Current Biomarkers of Apoptosis Process in Chronically Inflamed Nasal Sinus Epithelium: Representatives of BAX, Bcl-2 and miRNA Group

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Abstract

In chronic upper respiratory tract diseases, increased cell proliferative activity is observed, which is coordinated by Bcl-2 proteins as well as by small non-coding RNAs.

The aim of this study was to determine the expression of critical apoptosis markers at the mRNA and miRNA level in patients with chronic rhinosinusitis with nasal polyps (CSRwNP).

The study group consisted of 10 patients with CSRwNP and 10 healthy controls. TUNEL staining was performed to detect in situ apoptosis in the maxillary sinus mucosa. The levels of selected mRNA transcripts associated with cell survival and apoptosis: BAX, p53, p21, CASP3, CASP9, c-MYC, CCND1, BRIC5 and APAF1 and miRNAs: miR-17-5p, miR-145-5p, miR-146a-5p and miR-203a-3p were determined by RT-qPCR.

CSRwNP patients showed increased apoptosis determined by TUNEL assay accompanied by increased expression of BAX, P21, P53, CASP3, CASP9, c-MYC, APAF-1 transcripts and decreased mRNA levels of BCL-2 and BIRC5. There were increased expression levels of miR-203a-3p and decreased expression levels of miR-17-5p and miR-145-5p. These findings appear to be characteristic features of apoptosis in CRSwNP. The proapoptotic effect of miR-203a-3p may be crucial for future treatment strategies for CRSwNP.

1. Introduction

Apoptosis plays an important role in eliminating damaged cells and preventing uncontrolled cellular proliferation [1,2]. The inhibition of apoptosis can cause cancers or lead to development of autoimmune and inflammatory diseases. Genetic and epigenetic factors stimulate inflammatory responses in various autoimmune disorders, including chronic rhinosinusitis [3].

In chronic rhinosinusitis, the inflammatory process causes the stroma to swell and form polyps. Nasal polyps (NP) are benign changes that originate in the lining of the nasal sinuses. [4]

It was observed that patients with nasal polyps are characterized by increased epithelial cell proliferation induced inflammation associated with activation of epithelial repair mechanisms [5–7]. One of the hypotheses is that the formation of polyps is related to the abnormalities in cell proliferation and the inhibition of apoptosis [8,9]. Moreover, increased proliferative cellular activity has been noted in chronic upper respiratory tract diseases such as asthma and bronchitis [10].

The programmed cell death is a multifactorial process that can occur either by the intrinsic or extrinsic pathways, i.e. mitochondria apoptotic cascade or the death receptor-mediated pathway. The intrinsic pathway is coordinated by proteins of the Bcl-2(B-cell leukaemia/lymphoma 2) family. The proteins can act as pro-apoptotic or anti-apoptotic factors [11]. The important regulators of apoptosis are both BAX and BCL-2. BAX is an apoptosis-promoting protein that counteracts with the anti-apoptotic function of BCL-2

by binding to this molecule. The translocation of pro-apoptotic proteins e.g. BAX., to the internal mitochondrial matrix, releases cytochrome C, which binds to APAF-1 (apoptotic protease activating factor 1) inducing apoptosome formation [12]. The activation of caspase-3 (CASP3) within the apoptosome is indicative of the irreversible stage of apoptosis [13]. The increased BAX/BCL-2 ratio frequently correlates with higher expression of the P53 protein involved in inhibiting cell proliferation. Besides, this may increase the expression of cyclin-dependent kinase inhibitor P21 protein [14].

Small, non-coding RNA, namely miRNAs (18–24 nucleotides) play an essential role in regulating apoptosis, both intrinsic as well as an extrinsic pathway. These molecules act at the post-translational level, targeting pro-and anti-apoptotic genes. Recently, it was found that overexpressed miR-761 may alleviate chronic inflammation and induce remodelling of nasal mucosa in CRS mice. miR-761 targeted LCN2 (lipocalin 2), decreasing its expression and inactivating the LCN2/Twist1 signalling pathway, accompanied by reduced apoptosis and inflammation. Moreover, Zhang et al. determining miRNA profile showed that miR-125b could be significantly up-regulated in eosinophilic CRSwNP. Thus, investigation of both miRNA/mRNA associated with apoptosis and inflammation characteristic in CRSwNP can be an essential step in identifying new therapeutic targets[15].

To our knowledge, this work is the first study analysing the expression of selected apoptotic markers at mRNA and miRNA level, that may have potential prognostic utility in CRSwNP.

2. Results

2.1. TUNEL assay

The analysis revealed induced apoptosis in CRSwNP tissue samples. The apoptotic cells (stained brown) were counted after the assay. They were significantly increased compared with the healthy control. The apoptotic index based on in situ staining correlated with transcriptome profile determined (Fig. 1).

