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ORIGINAL ARTICLE

The expression of cancerous inhibitor protein phosphatase 2A in chronic rhinosinusitis with nasal polyps

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ABSTRACT

Conclusion: The study suggests that cancerous inhibitor of protein phosphatase 2A (CIP2A) expression and eosinophilia associate with chronic rhinosinusitis with nasal polyps with aspirin exacerbated respiratory disease (CRSwNP + AERD). Further studies with a larger sample size are needed to evaluate further the role of CIP2A and related pathways in CRSwNP + AERD.

Objectives: Low prostaglandin E2 levels putatively associate with CRSwNP + AERD and decreased c-Myc levels. The aim of this study was to evaluate the expression and revision-predictive role of oncoprotein CIP2A, another c-Myc modulator, in CRSwNP with/without AERD, and in antrochoanal polyps.

Method: Ninety retrospective archival objective glasses of nasal polyp tissue from CRSwNP or ACP patients were used for assessing mucosal eosinophilia. Of this population, 90 archival nasal polyp specimens were available for immunohistochemical staining with a polyclonal anti-CIP2A antibody, together with 19 control nasal mucosa specimens. CIP2A staining intensity and tissue eosinophilia were assessed by two blinded observers with a light microscope. Subject characteristics from 90 patients and 19 controls were obtained from patient records and additionally by a questionnaire from controls. The follow-up data was available from patient records of 84 patients and 16 controls.

Results: The expression of epithelial CIP2A was detected both in control inferior turbinate mucosa and nasal polyps. The expression was significantly lower in the CRSwNP + AERD group compared to controls and CRSwNP without AERD ($p < 0.01$). High mucosal eosinophilia associated with CRSwNP ($p < 0.01$). Neither CIP2A nor eosinophilia predicted the need for revision surgery ($p > 0.05$), whereas previous surgery, allergic rhinitis, and use of corticosteroids did predict the need for revision surgery ($p < 0.05$).

Abbreviations: ACP: antrochoanal polyp; ASA: acetylic salicylic acid, aspirin; CIP2A: cancerous inhibitor of protein phosphatase 2A; COX-2: Cyclo-oxygenase 2; CRS: chronic rhinosinusitis; CRSsNP: chronic rhinosinusitis without nasal polyps; CRSwNP: chronic rhinosinusitis with nasal polyps; ESS: endoscopic sinus surgery; Ig: immunoglobulin; NP: nasal polyp; pAb: polyclonal antibody; PEG2: prostaglandin E2; Th1: T-helper cell 1; Th2: T-helper cell 2

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Introduction

Chronic rhinosinusitis (CRS) is a significant health problem with a prevalence of ~7–11% [1]. CRS with nasal polyps (CRSwNP) and without (CRSsNP) are considered to be phenotypes of CRS with possibly different aetiologies and pathomechanisms [1].

Sinus specimens obtained from patients suffering from CRSsNP are generally characterized by basement membrane thickening, goblet cell hyperplasia, subepithelial oedema, abundant mononuclear cells, and few eosinophils [1]. Histomorphological characterization of CRSwNP reveals epithelial damage, goblet cell metaplasia, a thickened basement membrane, and oedematous to sometimes fibro-inflammatory tissue. CRSsNP is characterized by a T-helper cell 1 (Th1)

polarization with high levels of interferon-gamma and transforming growth factor beta. Caucasian CRSwNP is characterized by a Th2-skewed eosinophilic inflammation with high interleukin 5 levels [2], and reduced transforming growth factor beta, whilst over 50% of East Asian polyps are characterized with a Th1/Th17, neutrophilic dominant pattern [3].

Antrochoanal polyps (ACP) are benign polypoid lesions arising from the maxillary antrum and which extend into the choana. They occur more commonly in children and young adults, and are almost always unilateral [4]. Human papillomavirus type 16 has been detected at higher frequencies in ACP [5].

Aspirin exacerbated respiratory disease (AERD) is characterized by severe eosinophilic hyperplastic inflammation of

all sinuses and nasal passages as well as of the lower airways. AERD is characterized by higher than usual severity of disease with recurring polyps and frequent need for sinus surgery [6]. The pathogenesis of chronic eosinophilic inflammation of the airway mucosa and nasal polyps in AERD patients would seem to be the coincidence and possible overlap of non-steroidal anti-inflammatory drugs (NSAIDs) induced acute hypersensitivity and chronic eosinophilic mucosal inflammation. AERD has been documented to have a distinctive cytokine expression and upregulation profile, which has led to the postulation of a possibly different pathomechanism than in aspirin (ASA) tolerant patients [1,6].

