

Production and significance of CCAAT enhancer binding proteins alpha and beta in sinonasal inverted papilloma

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Summary. Sinonasal inverted papilloma (SIP) is a rare benign tumor featuring increased cell proliferation, a tendency toward squamous differentiation, recurrence and malignant transformation. The CCAAT enhancer binding proteins, C/EBPs, are transcription factors regulating the proliferation and differentiation of various types of cells, including epithelial cells. We prospectively investigated the production of these transcription factors and the related proliferation and differentiation targets, keratin-10, keratin-15 and cyclin-D1, in 26 SIP patients and 8 sinonasal polyposis cases suspected for SIP. Ten of these patients had one or more recurrences over follow-up periods of one to eight years. C/EBP-alpha and C/EBP-beta proteins were not found in normal-looking sinonasal epithelial cells. The proteins and RNAs were detected in SIP and, occasionally, in polyposis tissues. The production of these factors was not significantly correlated with age, sex, site, tumor size or medical history. By contrast, correlations were found between the levels of C/EBP-alpha and keratin-10 levels and between those of C/EBP-beta and keratin-15. C/EBP-alpha levels were also significantly correlated with cyclin-D1 levels. These data suggested that the C/EBPs are implicated in the regulation of cell proliferation and differentiation in SIP. Finally, recurrent SIP produced significantly larger amounts of C/EBP-alpha than non-recurrent tumors. These results implicate CCAAT enhancer binding proteins in the pathogenesis of SIP and highlight the role of C/EBP-alpha as a candidate marker for tumor recurrence.

Key words: Sinonasal inverted papilloma, CCAAT/Enhancer binding proteins, Keratin-10, Keratin-15, Cyclin-D1

Introduction

Sinonasal inverted papilloma (SIP, WHO code 8121/1) is a rare tumor, accounting for 0.4% to 4.7% of all nasal neoplasms (Nwaorgu and Onakoya, 2002). Its etiology remains unclear, but occupational exposure, allergy, chronic inflammation and viral infection, with human papilloma viruses 16 and 18 in particular, have been identified as risk factors (Katori et al., 2006). The tumor is derived from the Schneiderian membrane in which the epithelium invaginates and proliferates into the underlying stroma. The tumor is often unilateral and locally aggressive, causing considerable destruction of the surrounding sinonasal bones. Up to 70% of cases present recurrences after surgical intervention (Barnes et al., 2005). Squamous cell carcinoma is associated with SIP in 10% of cases, although the frequency of metachronous carcinomas in SIP patients is often <4% (Von Buchwald and Bradley, 2007).

SIP is considered a true epithelial neoplasm, with patchy squamous metaplasia and a hyperplastic columnar epithelium. Keratin (K) analysis reveals the immunoexpression of respiratory epithelium markers, such as K7, K8 and K18, and aberrant production of the stratified squamous epithelium keratins, K13, K17 and rare K10 positive cells (Schwerer et al., 2001). Low levels of K14 are associated with a risk of recurrence in patients with these tumors (Gunia et al., 2008). These results are consistent with the morphological findings of squamous metaplasia in SIP, where the normal respiratory epithelium is replaced with stratified columnar/squamous epithelium. Focal surface keratinization may be observed in 10 to 20% of cases and 5 to 10% may present dysplastic changes (Barnes et al., 2005). Previous studies have reported an increase in the rate of cell proliferation in SIP, correlated with epithelial dysplasia and a risk of malignant transformation (Hoffmann et al., 2006). However, the mechanisms regulating tumor cell proliferation and

differentiation in SIP remain largely unknown (Altavilla et al., 2009).

The CCAAT enhancer binding proteins (C/EBPs) are transcription factors with a characteristic basic leucine zipper region necessary for dimerization and binding to target DNA. Six members of this family (alpha, beta, gamma, delta, epsilon and zeta), with similar sequences and functions have been identified. In the normal epidermis, C/EBP-alpha and C/EBP-beta transactivate epidermal differentiation genes, including K1 and K10 (Lopez et al., 2009) and the epidermal stratification marker K15 gene (Radoja et al., 2004). Mice with an epidermis-specific C/EBP-alpha knockout survive, with no detectable abnormalities in epidermal differentiation (Shim et al., 2005). However, inactivation of C/EBP-alpha confers susceptibility to UVB-induced skin squamous cell carcinomas (Thompson et al., 2011).

