

Role of the TAp63 Isoform in Recurrent Nasal Polyps

(nasal polyps / TAp6 / chronic rhinosinusitis)

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Abstract. The pathogenic molecular mechanisms underlying the insurgence of nasal polyps has not been completely defined. In some patients, these lesions can have a recurrence after surgery removal, and the difference between recurrent and not recurrent patients is still unclear. To molecularly characterize and distinguish between these two classes, a cohort of patients affected by nasal polyposis was analysed. In all patients we analysed the p63 isoform expression using fresh tissues taken after surgery. Moreover, confocal immunofluorescence analysis of fixed sections was performed. The results show high $\Delta Np63$ expression in samples from the nasal polyps of patients compared to the normal epithelia. Analysis of the expression level of the TAp63 isoform shows dif-

ferential expression between the patients with recurrence compared to those not recurring. The data, considered as the $\Delta N/TAp63$ ratio, really discriminate the two groups. In fact, even though $\Delta Np63$ is expressed in non-recurrent patients, the resulting ratio $\Delta N/TAp63$ is significantly lower in these patients. This clearly indicates that the status of TAp63 expression, represented by the $\Delta N/TAp63$ ratio, could be considered a prognostic marker of low recurrence probability. In these samples we also investigated the expression of OTX2 transcription factor, known to be a selective activator of TAp63, detecting a significant correlation. Database analysis of HNSCC patients showed increased survival for the patients presenting OTX2 amplification and/or overexpression. These results, together with the fact that TAp63 can be selectively upregulated by HDAC inhibitors, open the possibility to consider local treatment of recurrent nasal polyps with these molecules.

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Abbreviations: AERD – aspirin-exacerbated respiratory disease, COX-1 – cyclooxygenase-1, CRS – chronic rhinosinusitis, CRSs-NP – chronic rhinosinusitis without NPs, CRSwNP – chronic rhinosinusitis with NPs, CT – computed tomography, ECM – extracellular matrix, EPOS – European position paper on rhinosinusitis and nasal polyps, HA – hyaluronic acid, HDAC – histone deacetylase, HNSCC – head and neck squamous cell carcinomas, LTC4 – leukotriene C4 synthase, NPs – nasal polyps, RTqPCR – real-time quantitative PCR; Th2 – T-helper 2, TP63 – tumour protein p63.

Introduction

Chronic rhinosinusitis (CRS) is a widespread clinical condition that affects approximately 5–15 % of the European and American population. A phenotype classification, based on endoscopic examinations of the nasal cavity or imaging procedures, differentiates CRS into chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP) (Koennecke, et al., 2018).

Nasal polyps (NPs) are inflammatory outgrowths of sinonasal tissue, which affect approximately 1–4 % of the general population, more likely male patients, and the typical age of diagnosis goes from 40 to 60 years. Usually, nasal polyps present as bilateral inflammatory lesions originating in the ethmoid sinuses and projecting into the nasal airway beneath the middle turbinate (Stevens et al., 2015).

In addition to obstruction/congestion or nasal discharge (anterior/posterior rhinorrhoea), reduction or loss of smell and facial pain/pressure, CRSwNP patients often comorbid other important medical conditions that can influence the disease severity, such as atopy, asthma, aspirin-exacerbated respiratory disease (AERD), Wegener's granulomatosis, and pathologies characterized by excessive inspissated mucus (cystic fibrosis) and mucociliary-transport alterations (e.g., Kartagener syndrome). The percent of allergic rhinitis patients with nasal polyps is similar to that of the general population (0.5–4.5 %) (Fokkens et al., 2012b). On the other hand, 51–86 % of CRSwNP patients are sensitized to at least one aeroallergen.

Sharing similar features of inflammation and remodelling, asthma and CRSwNP frequently coexist: CRSwNP is estimated to occur in 7 % of all asthmatics, while asthma is reported in 26–48 % of patients with CRSwNP (Langdon and Mullol, 2016). AERD (originally Samter's triad: nasal polyps, asthma and aspirin sensitivity) is defined as a clinical condition characterized by development of upper and/or lower respiratory tract symptoms following ingestion of medications that inhibit the cyclooxygenase-1 (COX-1) enzyme. Among 10–30 % of patients with CRSwNP present AERD; in those cases, nasal polyps are multiple, characterized by rapid growth and in the absence of medical management, a high recurrence after surgery (Steinke and Borish, 2015).

To date, the underlying mechanisms that contribute to the chronic inflammation of the sinonasal mucosa have not been completely defined, but the interactions between epithelial cells, the host immune system and pathogens certainly play an important role in CRSwNP pathogenesis (Koennecke et al., 2018).

A recent classification of chronic rhinosinusitis with nasal polyposis has been described by Dennis et al. (2016) (Table 1). Based on the endotype approach, there

are four subtypes: type-2 cytokine, characterized by a high presence of eosinophils, mast cells, basophils, and T-helper 2 (Th2) cells with a high level of IL-5, typically in nasal polyposis (Tomassen et al., 2016); eosinophil-based, with a predominant presence of eosinophils; IgE-based, with elevated levels of IgE (except AERD) and cysteinyl-based, characterized by a high level of cysteinyl leukotriene (CysLT), especially in AERD patients (Dennis et al., 2016). Because CysLT is metabolized and excreted through the urine, the 24-h urinary measurement of leukotriene C4 synthase (LTC4) has been suggested as a means to identify CRSwNP patients who have the AERD variant (Divekar et al., 2016). To date, no large-scale studies have been performed to identify genetic linkages and polymorphisms related to CRS pathogenesis (Schleimer, 2017).