Oncoprotein c-Myc is a multi-functional nuclear phosphoprotein driving a number of cellular responses including cell cycle progression, apoptosis, and cellular transformation, depending on cellular context. *In vitro* and animal experiments demonstrate that regulation of c-Myc is essential for normal cell proliferation and prevention of neoplastic changes [7]. Protein phosphatase 2 (PP2A) suppresses c-Myc through dephosphorylation of c-Myc's serine 62 [8]. Protein phosphatase is a serine/threonine phosphatase that dephosphorylates a number of kinases driving inflammatory cell signalling [9]. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncoprotein that inhibits PP2A [10]. CIP2A stabilizes c-Myc, thus promoting cell growth, metabolism, proliferation, and inhibition of apoptosis [7].

Previously, we have demonstrated that CIP2A expression is increased in metastasized tongue cancer and tongue hyperplasia [11]. The aim of our study was to investigate the association and predictive role of CIP2A in CRSwNP, which is another common benign lesion affecting the head and neck region. We hypothesized that CIP2A associates with the AERD phenotype and might have a role as a predictive marker.

Patients and methods

This retrospective follow-up study was performed at the Departments of Otorhinolaryngology and Pathology, Tampere University Hospital and Haartman institute, University of Helsinki, Finland. The study protocol was approved by the Hospital's Ethical Committee (nro. R07039). We collected CRSwNP patients' samples who had undergone polyp sampling for diagnostic purposes at the Tampere University Hospital or Tampere City Hospital between 2001–2006. The histopathological diagnosis was made in Fimlab Laboratories Ltd, Tampere, Finland. Patients were Caucasians, aged at least 16 or over. The inclusion criteria of patients were: diagnosis of CRSwNP or ACP based on the European position paper on rhinosinusitis and nasal polyps criteria of symptoms, endoscopic, and sinus computed tomography findings based on the patient records [1]. The exclusion criteria were cystic fibrosis, primary ciliary dyskinesia, and diseases with a severe impact on general immunity. Ninety patients were included as they had retrospective archival objective glasses of nasal polyp tissue available in the archives, for tissue eosinophilia assessment by microscopy.

Table 1. Characteristics of patients from whom nasal cavity specimens were taken.

	Control (n = 19)		CRSwNP (n = 59)		CRSwNP- + AERD (n = 21)		ACP (n = 10)		p-value
	n	%	n	%	n	%	n	%	
Gender									
Male	9	47.4	36	61.0	12	57.1	7	70.0	0.670
Female	10	52.6	23	39.0	9	42.9	3	30.0	
Age									
< 40 years	16	84.2	13	22.0	3	14.3	4	40.0	<0.001
≥ 40 years	3	15.8	46	78.0	18	85.7	6	60.0	
Allergic rhinitis									
No	7	36.8	37	68.5	12	57.1	8	88.9	0.031
Yes	12	63.2	17	31.5	9	42.9	1	11.1	
Asthma									
No	17	89.5	45	77.6	1	4.8	10	100	0.006
Yes	2	10.5	13	22.4	20	95.2	0	0	
Smokers									
No	18	94.7	42	75.0	17	81.0	7	87.5	0.290
Yes	1	5.3	14	25.0	4	19.0	1	12.5	
Previous sinonasal operation(s) ^a									
No	19	100	31	73.8	7	53.8	10	100	0.047
Yes	0	0	11	26.2	6	46.2	0	0	
Current sinonasal operation									
No (only sampling)	19	100	10	16.9	2	9.5	2	20.0	<0.001
Only polypectomy	0	0	9	15.3	4	19.0	4	40.0	
ESS + polypectomy	0	0	40	69.0	15	71.4	4	40.0	
Medication									
No	19	100	28	52.8	4	19.0	6	66.7	<0.001
IN CCS	0	0	23	43.4	8	38.1	3	33.3	
PO CCS ± IN CCS	0	0	2	3.8	9	42.9	0	0	

Control: mucosa from inferior turbinate; CRSwNP: chronic rhinosinusitis with nasal polyps; CRSwNP + AERD: chronic rhinosinusitis with nasal polyps and with aspirin exacerbated respiratory disease; ACP: antrochoanal polyp; ESS: endoscopic sinus surgery; CCS: corticosteroid; in: intranasal; PO: peroral.