These factors also regulate epithelial proliferation in normal and tumor tissues. C/EBP-alpha is thought to negatively regulate the cell cycle by binding to CDK4 and CDK2 and preventing complex formation with cyclin-D and cyclin-E, respectively. C/EBP-alpha can also inhibit E2F transcription activity and stimulate the activity of the CDK inhibitor p21 (Lopez et al., 2009). The forced expression of C/EBP-alpha in normal and tumor keratinocytes therefore leads to cell cycle arrest. C/EBP-alpha knockout animals are highly sensitive to chemical carcinogenesis, suggesting that this factor may have a tumor suppressor function in keratinocytes (Loomis et al., 2007). By contrast, C/EBP-beta may stimulate keratinocyte proliferation and this protein is overproduced in several malignant tumors, including breast cancer (Zahnow, 2009). In mice, C/EBP-beta has been identified as a mediator of cell survival and tumorigenesis, through the suppression of P53 and apoptosis (Ewing et al., 2008). Inhibiting the C/EBP family of transcription factors in transgenic mice skin was studied using a conditionally express dominant negative A-C/EBP protein that inhibits the DNA binding of C/EBP family members to their targets. During a two-step carcinogenesis experiment, A-C/EBP expression in the skin inhibited papilloma formation, caused hyperplasia of the basal epidermis and increased apoptosis in the suprabasal epidermis (Oh et al., 2007). In SIP, expression of several C/EBPs target genes and proteins were reported, including P53, cyclin-D1 and p21. The increased expression of these proteins was

associated with epithelial dysplasia and poor prognosis (Altavilla et al., 2009). In contrast, expression of C/EBP-alpha and C/EBP-beta, which also regulates epithelial cell survival, proliferation and differentiation, was not previously investigated.

The production of C/EBPs in SIP may be associated with increased keratins of stratified squamous epithelium, K15, and early epidermal differentiation keratins, K1/K10, and could regulate cell proliferation through protein interactions with target proteins, including cyclin-D1.

We provide here the first evidence of production and levels of the transcription factors C/EBP-alpha and C/EBP-beta in SIP, the correlation of these levels with levels of cyclin-D1, K10 and K15, and the association between C/EBP-alpha immunoeexpression and tumor recurrence.

Materials and methods

Patients

In this prospective study we investigated 26 SIP patients and 8 cases of nasal polyposis initially suspected for SIP (Table 1). The study was conducted in accordance with French bioethics regulations. Written informed consent was obtained from all patients for tissue sampling. All patients admitted to the Department of Otolaryngology and Head and Neck Surgery of Bichat Hospital (Paris, France) between May 2003 and May 2010 and diagnosed as SIP were included in the study.

The clinical diagnosis of SIP was confirmed histopathologically. The age of patients ranged between 17 and 69 years (mean=52±10.14), and the male to female ratio was 21:13. The SIP patients were 9 females with a mean age of 59±6.96 years and 17 males with a mean age of 50±7.35 years. All patients complained of unilateral nasal obstruction. Extension to the neighboring sinuses occurred in 13 patients and one patient displayed nasal septum invasion. Maxillary sinus extension was found in eight cases, ethmoidal extension in five cases and frontal extension in one case. The SIP patients were followed up for periods of one to eight years (mean of 4.8 years). Ten patients had one or more recurrences of SIP at the site of the investigated tumor. None developed carcinoma during the follow-up period.

The eight polyposis patients were initially suspected

Table 1. Clinicopathological data of the patients.

Patients (n)	Age (M±SD)	Sex (M/F)	Site (Uni/Bi)	Sinus (M/E/F)	Recurrence (n)	Follow-up (M±SD)
SIP (26)	52.5±8.47	17/9	26/0	8/5/1	10	4.8±2.63
Polyposis (8)	49.9±14.82	4/4	5/3	1/2/0	-	3.5±3.16
Total (34)	52.0±10.14	21/13	31/3	9/7/1	10	4.5±2.76

M/E/F: maxillary/ethmoid/frontal sinuses; Uni/Bi: Unilateral and Bilateral involvement of sinonasal mucosa. Follow-up expressed as mean and standard deviation in years.

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for SIP. However, the histopathologic examination did not show evidence of invaginated tumor epithelium. The surface epithelium showed areas of stratification and transitional columnar metaplasia with mucous secreting cells. Normal seromucinous glands and areas of inflammatory cell infiltration were evidenced in the stroma. These patients served as a control for epithelial stratification in non tumor tissue. The patients were 4 females with a mean age of 48 ± 21.2 years and 4 males with a mean age of 52 ± 7.2 years. Three patients presented bilateral polyps of the nasal cavity. One of these patients had had two previous interventions for inflammatory polyps, though in different anatomical sites. Extensions to the maxillary and/or to ethmoid sinus were observed in three cases and a history of asthma was recorded in three cases. The polyposis patients were followed up for periods ranging from 2 to 6 years with an average of 3.5 years. No history of sinonasal tumor was recorded during this period.