The airway epithelium of the human nasal mucosa represents a barrier that protects against inhaled substances and pathogens via tight junctions; defective epithelial barrier with decreased expression of TJ proteins is found in patients with chronic rhinosinusitis (CRS) and nasal polyps (NPs) (Soyka et al., 2012; Kojima et al., 2013). Tumour protein p63 (TP63, p63) is one of the regulators of various cell-matrix and cell-cell adhesion complexes in the epidermis (Arason et al., 2014). Loss of Δ Np63 isoform significantly reduces epithelial proliferation and increases E-cadherin expression in human airway epithelial cells (Li et al., 2011). The human *p63* gene expresses at least three alternatively spliced C-terminal isoforms (α , β , γ) and is transcribed from two different promoters (Yang, et al., 1998), giving rise to two proteins that either contain (TAp63) or do not contain (Δ Np63) the N-terminal TA domain (Yang et al., 1998; Melino et al., 2003). The Δ Np63 isoform can exert dominant-negative effects over p53, p73 by either competing for DNA-binding sites or by direct protein interaction. Δ Np63 isoforms were also shown to directly

Table 1. Chronic rhinosinusitis with nasal polyposis (CRSwNP) endotype classification

Endotype approach	CRSwNP subtypes	Cellular markers	Molecular markers	Targeted treatment
Type 2 cytokine-based	CRSwNP associated with asthma and atopy	Eosinophils Mast cells Basophils T-helper 2	IL-5 IL-4 IL-13	Anti-IL5 (reslizumab, mepolizumab) Anti-IL4/13 (dupilumab)
Eosinophil-based	CRSwNP in AFRS (Allergic Fungal RhinoSinusitis) AERD (Aspirin-Exacerbated Respiratory Disease) EMCRS (not-otherwise-categorized eosinophilic rhinosinusitis)	Eosinophils	AFRS: fungi, IL-4 AERD: IL-4 EMCRS: IL-5, IL-13	Anti-IL5 Ligands for Siglec-8
Immunoglobulin IgE-based	All CRSwNPs except AERD	Lymphocytes B	Ig-E	Anti-Ig E (omalizumab) Anti-GATA 3
Cysteinyl-based	AERD (polyps + asthma + intolerance to aspirin and other inhibiting COX1)	Eosinophils Mast cells	CysLT	Leukotriene receptor antagonists (montelukast) 5-lipoxigenase inhibitor (zileuton) Platelet-targeted

activate specific gene targets not induced by TA isoforms (Dohn et al., 2001; Wu, et al., 2003). Δ Np63 is the predominant isoform crucial for the maintenance of epithelial cell regenerative potential (Mills et al., 1999; Yang et al., 1999; Candi et al., 2006a,b; Carroll et al., 2006).

An increase in p63-positive cells is observed in the epithelium of NPs, and the expression of p63 in multiple cell layers is an important pathologic phenomenon in the epithelial remodelling seen in NPs (Hackett et al., 2009; Warner et al., 2013). Inhibitors of NF- κ B, HDACs and p38 MPAK, and RSV infection prevented p63 expression and induced TJ proteins, p63-negative regulation of the epithelial barrier and ciliogenesis of the nasal epithelium (Kaneko et al., 2017).

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from the lysine residues of target proteins, thereby promoting chromatin condensation and reduced transcription. Inhibition of HDAC activity reconstitutes a defective barrier by increasing TJ protein expression (Wawrzyniak et al., 2017). Δ Np63, HDAC1 and HDAC6 seem to be upregulated and CLDN-1 and -4 downregulated in the epithelium of sinusitis and probably in NPs. Knockdown of Δ Np63 induced expression of CLDN-1 and -4, enhancing the barrier and fence functions, and increased the number of microvilli on the cell surface.

Unlike p53, p63 is rarely mutated in human cancers, but it is involved in these pathological conditions, controlling cell cycle arrest and apoptosis (Melino et al., 2003; Wu et al., 2003). The balance between TAp63 and Δ Np63 isoforms appears to dictate the different cellular endpoints, survival and transformation versus cell death, although their precise roles in tumorigenesis remain unclear. The majority of tumours maintain Δ Np63 expression, and in many cases it appears to be over-expressed or its locus is amplified, consistent with a potential p63 pro-proliferative or oncogenic role (Mills, 2006; Candi et al., 2007). p63 is a target of genomic amplification and/or over-expression in > 80 % of primary head and neck squamous cell carcinomas (HNSCC) as well as other squamous epithelial malignancies (Hibi et al., 2000; Massion et al., 2003; Sniezek et al., 2004; DeYoung, et al., 2006). Recent reports show that Δ Np63 α expression directly correlates with a poor clinical response to cisplatin in patients with head and neck tumours (Zangen et al., 2005). TAp63 has been found to play a critical role in the development and function of the heart (Rouleau et al., 2011) and oocytes (Suh et al., 2006; Gonfloni, et al., 2009), and in the differentiation of cochlear neuroepithelium via regulation of the Notch pathway (Terrinoni et al., 2013). Δ Np63 proteins are involved in the early stages of skin development and are rapidly degraded upon induction of keratinocyte differentiation (Romano et al., 2012). The hypothesis is that TAp63 and Δ Np63 isoforms work in competition, and the Notch signalling pathway is very important in epidermal stratification and keratinocyte differentiation (Nickoloff et al., 2002).