^aESS and/or polypectomy, p-values by Fisher's exact test.

Of the 90 patients included, we searched and collected the available formalin-fixed paraffin-embedded polyp samples ($n = 55$) from the archives of the Fimlab Laboratories as previously described [12]. Subject data from the 90 patients was collected from patient records (Table 1), in which diagnosis of allergic rhinitis was based on skin prick test positivity and report of typical symptoms, and asthma was based on clinical features and diagnostic pulmonary function tests, corresponding with the Global Initiative for Asthma diagnostic criteria and AERD on a history of wheezing or asthma attacks precipitated by non-steroidal anti-inflammatory drugs.

Control inferior turbinate samples ($n = 19$) were collected prospectively from healthy non-smoking volunteers in 2005–2007 as previously described [12]. All subjects were Caucasian. The exclusion criteria of control subjects were: sinonasal disease (except mild allergic rhinitis) or any other disease requiring constant medication. Diagnosis of allergic rhinitis was based on skin prick test positivity during the time of sampling and typical symptoms. Other control subject data was collected by a questionnaire (Table 1).

The follow-up data on sinonasal operations and time of surgery was collected from patient records of the Tampere University Hospital or Tampere City Hospital between 2014–2015, in average 9 years after the time of sampling. The follow-up data was available from 84 patients and 16 controls. None of the subjects had undergone aspirin desensitization, allergen immunotherapy or anti IgE therapy prior

to, during sampling, or during follow-up. None of the control subjects had undergone sinonasal operations or had been diagnosed with CRS(wNP) during follow-up.

Tissue handling and immunohistochemistry

CIP2A immune staining was performed in 74 samples. The samples were cut into 4–5 µm thick sections and were placed on Superfrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Fully automated immunostaining was performed by Ventana BenchMark LT Automated IHC Stainer (Ventana Medical System, Tucson, AZ). An Ultraview Universal DAB detection kit (catalog No. 760-500, Ventana Medical System) was used. Ventana EZ Prep solution (catalog No 950-100, Ventana Medical System) was used for deparaffinization. For epitope retrieval CC1: Tris -EDTA buffer pH 8.0 (catalog No 950-124, Ventana Medical System) was used at 95–100 °C for 30 min with paraffin embedded tissue sections. Endogenous peroxidase was blocked with UV-Inhibitor 3% H2O2 (Ventana Medical System) for 4 min at 37 °C. Tissue slides were rinsed between steps with Ventana Tris-based Reaction buffer (catalog No. 950-300, Ventana Medical System). Slides were incubated at 37 °C for 32 min with pAb anti-CIP2A (1:100, catalog No. IHC-00266, Bethyl Laboratories Inc., Montgomery, TX) followed by application of Ventana Ultraview HRP Universal Multimer (8 min at 37 °C). 3,3'-diaminobenzidine was used as a chromogen and haematoxylin as a nuclear stain. Known positive tissue samples (oesophagus adenocarcinoma) were also used to confirm the staining reliability of all separate staining patches, as previously described [11]. The specificity of immunohistochemistry was controlled by omitting the primary antibodies or replacing them with irrelevant antisera.

Light microscopic evaluation

All samples were reviewed by a pathologist. CIP2A staining intensity was evaluated semi-quantitatively with an Olympus BH2 light microscope (Olympus Optical Co. Ltd, Japan) by two independent observers (ET and STS) blinded to the experimental conditions. The observers scored the stained sections independently. The observers discussed their opinions, and the consensus score was used for analyses.

CIP2A staining intensity was determined by assessing the staining intensity score so that the value 0 represented a stainless sample and the value 3 represented strongest staining. CIP2A staining intensity was scored semiquantitatively as: 0 = no, 1 = weak, 2 = moderate, 3 = strong staining. The relative density of eosinophils to all leukocytes was determined from 104 samples (by AL and TP) stained with Hematoxylin & eosin, from at least five inflammatory hot-spot fields; and was evaluated as follows: low = less than 20% eosinophils; high = 20% or more eosinophils.