2.2 The level of genes associated with apoptosis in CRSwNP patients

The analysis of qRT-PCR expression revealed that tissue samples from CRSwNP patients exhibited significantly higher mRNA expression of pro-apoptotic genes, including *BAX*, *P53*, *P21*, *c-MYC*, *CASP3*, *CASP9* and *APAF1*. Increased expression of these transcripts correlated with lower expression of anti-apoptotic Bcl-2 as well as with survivin (*BIRC5*) and cyclin D (*CCND1*). The analysis of miRNA levels revealed increased expression of miR-203a-3p and decreased expression of miR-17-5p and miR-145-5p. In both analyzed groups, we found no significant differences between miR-146a-5p levels (Fig. 2–4)

3. Discussion

In most oncological diseases, overexpression of the anti-apoptotic protein BCL-2 inhibits the BAX protein and its activators, leading to tumour growth and its resistance to the applied therapy[16–20]. Homolog cell

production from viruses, similarly to Bcl-2 proteins, inhibits apoptosis and death of infected cells [21]. Cells missing both Bak and BAX proteins are resistant to cytochrome C promotion and apoptosis [22]. At the moment, studies concerning BAX activation substances in acute leukaemia are still in progress [23]. Disturbances in apoptosis, guided by increased accumulation of BCL-2, are responsible not only for oncological but also for autoimmunological diseases [22,24,25]. Mason et al. indicated that the absence of BAK/BAX expression in mice promotes a deadly disease similar to lupus [24]. Less severe symptoms were noted in mice with an isolated lack of BAK, though never in those with an isolated lack of BAX. Regulation of apoptosis in inflammatory diseases by intracellular peptides and signalling proteins is not fully understood yet [26]. Fan et al. examined the eosinophilic apoptosis in the mucous membrane of patients with chronic allergic rhinosinusitis with increased BAX expression. The authors observed no differences in the superficial and deep layers of the mucous membrane [27]. The role of BCL-2 and BAX in sinus mucosal inflammation was also studied using Staphylococcus aureus, confirming their importance in the bacteria-induced apoptosis of olfactory cells [28]. Tesfaigzi et al. observed that respiratory tract exposure to environmental allergens or toxins might cause mucous membrane damage, followed by inflammatory response and lower expression of BCL-2 protein [2]. Recovery means reducing the number of cells to the state before damage/inflammation, with temporarily decreased expression of Bcl-2 as an apoptosis inhibitor [29]. Cohen et al. examined decreased levels of Bcl-2 in patients with bronchial asthma and apoptosis inhibition [10]. We also reported increased levels of BAX mRNA and decreased levels of Bcl-2 in mucous membrane of patients with CRSwNP, which confirms sinus mucosa damage, followed by inflammatory process and apoptosis inhibition.

The BAX/BCL-2 ratio is considered a prognostic biomarker for cancer aggressiveness[5,30–32]. The authors examining melanoma progression found reduced apoptosis, overexpression of BCL-2, low expression of the BAX protein, and a lack of mutation in coding sequence proteins [32]. The increased BAX/BCL-2 ratio has also been used as an independent predictive marker in experimental studies evaluating damaged nervous tissues in patients with *miastenia gravis* [33,34]. In our examined patients with CRSwNP, the BAX/BCL-2 ratio increased, which may be associated with increased apoptosis.

The tumor suppressor oncoprotein-p53, known as “the guardian of the genome,” regulates gene transcription responsible for, e.g., DNA repair, cell cycle, ageing and proliferation processes[35–37]. Cell cycle repression and apoptosis play a similar role in oncogenesis suppression. Due to proteins antagonistic bifunctionality, a single cell action enables an accurate reaction for changing environmental conditions [38]. Mutation in the p53 genome is characteristic of most cancers [39]. However, Ingle et al. suggested that in benign epithelial lesions of the upper respiratory tract, the accumulation of p53 protein can occur without gene mutation[37]. We found higher levels of P53 transcripts in patients with CRSwNP, which corresponded with increased proliferation of epithelial cells.

The p21 is a multifunctional protein regulating the cell cycle and ensuring genome stability in response to various stimuli, including DNA damage [40]. The transcription of p21 is regulated by p53-dependent and -

independent pathways [41]. The mutation of the p21 genome is rarely detected [42]. In oncological patients, an analysis of P53 and P21 expression in predicting the effects of treatment was important [43]. An increased vulnerability to apoptosis with p53 in cells lacking p21 was observed in colorectal cancer [44].

A higher expression of p53 in CRSwNP was reported in 1999 by Lavezzi et al.[45]. Garavello et al. noted overexpression of p53 and Bcl-2 and increased apoptosis in mucous membranes with polyps, compared to the healthy sinus mucous membranes with an unchanged expression of the p21 protein [46]. Chalastras et al. examined patients with chronic rhinosinusitis and reported an increased tendency toward proliferation and decreased apoptosis in epithelial cells, increased expression of p53 and decreased levels of Bcl-2 in hyperplastic polyps[36]. On the other hand, Küpper et al. reported decreased expression of p53 in patients with CRSwNP [9]. Therefore, the expression of p53 in CRSwNP varies greatly. The reason may be, in some experiments authors using as study control, fragments of tissue collected from different anatomical locations (e.g., sinuses, nasal cavity, inferior nasal turbine) or perhaps because different diagnostic methods were used [9,36,45]. When p53 protein accumulation is reported with immunohistochemical studies, it does not always respond with its genome mutation. The accumulation of p53 antigen in healthy cells may be very discreet and difficult to estimate [36,37]. We observed a higher expression of the p53 and p21 proteins in patients with CRSwNP, but the p53/p21 ratio was similar in both study groups.