In a recent study it has been demonstrated that one of the transcriptional activators of TAp63 is OTX2. OTX2

is able to transactivate TAp63 via a responsive element located in intron 1 of the gene, whereas there is no transcriptional regulation of Δ Np63. These results can identify a regulatory cascade going from OTX2 to TAp63, arriving to the Notch pathway (Palombo et al., 2015).

OTX proteins are an important class of homeodomain-containing transcription factors with a major role in embryonic morphogenesis. Analysing the role of the OTX gene in cancerogenesis, OTX1 has been demonstrated to be involved in breast cancer physiology, being able to interact with wild-type p53, suggesting that p53 and OTX1 over-expression represent an attempt to force the neoplastic cells to differentiate (Terrinoni et al., 2011). Importantly, the presence of OTX1 and OTX2 proteins has been demonstrated in normal sinonasal mucosa and in different epithelial and neuroectodermal nasal neoplasms of adult subjects (Pirrone et al., 2017). There is significant modulation in the expression of OTX1 and/or OTX2 in neoplastic tissue compared with normal tissue, suggesting that the activation/inactivation of OTX factors is a significant event in the response to sinonasal neoplasm development (Pirrone et al., 2017).

NP recurrence after surgery is well known and documented. However, prevention and prediction of recurrence is still a subject of research and debate; in fact, a potential relationship between the clinical, radiological, immunological, molecular factors and polyp recurrence remains undetermined (Bruno et al., 2002, 2004; Young et al., 2007). Patients presenting with extensive disease, suggested by CT scan staging, seem to have a higher risk for the development of recurrences after endonasal surgery for nasal polyps (Akhtar et al., 2010). Various studies show that patients with both CRSwNP and AERD are at risk for the development of recurrences after endonasal surgery for nasal polyposis (Albu, et al., 2004; Akhtar et al., 2010).

Such experimental findings may confirm the hypothesis that NPs share biological pathways with neoplastic forms. In fact, the clinical behaviour of NP regarding recurrence after surgery is various, being represented by a low, medium or high risk rate of incidence.

The aim of this work was to increase the knowledge of the underlying mechanism or markers useful to characterize patients with different clinical outcomes. We evaluated, according to the items expressed before, expression of the two isoforms of transcription factor p63 in cases of recurrence and non-recurrence.

Material and Methods

All patients have been treated according to the European position paper on rhinosinusitis and nasal polyps (EPOS 2012, Fokkens et al., 2012a) and underwent preliminary study with clinical and radiological investigation before surgery, including nasal endoscopy and axial and coronal computed tomography (CT) scanning. All patients underwent surgical treatment by FESS under general anaesthesia after obtaining informed con-

sent. Standard surgical steps were applied in each case according to the extent of the disease.

Immunofluorescence analysis

A previously described protocol (Palombo et al., 2015, 2016; Terrinoni et al., 2018) was used. The following primary antibodies were used: mouse polyclonal anti-p63 (Abcam Ab735, Cambridge, UK) and rabbit polyclonal anti-K5 (Covance PRB-160P, Princeton, NJ). The following secondary antibodies were used: Alexa fluor[®]488 goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) and Alexa fluor[®]568 goat anti-mouse IgG (H+L) (Invitrogen). DAPI was used for visualization of nuclei. Primary and secondary antibodies were prepared in blocking buffer. Sections were covered by Prolong Antifade reagent (Invitrogen) and observed using an A1 Nikon confocal laser microscope system and software NIS Element AR4.00.04 (Nikon, Tokyo, Japan).

RTqPCR

Samples of resected patient polyps were mechanically homogenized in RLT buffer of RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was isolated following the manufacturer's protocol of this kit. Five hundred ng of total RNA was used for reverse transcription using the GoScript[™] Reverse Transcription System (Promega, Madison, WI). The expression levels of TAp63, ΔNp63, keratin 5 and OTX2 were determined using the real-time quantitative PCR technique (RTqPCR) that allows selective amplification and visualization of PCR products in real time. The GoTaq[®] qPCR Master Mix (Promega) was used with specific primers to amplify each gene: *TAp63* forward 5'-GGA CTGTATCCGCATGCAG-3', reverse 5'-GAGCTGGG CTGTGCGTAG-3', *ΔNp63* forward 5'-GAAGAAAGG ACAGCAGCATTGAT-3', reverse 5'-GGGACTGGTG GACGAGGAG-3', keratin 5 forward 5'-TGGACCTG GATAGCATCATC-3', reverse 5'-CATTGTCAATCT CGGCTCTC-3', and *OTX2* forward 5'-TCGAAGAG CTAAGTGCCGCC-3', reverse 5'-GGCAATGGTTCGG GACTGAGG-3'. The analysis was performed using the Sybr-Green reagent, the results were normalized using actin as endogenous control (forward 5'-CTGGCA CCACACCTTCTACAATG-3', reverse 5'-GTTGCTAT CCAGGCTGTGCTA-3'). The amplification was performed by a 7500 Real Time PCR System (Applied Biosystems, Thermo Scientific, Waltham, MA) and data analysed with 7500 Software v2.3.