Data analysis

Descriptive statistics for subject characteristics were presented in different groups. Fisher's exact test (2-tailed) was

used to compare patient characteristics, and the correlations were analysed by the Spearman rank correlation test. The comparison of CIP2A staining intensity in the different groups were made by Fisher's exact test, Kruskal-Wallis test (more than two groups) and by the Mann-Whitney U-test (two groups). The time periods between the initial study operation and second surgeries were analysed with the Kaplan-Meier method, and compared with the log-rank test. Survival was calculated from the date of the initial study operation to first follow-up revision sinus surgery and/or nasal polypectomy/death/end of March 2015, whichever came first. Two-tailed *p*-values of <0.05 were considered statistically significant. Statistical analysis was carried out by the SPSS Base 15.0 Statistical Software Package (SPSS Inc., Chicago, IL).

Results

Patient characteristics

The characteristics are shown in Table 1. The patient groups differed significantly in terms of age, presence of AR, asthma, previous sinonasal operation, current operation during sampling, medication, and mucosal eosinophilia (*p* < 0.05, Table 1). In terms of other patient history, data did not differ significantly (*p* > 0.05).

CIP2A staining intensity

CIP2A immunostaining was performed on nasal mucosal specimens of 19 controls; and on nasal polyp specimens of 33 patients with CRSwNP, 13 patients with CRSwNP + AERD, and nine patients with ACP (Figure 2). CIP2A expression was detected in the cytoplasm of epithelial cells and in glandular cells of the nasal mucosa and nasal polyp tissue (Figures 1 and 2). We observed the CIP2A staining intensity semi-quantitatively with a scoring from 0–3. A lower CIP2A staining intensity associated with CRSwNP + AERD patients compared to control and the CRSwNP group (*p* < 0.01, Figure 3(A)). The result remained similar when observing only patients without corticosteroid medication prior to sampling (*p* < 0.05, Figures 3(B–D)). When performing pair-wise comparisons based on the corticosteroid treatment, lower CIP2A staining intensity was associating with the patient group with peroral corticosteroid ± intranasal corticosteroid medication prior to sampling (*n* = 5), compared to the group with no corticosteroid treatment prior to sampling (*n* = 19) (*p* = 0.018). CIP2A staining intensity did not differ when comparing the group having only intranasal corticosteroid treatment prior to sampling (*n* = 26), to either of the previously mentioned groups (*p* > 0.05, both, by Fisher's exact test).

Tissue eosinophilia

Nasal mucosal or polyp eosinophilia was determined from 19 controls, 59 patients with CRSwNP, 21 patients with CRSwNP + AERD, and 10 patients with ACP (Table 1).

Figure 1. CIP2A expression in nasal specimens. (A) Antrochoanal polyp (ACP) as a negative control. (B) No CIP2A expression in control inferior turbinate (score =0). (C) Weak CIP2A expression in control inferior turbinate (score =1). (D) Moderate CIP2A expression in control inferior turbinate (score =2). (E) Strong CIP2A expression in control inferior turbinate (score =3). (F) No CIP2A expression in polyp tissue from a patient with chronic rhinosinusitis with nasal polyps and with aspirin exacerbated respiratory disease (CRSwNP + AERD) (score =0). (G) Mild CIP2A expression in CRSwNP + AERD (score =1). (H) Moderate CIP2A expression in polyp tissue from a patient with chronic rhinosinusitis with nasal polyps (CRSwNP) (score =2). (I) Strong CIP2A expression in ACP (score =3). (J) Strong CIP2A expression in CRSwNP (score =3). Original magnification was $\times 400$.

Figure 2. (A) Moderate CIP2A expression in the cytoplasm of epithelial cells of the nasal polyp tissue from a patient with chronic rhinosinusitis with nasal polyps (score =2). (B) Strong CIP2A expression in glandular cells of the nasal polyp tissue from a patient with chronic rhinosinusitis with nasal polyps (score =3). Original magnification was $\times 600$.