Elevated levels of BAX, P53, P21 genes and reduced levels of Bcl-2 evidenced increased mucosal apoptosis in patients with CRSwNP. Disorders of apoptosis may include the oncogenic c-MYC, which codes for transcription factors, regulates processes required for cell growth and oncogenesis, and affects Bcl-2 expression levels. According to Jung, c-MYC activation is correlated with increased expression of CCND1 and lower expression of Bcl-2 and p21[47]. Our study reported higher expression mRNA levels of c-MYC and p21 and lower expression levels of Bcl-2 and CCND1. According to literature data, these findings are, however partially contradictory.

We also detected a significantly higher expression of Apaf-1, CASP3 and CASP9, contributing to the induction of apoptotic processes in CRSwNP. APAF-1 is a cytosolic protein that activates caspase-9, a key factor in the apoptotic pathway of mitochondrial cells [48]. In healthy cells, APAF-1 remains in its autoinhibited form, and CASP-3 and CASP-9 remain inactive[49]. Cho et al. reported no apparent difference in CASP3 expression levels between normal mucosa and mucosa with nasal polyps[50]. CRSwNP can be, however, compared to other inflammatory processes. Hou and Xing found increased expression levels of p53, CASP3 and CASP9 and decreased expression levels of Bcl-2 in patients with RA [51]. Our results suggest a similar inflammation pattern.

BIRC5 is a gene (survivin), which acts as a prognostic biomarker in neoplastic diseases, indirectly inhibits apoptosis and promotes cell proliferation. Qui et al. found that its overexpression, associated with immune response [52,53], may play a key role in nasal polyps development [54]. However, these authors examined mucosal samples from various locations (turbinate, tissue surrounding polyps and nasal

polyps) [54]. Cho et al., who examined mucosa from the inferior turbinate, did not report higher survivin expression in healthy control and in patients with nasal polyps [50]. We reported lower expression levels of *BIRC5*, which is characteristic of increased apoptosis. Due to its immunomodulatory effect, *BIRC5* may be a potential therapeutic target whose activity can be modified by an external stimulus. However, this hypothesis requires further research.

The advantage of our study relies on tissue sampling, which allows us to compare the levels between healthy patients and CRSwNP patients. In our study, tissue samples were taken from the exact location, i.e. the maxillary sinus mucosa. The study may be limited by the small sample size, also from healthy participants. This group is however rarely accessible to examination. Increased levels of the *BAX* transcripts and decreased levels of *BCL-2* indicated increased apoptosis in the inflamed mucous membrane of patients with CRSwNP. A comparison of p53 and p21 in the healthy control and the study group suggested disturbances in apoptosis regulation.

The small molecules, RNAs or microRNAs (miRNAs), play a crucial role in many pathways that regulate apoptosis, as in cellular proliferation, differentiation and ageing Yang. Moreover, these molecules regulate gene expression in human diseases, serving as valuable indicators of cancer progression [55,56] and in diagnosing other diseases with excessive apoptosis. Senescence strongly inhibits cell proliferation and induces IL-6 and IL-8 secretion. Bhaumik et al. showed that increased expression of miR-146a/b inhibits an excessive secretion of inflammatory cytokines [57]. Sun et al. analysed miR-125-5p and miR-143/145 expressions as potential biomarkers for ischemic stroke [58]. Tang et al. examined the expression level of miR-145-5p and found that it was decreased in synovial tissues of RA patients [59]. Moreover, miRNA molecules are used in therapy for many diseases. Zhao et al. treated patients with traumatic brain injury with miR-203 inhibitors and reduced neuronal apoptosis by inhibiting CASP3 activity and increasing Bcl-2 expression [60].

Cimmino et al. demonstrated that miR-15a and miR-16-1 negatively regulate Bcl-2 at the post-transcriptional level [61]. Moreover, it has been found that miRNAs (miR-106b or miR-29 cluster) can regulate the activity of p53 and p21 proteins in the processes of DNA damage [62,63]. Liu et al. reported an association between miR-125b, miR-133, miR-146a, and miR-203 with acute exacerbation risk, inflammation, and severity of the chronic obstructive pulmonary disease. The authors reported a positive correlation with the level of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17 and IL-23) [64]. Taganov et al. suggested that miRNAs could regulate cellular immune response to particular pathogens. They found that miRNAs acted as potentially negative regulators of inflammation and showed increased expression levels of miR-132, miR-146 and miR-155 in monocytic cells as a response to bacterial infection [65]. Faraoni et al. described the role of miR-155 in the immune response to inflammation, e.g., in viral infections caused by DNA viruses. Furthermore, miR-155 is involved in hematopoietic cell differentiation, cancer, and cardiovascular disease [55]. The introduction of miRNA inhibitors into cells regulates response to inflammation by protecting cells against cytokine-induced death[55,66].