cBioPortal survival analysis

The Comprehensive TCGA PanCanAtlas (<https://gdc.cancer.gov/about-data/publications/pancanatlas>) data from 11k cases and all TCGA tumour types (PMID: 29625048, 29596782, 29622463, 29617662, 29625055, 29625050) was analysed using OTX2 as search key. We selected the Putative copy-number alterations from GISTIC and mRNA Expression Z-scores, RSEM (Batch normalized from Illumina HiSeq_RNASeqV2), with a Z-score threshold ≥ 2 .

Results

Patients

Our study project analysed a group of 28 patients affected by chronic rhinosinusitis (Fig. 1) and referred to our Otolaryngology Unit from 2014 to 2016. All these patients underwent surgical treatment by functional endoscopic sinus surgery (FESS). The clinical diagnosis was confirmed by the definitive histopathological examination and in 10 cases it was nasal polypoid rhinosinusitis, while in five cases it was an anthro-choanal polyp and in the remaining 13 a simple hyperplastic rhinosinusitis. Therefore, the group of 15 patients with polyposis was subsequently subjected to 6-monthly clinical checks in the two years following surgery. From the follow up it emerged that five cases of nasal polyposis were relapse.

Analysing this group of patients affected by nasal polypoid rhinosinusitis, further distinguishing features emerged between the two groups of recurrent and non-recurrent cases. In particular, the group of patients suffering from recurrent polyposis had a mean age of 63 years, they were non-smokers, not allergic. The group of non-recurrent patients was characterized by an average age of 67 years, non-allergic. In both groups, the patients were not affected by relevant comorbidities.

Based on the knowledge of transcription factor p63 and its role in the development of epithelial cells, we expanded the study by also analysing the OTX2 factor, which, although currently demonstrated in the context of cochlear and macular development, has a fundamental role in the regulation of p63 and in particular of the TAp63 isoform (Terrinoni et al., 2013; Palombo et al., 2015), and has recently been found to be expressed in nasal polyps (Pirrone et al., 2017).

Molecular analysis

To analyse expression of both isoforms of p63 in the polyps of our patients, we first used a molecular approach consisting in extracting RNA from part of fresh polyps biopsies, followed by RTqPCR. Using this method we selectively amplified TAp63, ΔNp63 and keratin 5. The results were analysed as fold over control with respect to the normal nasal mucosa.

In our study samples, the patients with nasal polyposis recurrence were characterized by higher expression levels of ΔNp63, compared to those that had not relapsed (Fig. 2A). Furthermore, in these cases, a high level of expression of epithelial basal cytokeratin K5 was detected. The expression levels of the latter are concordant with the ΔNp63 expression observed (Fig. 2B). In contrast, in the group without recurrence of polyposis history, the levels of ΔNp63 remained lower, similar to that observed in the turbinate epithelium used as normal control (Fig. 1A). The indicator of basal epithelium, cytokeratin K5, also remained lower expressed (Fig. 1B). Since the ΔNp63 action seems to be counteracted by the TA isoform, we also analysed its expression by RTqPCR.