When performing pair-wise comparisons between two groups, the proportion of subjects with elevated mucosal eosinophilia (20% or more) was significantly higher in the CRSwNP groups compared to the control group ($p=0.002$, by Fisher's exact test) (Table 1) and in CRSwNP + AERD compared to the control group ($p<0.001$, Table 1). Other pair-wise comparisons did not differ significantly (data not shown). The high number of eosinophils did not associate with CIP2A staining intensity in the tissue when observing all groups ($p>0.05$ by Fisher's exact test), or only patients without controls ($p>0.05$).

Follow-up time

The follow-up data of sinonasal operations was collected from patient records. There were six deaths (three CRSwNP,

three CRSwNP + AERD) during follow-up. None of the deaths were caused by CRS or CRS surgery. None of the patients had undergone revision CRS surgery during follow-up prior to death. The mean (min-max) follow-up of all subjects was 9.4 (3–14) years. The mean (min-max) follow-up of the subjects that were living at the end of follow-up was 9.3 (7–14) years. During follow-up, none of the control patients were diagnosed for CRS, nor did they undergo sino-nasal operations.

Predictive relevance of CIP2A and other factors

We evaluated the effect of several factors to the time to first revision polypectomy $\pm$ endoscopic sinus surgery (ESS) in nasal polyp patients during follow-up of in average 9 years. Polypectomy with additional ESS at the time of

Figure 3. CIP2A staining intensity in the different patient groups. The y-axis indicates the percentage of patients in different CIP2A staining intensities. (A) CIP2A staining intensity in all patients. (B) CIP2A staining intensity in controls and in the patients without peroral corticosteroids prior to sampling. (C) CIP2A staining intensity in controls and in the patients without peroral and/or intranasal corticosteroids prior to sampling. (D) CIP2A staining intensity in the patients without peroral corticosteroids, with intranasal corticosteroids prior to sampling. Control: inferior turbinate tissue from healthy controls; CRSwNP: polyp tissue from patients with chronic rhinosinusitis with nasal polyps; CRSwNP + AERD: polyp tissue from patients with chronic rhinosinusitis with nasal polyps with aspirin exacerbated respiratory disease; ACP: polyp tissue from patients with antrochoanal polyp; n: number of patients. P-values by Fisher's exact test. Only p-values <0.05 are shown.

sampling associated significantly with the need for revision polypectomy ± ESS, when comparing to patients undergoing only polypectomy or biopsy at the time of sampling (Figure 4(A)). The result remained the same, when observing only the patients who underwent surgery (polypectomy ± ESS) at the time of sampling (Figure 4(B)). Report of previously performed polypectomy (one or more) and/or ESS associated significantly with revision polypectomy ± ESS compared to those without a history of previous sinonasal surgery (Figure 4(C)). The result remained the same, when observing only the patients who underwent surgery at the time of sampling ($p = 0.002$). Use of peroral corticosteroids at the time of sampling and a patient history of AR associated significantly with the need

for revision polypectomy ± ESS (Figures 4(D–E)). Fifty-five per cent of patients currently using peroral steroids needed revision surgery during the follow-up of 9 years, when the respective figure for those not using peroral steroids was 85%. The result remained the same, when observing only the patients who underwent surgery at the time of sampling ($p = 0.002$, $p = 0.011$, respectively). There was a trend that a patient history of AERD associated with revision polypectomy ± ESS (Figure 4(F)), which remained also the same when observing only the patients who underwent surgery at the time of sampling ($p = 0.073$). Age, gender, asthma, smoking, use of intranasal corticosteroids, CIP2A expression or tissue eosinophilia did not associate with revision surgery during the follow-up (data not shown).

Figure 4. Predictive effect of different factors to the time until revision surgery (polypectomy and/or endoscopic sinus surgery (ESS)) of polyp patients according to the Kaplan-Meier method. The follow-up data was available from patient records of 84 patients. (A) Predictive effect of current ESS + polypectomy ($n = 82$, because 82/84 patients had the surgery information available in the patient records). (B) Predictive effect of current ESS + polypectomy only in the group of patients who underwent surgery at the time of sampling ($n = 72$). (C) Predictive effect of previously performed ESS and/or polypectomy ($n = 59$). (D) Predictive effect of use of peroral corticosteroids at the time of sampling ($n = 78$). (E) Predictive effect of a patient history of AR ($n = 80$). (F) Predictive effect of AERD ($n = 84$). p -values by log rank test. AR: allergic rhinitis; AERD: aspirin exacerbated respiratory disease.