The similar levels of miRNA-146a in patients with CSR and healthy control may explain the results from Bhaumik et al. [57], who suggested that delayed miR-146a/b induction might be a compensatory response to restrain inflammation [57]. So far, no studies have investigated the role of miRNAs in CRSwNP. miRNA-203a overexpression and lower expression levels of miR-17-5p and miR-145-5p can promote increased apoptosis. The selected miR-17 was considered as a biomarker of chronic immune, cardiovascular and neurodegenerative diseases, and its overexpression is associated with autoimmunity [67]. Selected miR-145-5p and miR-146a 5p exhibit anti-apoptotic roles[68–70] and MiR-203a-3β overexpression is associated with increased apoptosis [71]. miRNAs expression levels are variable and based on cell-specific gene expression [68]. However, there is clear compliance of the direction of changes with those described in the literature.

miR-203a-3p gene, which exhibits the most potent pro-apoptotic effect, inhibits the proliferation, migration and invasion of cancer cells [72]. Lee et al. suggested a link between miR-203-3p expression and human lifespan [73]. Han et al. showed that higher levels of miR-203-3p expression inhibit pathological retinal angiogenesis [74]. However, this does not satisfactorily explain increased apoptosis and polypoid hyperplasia in patients with CRSwNP. In the course of this disease, both processes may not be sufficiently inhibited. We suppose that miR-203a-3p, due to its pro-apoptotic effect, may play a key role in developing new treatment strategies for CRSwNP, but our hypothesis requires further research.

ConclusionsTUNEL-demonstrated significantly increased apoptosis in sinus mucosa in patients with CRSwNP was evidenced by higher mRNA levels of *BAX*, *P53*, *P21*, *CASP3*, and *CASP9*, *APAF-1* and *c-MYC*, and lower levels of *BCL-2* and *BIRC5* transcripts.

The increased level of miR 203a-3p and decreased levels of miR 17-5p and miR-145-5p are also characteristic features of apoptosis in CRSwNP.

Due to its pro-apoptotic activity, miR 203a-3p may play an essential role in developing new treatment strategies for CRSwNP, but this hypothesis requires further studies.

In opposite, comparable levels of miR-146a in healthy patients and controls exclude its possible utility as a biomarker of CRSwNP.

4. Methods

4.1 Patients

The study group consisted of 10 patients with CRSwNP (n = 10), including 7 women and 3 men (mean age = 54.5 years; SD = 14.19; min. = 27; max. = 69). In each case, symptoms of rhinosinusitis persisted for more than one year. Based on anamnesis, treatment history, the results from available endoscopic studies (rigid endoscope with 00 and 300 direction of view, before and after decongestion of the mucosa), and craniofacial computer tomography, all patients were qualified according to EPOS 2012

guidelines 15 for functional endoscopic sinus surgery (FESS) in the ENT Department. One month before FESS, no steroids or antibiotics were used. During the procedure, a fragment of the inflamed mucosa was taken from the maxillary sinus. Histopathological examination always confirmed the diagnosis of chronic eosinophilic sinusitis.

The control group was made up of 10 generally healthy people with only various orthognathic defects (4 women and 6 men) (mean age = 27 years; SD = 3.46; min. = 23; max. = 34). The main exclusion criteria were any pathologies of sinuses. Patients qualified for Lefort I level osteotomy were treated at the Department of Maxillofacial Surgery at the local Medical University. During hospitalisation, blood tests were performed, particularly to measure inflammatory markers (blood cell count, C-reactive protein [CRP], procalcitonin, and fibrinogen levels), along with CT and nasal endoscopy before and after surgery. Excess mucosa of maxillary sinus, obtained each time, was examined.

The study was approved by the Bioethics Committee at the local Medical University (KB-545/2015). Each patient and healthy controls signed an informed consent form and decided freely to participate in the study. The study was conducted in compliance with the principles of the Declaration of Helsinki.

4.2 TUNEL-assay

Apoptotic cells in tissue samples were determined with the TUNEL Apoptosis Detection Kit (Cat # ab206386, Abcam, Cambridge, UK). Obtained paraffin blocks were cut into slices with 4-µm thickness. The tissue samples were washed 3 times with HBSS and fixed in 4% paraformaldehyde (PFA). Paraffin-embedded tissues were stained using TUNEL assay according to the manufacturer's instructions. Slides were deparaffinised in xylene (Sigma Aldrich, Munich, Germany) and rehydrated in ethanol series (concentration from 100–70%). Then, specimens were permeabilised using a Proteinase K solution, and endogenous peroxidases were inactivated with 3% H2O2. Tissues were equilibrated in TdT Equilibration Buffer and labelled with a TdT Labeling Reaction Mixture for 1.5 hours at room temperature. Subsequently, the specimens were covered with Blocking Buffer and then a Conjugate solution was added and incubated for 30 min in a humidified chamber at room temperature. Subsequently, DAB Solution was added for 15 min. Specimens were counterstained with Methyl Green Counterstain solution, mounted with DPX Mounting Media (Aqua-Med, Łódź, Poland) and analysed using epi-fluorescent microscopy (Zeiss, Axio Observer A.1). The signals obtained after staining were determined using ImageJ and Pixel Counter plugin (version 1.6.0, U. S. National Institutes of Health, Bethesda, MD, USA) as described previously.

