences had IFN $\gamma$ R1/IFN $\gamma$ R2 < 0.5 in lesion

**Tabela 3.** Statystyczna analiza liczby kopii transkryptów mRNA zależnych od IFN $\gamma$ R1/IFN $\gamma$ R2, osteolizy kości, wycinku, i wyniku badań histopatologicznych. Wszyscy pacjenci z nawrotami mieli IFN $\gamma$ R1/IFN $\gamma$ R2 < 0,5

	Lesion		Margin		Control	
Parameter	Rec.	No rec.	Rec.	No Rec.	Rec.	No Rec.
Number	11	139	11	139	11	139
BCL-2	ns. (p = 0.22)		ns. (p = 0.82)		ns. (p = 0.62)	
BAX	ns. (p = 0.54)		ns. (p = 0.06)		ns. (p = 0.19)	
BCL-2/BAX	ns. (p = 0.41)		ns. (p = 0.12)		ns. (p = 0.14)	
Histon H3	ns. (p = 0.92)		ns. (p = 0.31)		ns. (p = 0.69)	
IFN $\gamma$ R1	ns. (p = 0.76)		ns. (p = 0.52)		ns. (p = 0.47)	
IFN $\gamma$ R2						
Median	2329	2458	1519	3296	1312	3997
Lower Quartile	763	879	346	1295	795	1779
Upper Quartile	3246	11566	2127	7275	3429	10849
U Mann-Whitney test	ns. (p = 0.54)		ns. (p = 0.08)		p = 0.04	
IFN $\gamma$ R1/IFN $\gamma$ R2						
Median	0.20	0.10	0.19	0.13	0.26	0.17
Lower Quartile	0.16	0.05	0.17	0.07	0.25	0.07
Upper Quartile	0.38	0.30	0.46	0.23	0.44	0.27
U Mann-Whitney test	ns. (p = 0.10)		p = 0.03		p = 0.02	
IFN $\gamma$	ns. (p = 0.13)		ns. (p = 0.69)		ns. (p = 0.96)	



help decrease the risk of recurrence and the need for another surgical procedure. Morphological similarity between giant cell tumours and sarcomas was discussed by *Hattowska and Stelińska* [16]. Pathomorphologists deny that epuli could undergo malignant transformation; few cases mentioned in literature have not been fully documented [17–19].

The sparing procedure (without teeth removal and base coagulation) has not found recognition in Poland although it has been popular abroad. Some epuli, especially giant cell lesions with rich

vascularization, were effectively treated with local injections of trichloroacetic acid [20], sclerotizing agents [21], penicillin G [22], corticosteroids [23, 24] or subcutaneous calcitonin, recommended during pregnancy [25–27].

Several questions concerning epuli have not been elucidated yet, and among those why osteolysis or recurrence develop. Our studies have suggested that the processes can be accurately predicted by molecular analysis of biopsies, also those collected from healthy tissue.

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Received: 20.06.2011

Revised: 16.09.2011

Accepted: 16.09.2011

Praca wpłynęła do Redakcji: 20.06.2011 r.

Po recenzji: 16.09.2011 r.

Zaakceptowano do druku: 16.09.2011 r.