Discussion

This study was implemented to evaluate differences in CIP2A expression in nasal biopsies of patients with CRSwNP and ACP. We found that CIP2A was expressed in the cytoplasm of epithelial cells and in glandular cells of the nasal mucosa and in nasal polyp tissue. According to the literature, CIP2A is rarely present in non-transformed/non-malignant cells [13]. However, to this date CIP2A has already been detected in tongue hyperplasia, testis, prostate, cerebellum, brain, and bone marrow [7,11].

The most significant finding was that no or only weak CIP2A expression might associate with CRSwNP + AERD. Yet, we were not fully able to evaluate the putative confounding effect of peroral corticosteroid treatment to the result. CIP2A inhibits the suppressor of c-Myc, thus stabilizing c-Myc, which promotes cell growth and proliferation. Whether the c-Myc pathway is affected in CRSwNP + AERD and whether low CIP2A could have immunomodulatory and polyp growth reducing functions requires further studies.

It has been suggested that a deficit in prostaglandin E2 (PGE2) might be responsible for the chronic inflammation in AERD [14]. Interestingly in malignancies, c-Myc has been studied and found to be an early target of COX-2 inhibitors, as in leukaemia [15]. The inhibition of the COX-2/PGE2 axis

has been found to have anti-proliferative effects in Kaposi's sarcoma-associated herpes virus and related B-cell malignancies [16]. Moreover, Ioannidis et al. [17] detected with quantitative PCR a trend that Epstein-Barr virus was higher and Human Herpes Virus-6 (HHV-6) lower in CRSwNP specimens compared to controls. The group did not perform analyses in the sub-group with CRSwNP + AERD. Although it has not been confirmed yet whether chronic viral infection is possibly a reason, consequence, or not associated at all with CRSwNP, it could be speculated that in CRSwNP + AERD, herpes viruses could putatively be able to chronically induce c-Myc. This could lead to reduction of PGE2 and CIP2A as a negative feedback loop. Further studies, however, are mandatory to prove this.

Only few previous studies have addressed CIP2A expression in benign tissues [11]. We detected CIP2A expression in control inferior turbinate, which is in line with our previous observation that CIP2A is expressed in tongue hyperplasia [11]. It could be possible that CIP2A has a different, yet unknown, functional role in benign tissues compared to cancer. PP2A, the target of CIP2A, has been found to be a master controller of inflammatory signalling pathways, and has recently emerged as a target in asthma [18]. More studies would be needed on CIP2A, PP2A, and c-myc in terms of controllers of inflammation in CRSwNP.

In our study, high tissue eosinophilia associated with both the CRSwNP and CRSwNP + AERD groups compared to controls, which is in line with previous studies [1]. Our survival analyses showed that neither CIP2A nor eosinophilia associated with recurrent CRS surgery. Van Zele et al. [19] showed that total IgE, specific IgE to *Staphylococcus aureus* enterotoxin, eosinophilic cationic protein, and IL-5 were significantly increased and IFN-gamma decreased in the group with CRSwNP compared to the group with non-recurrent CRSwNP. The study group also showed that the CRSwNP + AERD phenotype associated with recurrent CRSwNP, which is in part in line with previous observations and our observation of a trend that AERD associates with recurrent surgery [20]. Our findings that previous CRS surgery, allergic rhinitis, need for peroral corticosteroids, associated with recurrent CRS surgery might reflect to a phenotype of uncontrolled and progressive CRSwNP. Still, more studies with increased sample sizes are mandatory to prove this.

We acknowledge that the number of patients was too small to fully evaluate the effect of atopy, corticosteroid treatment, and other confounding factors on the results. This study set-up is not able to demonstrate a causal association between low CIP2A expression and polyp formation. Other limitations were that there were no corresponding samples available between polyp tissue, middle turbinate, and sinus cavity in the same patient, and that control nasal cavity samples were from inferior instead of middle turbinate.

Conclusion

The study suggests that low CIP2A expression and high eosinophilia associate with CRSwNP + AERD. Further studies are needed to show whether low expression of CIP2A is connected to low PGE2 levels, as both seem to associate with AERD and can affect c-Myc levels.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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