4.3 The analysis of gene expression: Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) for the detection of mRNA and miRNA levels

The expression of apoptosis-related markers was analyzed on the mRNA and microRNAs levels. The tissue samples were washed three times with Hanks' Balanced Salt Solution (HBSS) and cut into small

pieces using a scalpel. For homogenisation, we used 1 ml of Extrazol® (Blirt DNA, Gdansk, Poland). The total RNA was isolated with protocols provided by the manufacturer by using the phenol-chloroform method described by Chomczyński and Sacchi 16. Obtained RNA was diluted in 30 µL of DEPC-treated water. Quantity and purity of RNA were evaluated using a spectrophotometer (Epoch, Biotek, Bad Friedrichshall, Germany) at 260 and 280 nm wavelengths. Before reverse transcription, total RNA (500 ng) was treated with DNase I (Primerdesign, BLIRT S.A, Gdansk, Poland) to remove genomic DNA. cDNA synthesis was carried out using Tetro cDNA Synthesis Kit (Bioline Reagents Limited, London, UK). The gDNA digestion and cDNA transcription were performed using T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocols and the previously established methods. Additionally, for evaluation on miRNA level, after digestion, cDNA was synthesised using 375 ng of total RNA with Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio Europe, Saint-Germainen, Laye, France). The obtained matrices were used for quantitative PCR using the SensiFAST SYBR®&Fluorescein Kit (Bioline Reagents Ltd., London, United Kingdom). Each reaction was performed in the final volume of 10 ul, where 1 ul of cDNA was used and the concentration of primers was 0.5 µM. Quantitative PCR was performed in in CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The cycling conditions used for transcript detection were as follows: initial denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 15 seconds, annealing for 15 seconds, and elongation at 72°C for 15 seconds with a single fluorescence measurement. The accuracy of the PCR reaction was evaluated by an analysis of the dissociation curve of the amplicons. The melting curve was performed using a gradient program in the range of 55–95°C at a heating rate of 0.2°C/s and with continuous measurement of the fluorescence. Primer sequences are summarized in Table 1.

Table 1. Primer sequences used for the detection of mRNA and miRNA and miRNA levels.

Gene name	Gene	Primer	Sequence 5'-3'	Amplicon	Annealing	Accesion no.
Caspase-3	*CASP3*	F:	GCGGTTGTAGAAGTTAATAAAGGT	232	55	NM_001354784.1
		R:	CGACATCTGTACCAGACCGAG			
Caspase-9	*CASP9*	F:	AGGCGGTGACCCCAGAATTG	241	59,3	NM_032996.3
		R:	AAGGAAGAGCCTGACTCCCTC			
Cellular tumor antigen p53	*P53*	F:	AGATAGCGATGGTCTGGC	381	57	NM_000546.6
		R:	TTGGGCAGTGCTCGCTTAGT			
Cyclin-dependent kinase inhibitor 1	*P21*	F:	AGAAGAGGCTGGTGGCTATTT	169	57	NM_001220777.2
		R:	CCCGCCATTAGCGCATCAC			
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	F:	GTCAGTGGTGGACCTGACCT	256	60	NM_001357943.2
		R:	CACCACCCTGTTGCTGTAGC			
Apoptosis regulator BAX	*BAX*	F:	ACCAAGAAGCTGAGCGAGTGTC	414	59,3	NM_001291428.2
		R:	ACAAAGATGGTCACGGTCTGCC			
Apoptosis regulator Bcl-2	*BCL-2*	F:	ATCGCCCTGTGGATGACTGAG	129	58,6	NM_000633.3
		R:	CAGCCAGGAGAAATCAAACAGAGG			
Apoptotic protease-activating factor 1	*APAF1*	F:	CTTCTTCCAGTGTAAGGACAGT	243	58,8	NM_013229.3
		R:	CTGAAACCCAATGCACTCCC			
Baculoviral IAP repeat-containing protein 5	*BIRC5*	F:	ACCGCATCTCTACATTCAAG	113	55	NM_0010122702
		R:	CAAGTCTGGCTCGTTCTC			
G1/S-specific cyclin-D1	*CCND1*	F:	GATGCCAACCTCCTCAACGA	211	58,2	NM_053056.3
		R:	GGAAGCGGTCCAGGTAGTTC			
Myc proto-oncogene protein	*c-MYC*	F:	CTTCTCTCCGTCCTCGGATTCT	204	58,8	NM_002467.6
		R:	GAAGGTGATCCAGACTCTGACCTT			
hsa-miR-203a	*miR-203a*	F:	GTGAAATGTTTAGGACCACTAG	*	58,8	MIMAT0000264
h/e/m/r-miR-145-5p	*miR-145*	F:	GTCCAGTTTTCCCAGGAATCCCT	*	58,8	MIMAT0000437
h/e/m/r-miR-17-5p	*miR-17*	F:	CAAAGTGCTTACAGTGCAGGTAG	*	58,8	MIMAT0013084
h/m/r-miR-146a-5p	*miR-146*	F:	TGAGAACTGAATTCCATGGGTT	*	58,8	MIMAT0000449

* Due to poly (A) sequence it is not possible to predict the actual product size.

The expression of genes was calculated using RQMAX algorithm and converted into log2 scale as published previously. The normalization of detected transcripts was made in refrerence to the housekeeping gene – GAPDH (glyceraldehyde 3-phosphatedehydrogenase) for mRNA, and U6snRNA (Takara Bio Europe, Saint-Germainen, Laye, France) for miRNA.