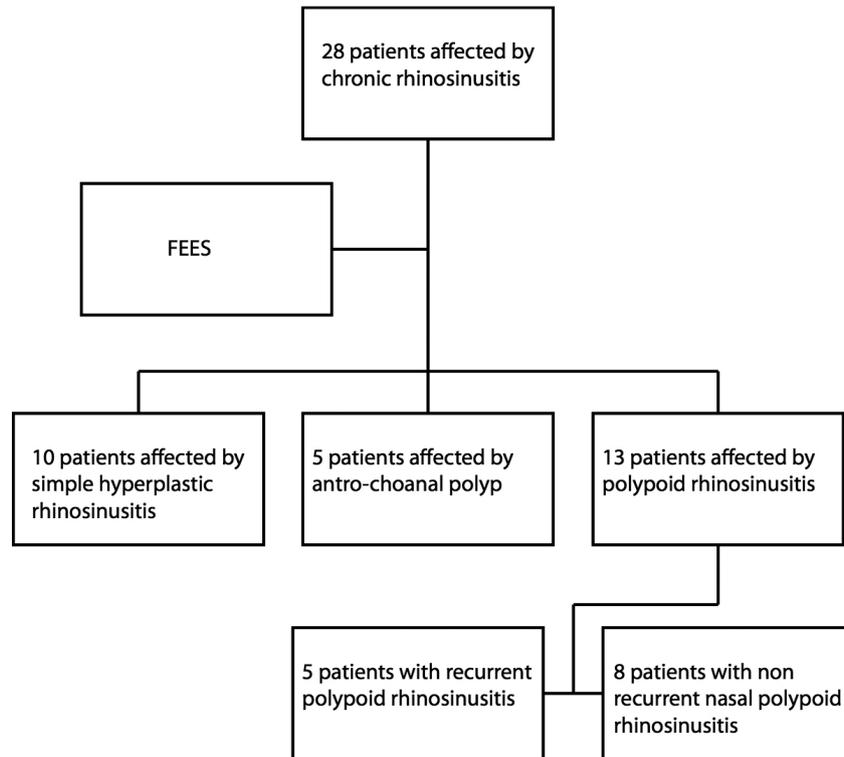


Fig. 1. Schematic layout of the study

The results showed variable TAp63 expression in both groups, but analysing the $\Delta N/TA$ ratio, again the patients were distinguished into two classes of relapsing and not-relapsing (Fig. 2C). Indeed, this ratio was higher in the groups of relapsing patients, confirming that the balance between these two transcription factors characterize the nature of the polyps. The box plot organization of the data clarifies well the differences between the two classes of patients (Fig. 2B, D, F). Moreover, according to the recently published data related to the possible induction of TAp63 expression by OTX2, we also tested the expression of the latter. Again, the data interpretation, due to the expression variability and the small number of patients analysed, are difficult to be graphically represented, but looking to the raw data (Fig. 2G), the indication is that OTX2 is concordant with TAp63 expression. These results confirm the previous proposed pathway (Palombo et al., 2015), in which the expression of OTX2 is able to transactivate TAp63, probably to counteract the ΔN isoform.

Histological analysis

To analyse the expression of p63 in samples from patients diagnosed with polyposis, we performed immune fluorescence confocal analysis of some surgical samples, using antibodies against p63 and keratin 5. In samples from patients with relapsed events, the primary polyp showed strong $\Delta Np63$ staining and numerous positive cells were disposed in multiple layers (Fig. 3, panels A, B, asterisks in D, E). Analysis of polyps from patients without relapse showed a characteristic one-layer dispo-

sition of p63 (Fig. 3, panels C, F), indicating the presence of active regenerating cells only in the basal compartment. This situation was confirmed by staining with K5 antibody, showing the presence of this keratin again in multiple cell layers (Fig. 3, panels G, H and L, M), where the distribution in the patients with no relapse was more concentrated in the basal layer (Fig. 3, panels I, N), similarly as in the normal epithelial compartment. The distribution of K5 clearly resembled that of p63, according to the well-known mechanism of K5 expression induction by the p63 ΔN isoform.

Database analysis

In the analysis of the OTX2 \rightarrow TAp63 axis, we tried to investigate the modification of the expression of this gene in public databases of HNSCC, containing available data characterizing the genomic and transcriptional status with follow up longer than the 24 months analysed in our patients. The data retrieved in "cBioPortal" (Cerami et al., 2012; Gao et al., 2013), analysing a HNSCC database (details in the Methods section), showed that there were a few patients, around 4 %, in whom amplification and/or OTX2 RNA over-expression (Fig. 4A) was detected. These patients seemed to have a longer survival time (Fig. 4B, upper and lower panel), also spanning a longer disease-free period (Fig. 4C, upper and lower panel). The total number of patients and the fact that the analysis was stopped at 140 months, do not permit to have a definitive statistic data, even if there is an indication of a major overall survival.

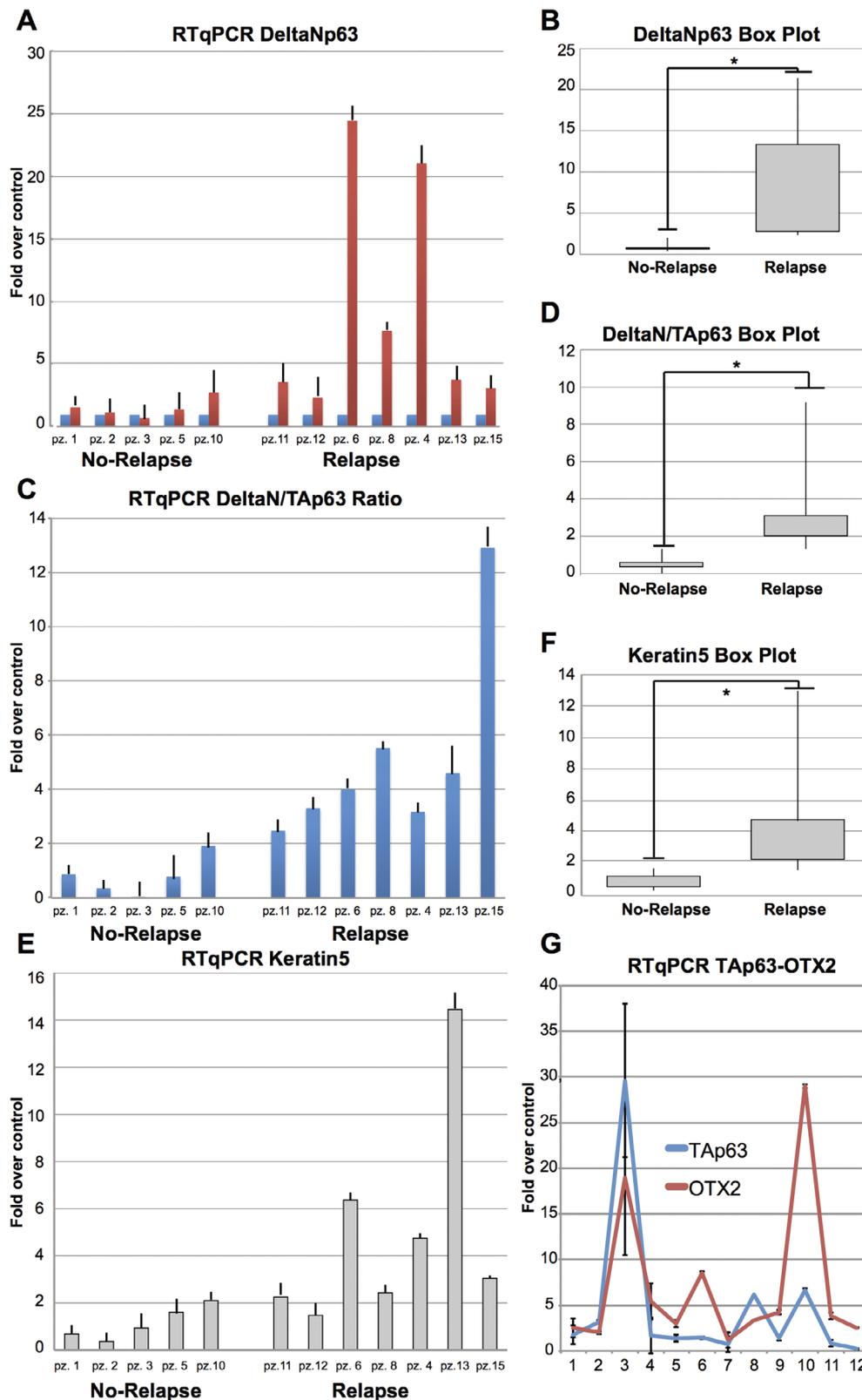


Fig. 2. Expression analysis

A. RTqPCR analysis showing the expression level of Δ Np63 evaluated as fold over the control compared to the normal sinonasal epithelium. **B.** Box-plot representation of the data. Data were clustered into two classes, relapsing and not relapsing. The differences between the two classes are significantly different (*indicates *t*-test with $P = 0.033$). **C.** Δ Np63/TAp63 ratio using RTqPCR expression data. **D.** Box plot showing that the ratio between the two p63 isoforms is different in patients presenting recurrence compared to those that do not (*indicates *t*-test with $P = 0.013$). **E, F.** The same analysis for keratin 5 expression, showing a behaviour similar to Δ Np63 (*indicates *t*-test with $P = 0.032$). **G.** Expression levels, in fold over control, of OTX2 and p63, showing a similar tendency in the patients.