4.4 Statistical Analysis

The obtained results are presented as the mean from at least three technical repetitions. Means are presented with standard deviation (± SD). Statistical comparison between the groups was determined using unpaired Student's t-test. The data were analyzed using GraphPad Prism 8 software (La Jolla, CA, USA). Differences with a probability of $p < 0.05$ were considered statistically significant.

Declarations

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Author contributions

Conception or design of the work: MMK, AS, KM, MB

Data collection: MMK, KN, KZ, KLM

Data analysis and interpretation: MMK, AS, KKM, KZ, KN, MB

Drafting the article: MMK, AS, MB,

Critical revision of the article: MMK, AS, TZ, MB

Final approval of the version to be published: MMK, AS, KLM, KM, KN, KZ, TZ, MB

Competing interests

The authors declare no competing interests.

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Figures

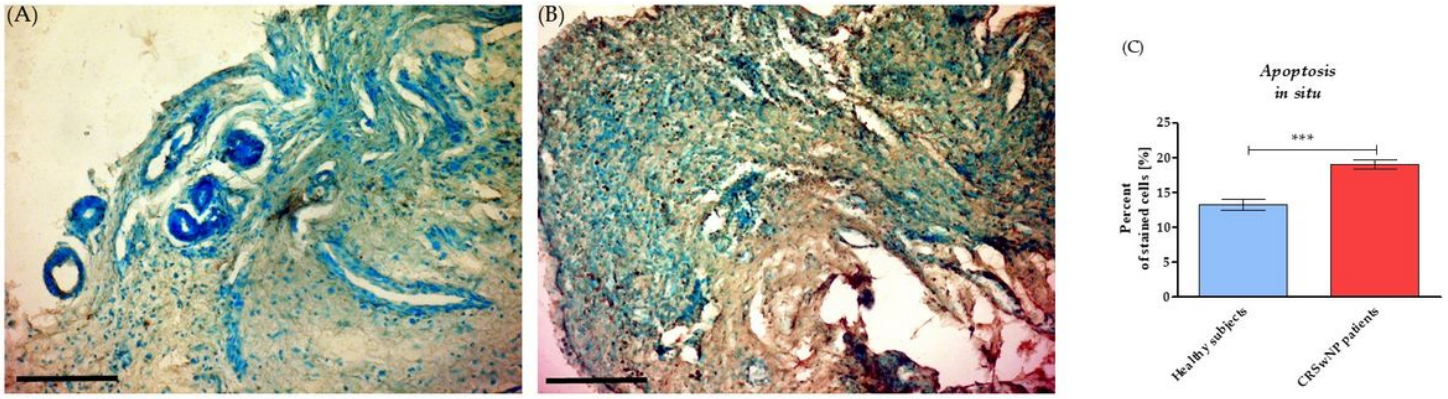

Figure 1

Apoptosis detection in tissue sections derived from healthy subjects (A) and CRSwNP patients (B). Scale bars, 200 μm. the results of statistical analysis showing increased occurrence of apoptotic cells in tissues from CRSwNP patients (C). Statistically significant differences were noted at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)