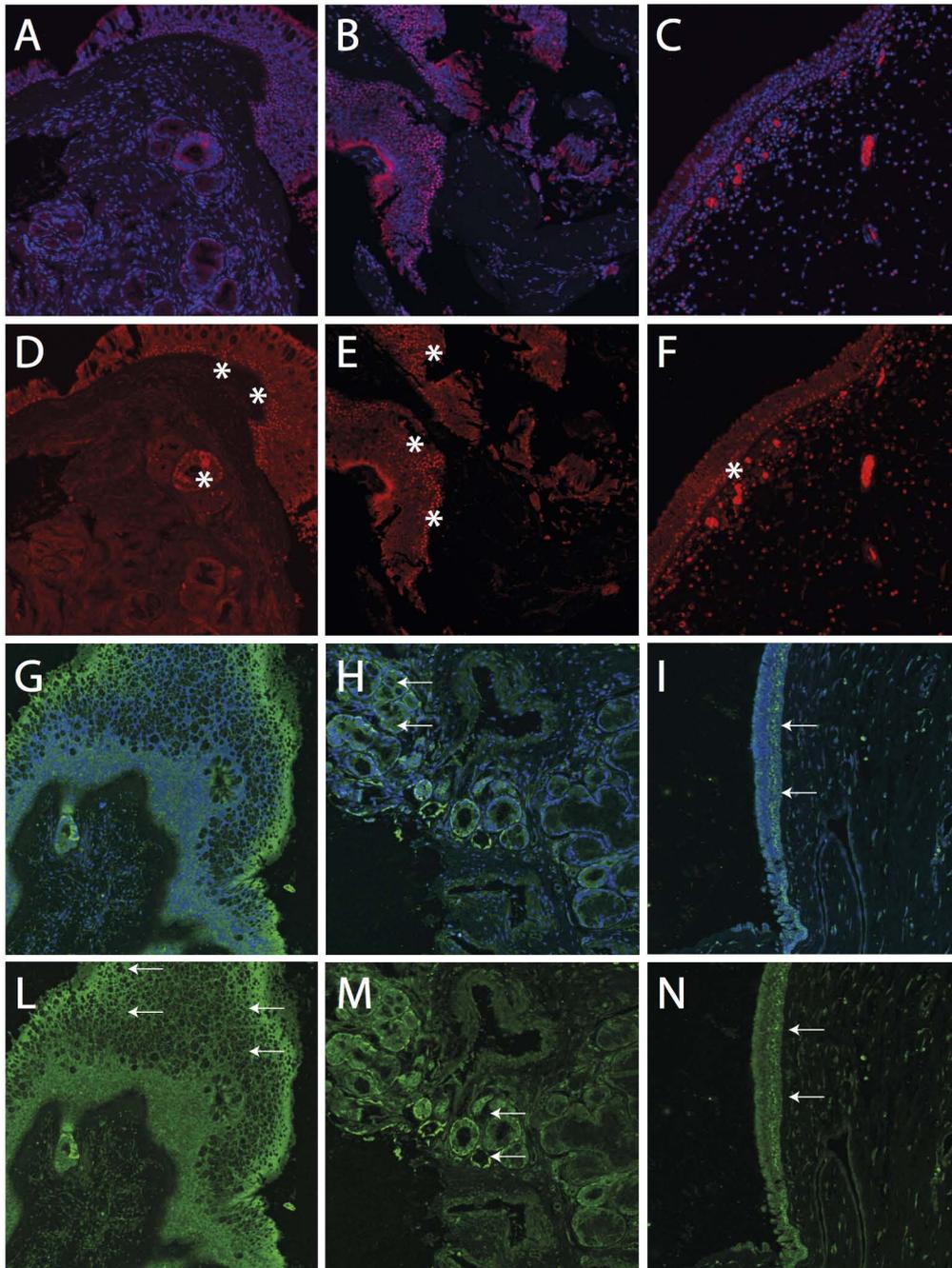


Fig. 3. Histological immunofluorescence confocal analysis of polyps

A, B. Examples of polyps from relapsing patients. **C.** Example of a polyp from a not relapsing patient (blue is DAPI and red is p63 staining). **D, E.** Staining with anti-p63 antibody shows the presence of a multilayer of positive cells. The epithelium seems thicker compared to that visible in **F**, from a non-relapsing patient, where a lower number of positive cells, mainly in the basal compartment, is visible. **G, H.** Keratin 5 staining (green) from two selected relapsing patients (blue is DAPI). **I.** Keratin 5 staining (green) from a selected non-relapsing patient (blue is DAPI). **J-L.** Keratin 5, normally present in the basal layer only, shows an evident multilayer distribution, indicating the presence of proliferating cells, whereas in **M**, the expression is limited to a more basal distribution.

Discussion

As it is known, the majority of tumours of epithelial derivation are characterized by high Δ Np63 expression, indicating a potential p63 pro-proliferative or oncogenic role (Mills, 2006; Candi et al., 2007). In squamous cell

carcinoma, Δ Np63 was the predominant isoform expressed at the protein level (Ye et al., 2014; Moses et al., 2019). Recent data also indicate Δ Np63 as capable of sustaining production of hyaluronic acid (HA), the major component of the extracellular matrix (ECM) in basal-like breast carcinoma (TNBC), favouring a HA-rich

A

Profiled in mRNA Expression z-Scores
(RNA Seq V2 RSEM)

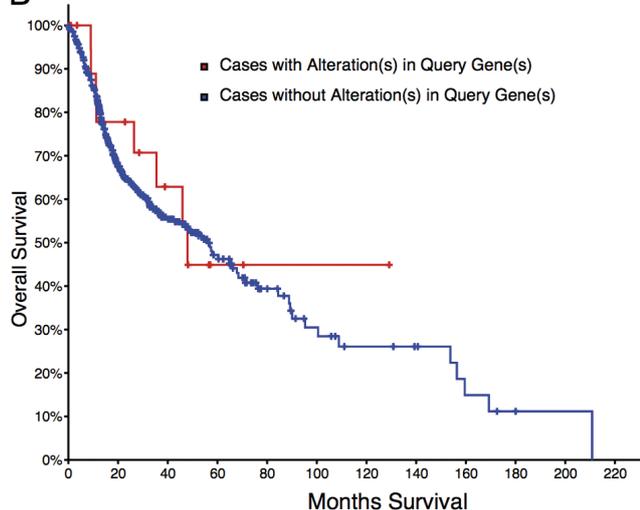
OTX2

Genetic Alteration ■ Amplification ■ mRNA Upregulation ■ No Alterations

Profiled in mRNA Expression z-Scores (RNA Seq V2 RSEM) ■ Yes ■ No

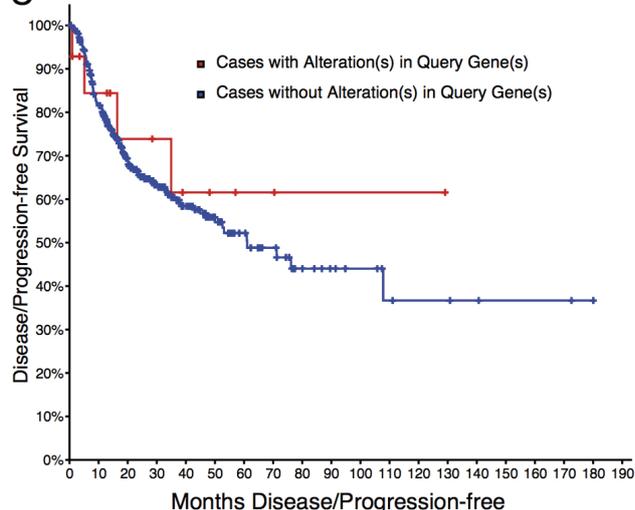


B



	Number of Cases, Total	Number of Cases, Deceased
Cases with Alteration in Query Gene	21	8
Cases without Alteration in Query Gene	481	207

C



	Number of Cases, Total	Relapsed/Progressed
Cases with Alteration in Query Gene	15	4
Cases without Alteration in Query Gene	364	137

Fig. 4. Database analysis

A. The analysis made using data from cBioPortal shows the presence of OTX2 amplified and/or over-expressed in 4 % of patients. B, C. These patients show an improved survival time and an increased progression-free disease period.

microenvironment, which can sustain epithelial cell proliferation (Compagnone et al., 2017). An increase in p63-positive cells has also been previously reported in the epithelium of rhinosinusitis patients (Hackett et al., 2009; Warner et al., 2013). The expression of p63 in multiple cell layers was found in our recurring NP patients, representing an important possible pathologic signature.

In fact, in our experiments, patients with recurrence showed high ΔN expression and high ΔN /TAp63 ratio. This clearly indicates that the TAp63 expression level is important in modifying the absolute ΔN p63 expression. The ratio between the two transcription factors can be considered a possible prognostic marker of high recurrence probability, even if a more extensive study should be performed to define its critical level. Indeed, the status of TAp63 expression is important, since it directly influences the balance and can well reflect the status of

polyp proliferation in the patients. It is conceivable that OTX2 expression in nasal polyps (Pirrone et al., 2017), specifically driving TAp63 expression (Palombo et al., 2015), may represent a response mechanism to the cell proliferation.

The hypothesis underlying these findings is that the role of OTX2 is to transactivate TAp63 and therefore to promote ΔN p63 inhibition and cell differentiation, thus reducing their proliferation potential. This should bring about some recovery of the disease. In patients in whom this pathway is low or not active, the ratio between the two isoforms of p63 (ΔN p63 over TAp63) is too high, this control is missing, and the patients experience recurrence. The analysis of the Head and Neck Squamous Cell Carcinoma database in cBioPortal (Cerami et al., 2012; Gao et al., 2013) gives interesting information on the possible protective role of OTX2 expression in HNSCC, which even if requiring more solid statistic

data leads to considering the OTX2->TAp63 axis as the possible modifier in non-relapsing nasal polyps.

Regarding the treatment of NPs, according to European and US guidelines, intranasal corticosteroids are recommended as initial medical treatment (Stevens et al., 2016). These topical drugs can improve the patients' quality of life by reducing sinonasal symptoms and decreasing the polyp size and can be managed better than oral corticosteroids (Lund et al., 1998; Rudmik et al., 2012), but are not curative.

The sinus surgery approach (usually FESS) should be considered for patients with severe sinonasal disease and those who failed medical treatments. However, as shown, nasal polyps can reoccur after surgery, especially in patients having both CRSwNP and asthma (Bhattacharyya, 2007; Young et al., 2007). In recent years, several biologics (such as omalizumab, mepolizumab and dupilumab) underwent clinical trials evaluating their safety and efficacy in CRSwNP. Although recent studies are promising, these drugs have not yet been approved for the treatment of nasal polyps (Gevaert, et al., 2011, 2013; Bachert et al., 2016).

The potential role of the OTX2/TAp63 axis in the differentiation between relapsing or not relapsing NP patients opens the possibility to use new drugs. HDAC inhibitors, largely used in carcinoma therapy, are able to induce TAp63 expression (Giacobbe et al., 2013; Terrinoni et al., 2013); their therapeutic mechanism is due to the inhibitory effect of this isoform on Δ Np63. Furthermore, as reported, the inhibition of HDAC1 and HDAC6 also induces expression of TJ proteins (Wawrzyniak et al., 2017), ameliorating the inflammation and restoring the normal basal lamina barrier.

This paper is a first step in the analysis, and more work should be done to characterize the pathway, obtaining data from a wider cohort of patients. This is important because if confirmed, the treatment with HDAC inhibitors such as hydroxamic acids and benzamides could also become a possibility in the topical treatment of nasal polyps to ameliorate the disease and counteract its recurrence.

Author contributions

A. T. wrote the paper, conceived and designed the project; R. P. performed RNA extraction and real-time PCR; C. P., S. C. performed immunofluorescence analysis; R. D. B., S. C., A. L., S. M. collected and clinically classified patients; G. M., S. B., M. M. interpreted the data and revised the paper; E. B. wrote the paper, conceived and designed the project.

Conflicts of interest

All authors disclose no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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