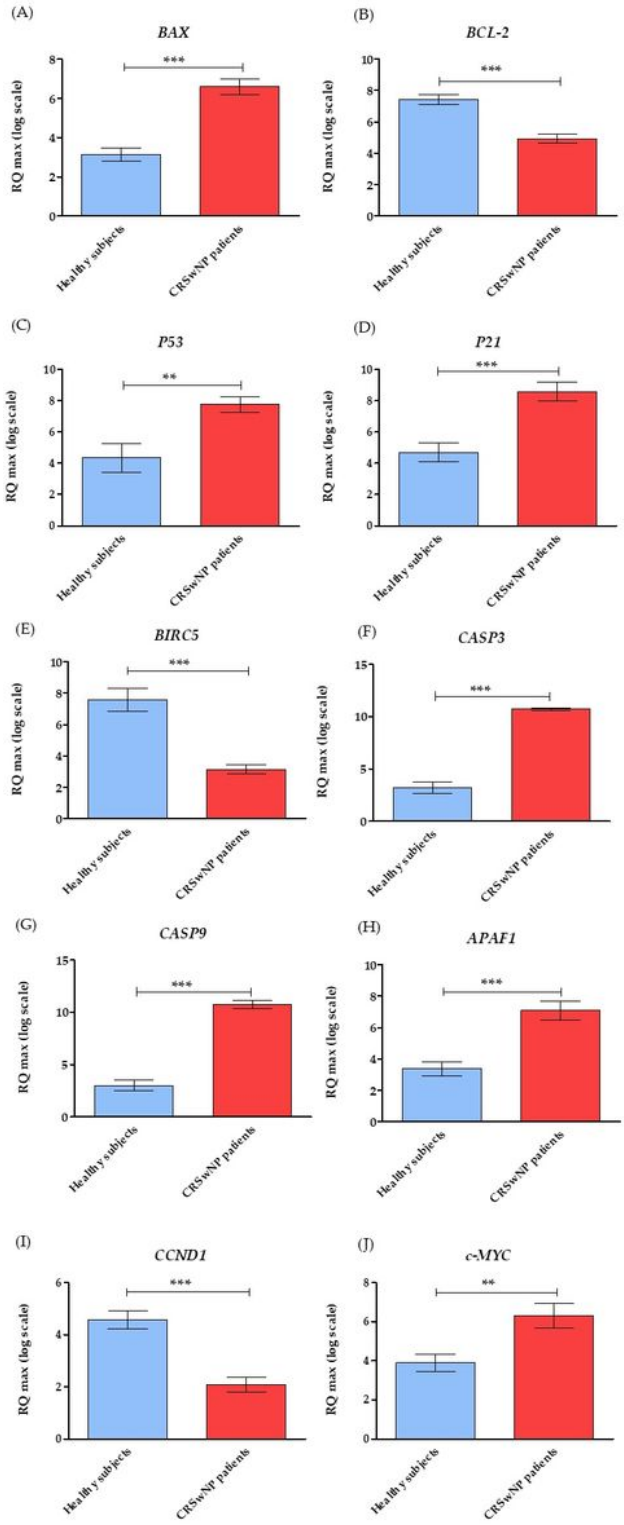

Figure 2

The expression profile of genes was associated with apoptosis and cell survival. Transcript levels were established for CRSwNP patients in relation to the healthy control. Following markers at mRNA were detected: BAX - apoptosis regulator BCL2 Associated X (A); BCL-2 - B-cell CLL/lymphoma 2 (B); P53 - Tumor Protein P53 (C); P21- cyclin-dependent kinase inhibitor 1A (D); BIRC5 - Baculoviral IAP repeat-containing protein 5 (E); CASP3 - Caspase 3 (F); CASP9 - Caspase 9 (G); APAF1 - Apoptotic protease-

activating factor 1 (H); CCND1 - G1/S-specific cyclin-D1 (I); cMYC - Myc proto-oncogene protein (J). The average fold change of the target genes was determined in relation to the housekeeping gene i.e., Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Statistically significant differences are indicated with an asterisk (*). Statistically significant differences were noted at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

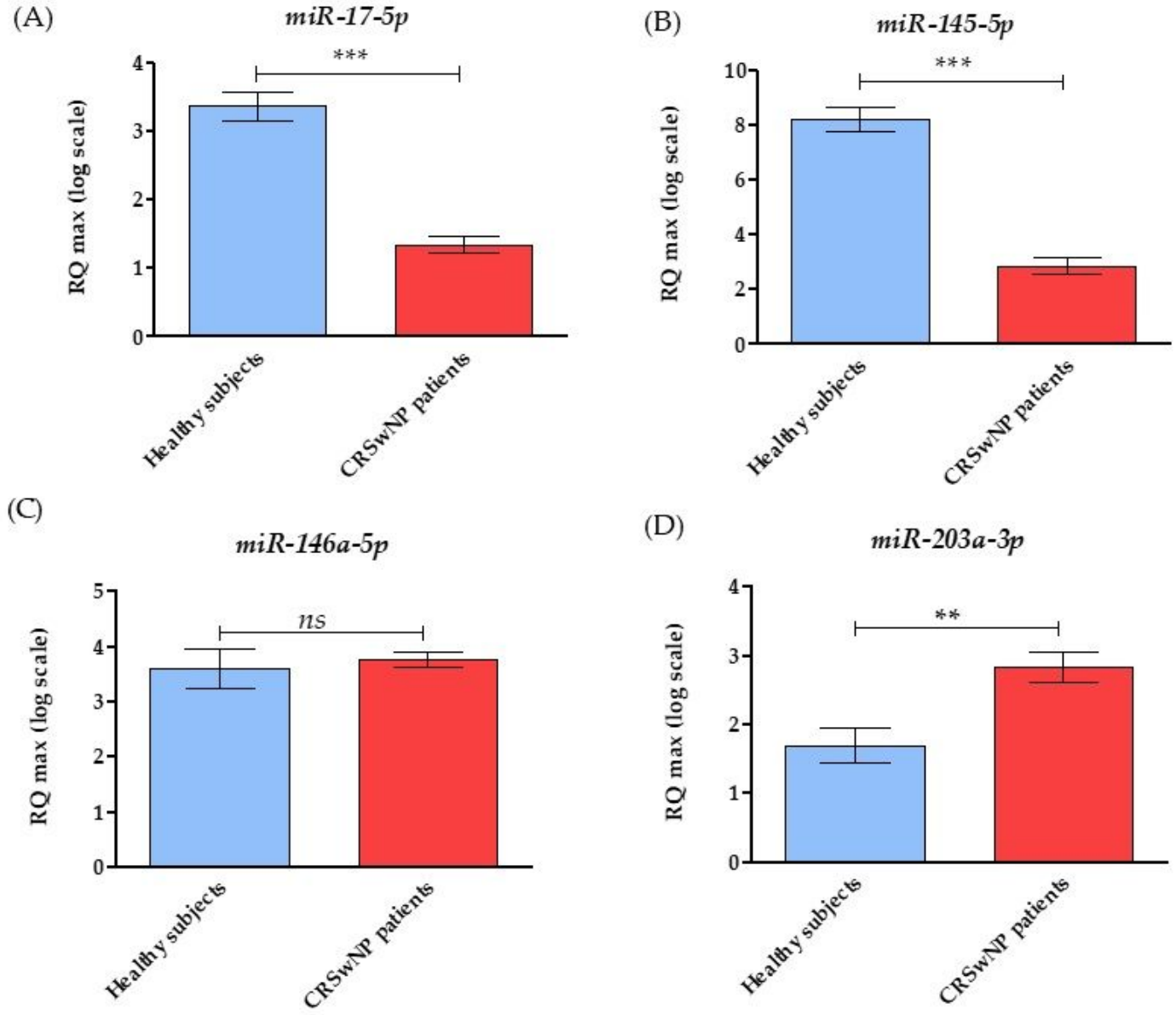

Figure 3

The miRNA levels were associated with apoptosis and cell survival. Transcript levels were established for CRSwNP patients in relation to the healthy control. Following miRNAs were measured: miR-17-5p (A); miR-145-5p (B); miR-146a-5p (C) and miR-203a-3p (D); The average fold change of the target genes was determined in relation to the housekeeping gene, i.e. small nuclear RNA U6 (snU6). Statistically significant

differences are indicated with an asterisk (*). Statistically significant differences were noted at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

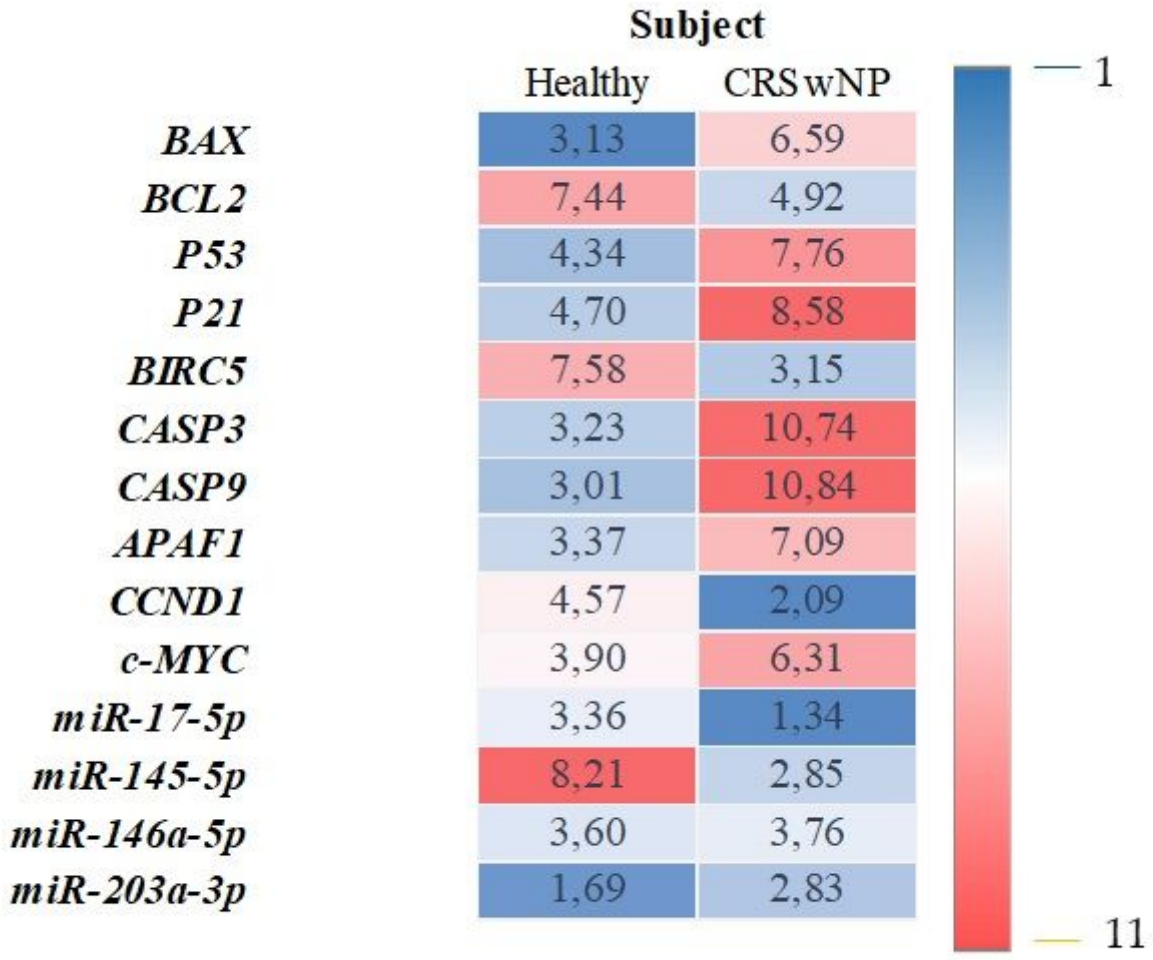

Figure 4

Heatmap showing the examined mRNA and miRNA levels in the healthy control and CRSwNP patients. The analysis showed the mean relative transcript amount. Levels of down expression (blue) or overexpression (red) are shown on a log2 scale from the high to the low expression of each gene.