Tissue specimens from 34 patients were fixed in 10% formal saline pH 7.4 at 24°C for 48h and embedded in paraffin before processing (see below) for histological staining and immunofluorescence labeling. In parallel, unfixed tissues fragments, $\approx 25 \text{ mm}^3$, from 22 of the patients were frozen in liquid nitrogen for the analysis of mRNA expression and to control the effects of fixation and paraffin processing on protein detection by immunofluorescence. Only 15 were retained for the study of RT-PCR, 11 SIP and 4 polyps. Exclusion was due to either insufficient RNA quality (3 cases) or that tissue fragment was not morphologically representative for the diagnosis (4 cases). The diagnosis reported by the pathologist was confirmed on the frozen tissues, by staining the first and last of the sections cut from each tissue fragment. Serial 6 μm -thick cryosections (LEICA CM3050S) were air-dried and stored at -80°C until use.

Immunolocalization

Indirect immunofluorescence techniques were used for protein detection and colocalization. Paraffin sections were heated with citrate buffer for 10 minutes in a microwave adjusted to 800W and permeabilized by incubation with 0.1% Triton x100 in PBS for 10 minutes at 24°C before blocking of non specific binding sites by incubation with 3% BSA in PBS for 30 minutes at 24°C. Frozen sections were permeabilized and blocked as above before applying the primary antibodies. C/EBP-alpha was detected with rabbit (sc-61) antibody and C/EBP-beta was detected with rabbit (sc-150) and with mouse (sc-7962) antibodies (the latter was used in C/EBPs colocalization study see Results), all from Santa-Cruz Corporation. Keratins were detected with monoclonal antibodies against K10 (LH1, Acris and DE-K10, DAKO) and K15 (XM26, NovoCastra). Cyclin-D1 was detected using the monoclonal DCS-6 (Santa-Cruz Corporation).

Primary antibody binding was detected by incubation with the appropriate secondary antibodies

labeled with Alexa Fluor-594 and Alexa Fluor-488 from Molecular Probes. All incubations with antibodies were carried out for one hour in a moist dark chamber. No staining was obtained if the primary antibody was replaced with non immune serum. Polyclonal antibodies for C/EBP-alpha and C/EBP-beta were controlled for specificity by pre-absorption with purified peptides for C/EBP-alpha (sc-61 P) and for C/EBP-beta (sc-150 P). Oral stratified squamous epithelium was used as a positive control for keratins' immunoexpression and mouse skin served as control for C/EBPs antibodies, while anti C/EBP-alpha labeled the dermal papilla and the outer root sheath of hair follicle, C/EBP-beta labeled the matrix, and inner and outer root sheaths. Double labeling was performed by simultaneous incubation with the primary antibodies after blocking with 3% BSA for 30 minutes and permeabilization in 1% EDTA in PBS at 24°C. Labeling was assessed with a Zeiss LSM 710 confocal laser microscope, with quantification based on the number of positive cells per 25x microscopic field. All evaluations of intensity and number of positive cells were performed independently and blindly by two observers (E.S. and J.D.). At least 5 representative fields per section were randomly chosen and evaluated for the number of positive epithelial cells. Labeling levels were considered strong if there were >50% positive cells, moderate if there were 25-50% positive cells, and weak if there were <25% positive cells. Staining was considered negative in the absence of a detectable signal at 1.5 sec exposure time, 25x objective lens in a dark room. Intensity was evaluated by the time of exposure necessary for fluorescence signal detection. Whilst negative reaction was recorded for exposure time >1.5 seconds, low intensity required, between 0.7 and 1.5 seconds and high intensity was detectable at shorter exposure time. Standard formalin-fixed, paraffin-embedded, hematoxylin- and eosin-stained sections were used to evaluate morphology.

RT-PCR

Frozen tissues were homogenized with an Ultra-Turrax T8 (IKA, Labortechnik, Staufen, Germany) in Tri-Reagent solution (Euromedex, France), according to the manufacturer's instructions. Total RNA concentration was determined on the basis of absorbance measurements (GeneQuant II, Pharmacia Biotech, England). RNA integrity was assessed by ethidium bromide staining of 18S and 28S ribosomal RNA and agarose gel electrophoresis. Cases yielding low-quality RNA were excluded.

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification procedures were performed as previously described (Papagerakis et al., 2009). Briefly, total RNA (1 μg) was reverse-transcribed with a cDNA kit (Invitrogen, France), according to the manufacturer's instructions. We then amplified 1 μl of the resulting complementary DNA in 25 μl of PCR buffer containing 0.025 U/ μl AmpliTaq polymerase. The thermocycler

(Perkin Elmer GeneAmp PCR System 2400, Norwalk, USA) was programmed for 35 cycles of 30 seconds each at the appropriate temperature for primer pairs. RNA that had not previously been subjected to reverse transcription was used as a control to check for an absence of genomic DNA contamination. Primers were synthesized by Genset SA, Paris and Oligo Express Montreuil, France. The primers used for the amplification of C/EBP-alpha (GI, 28872793) were: forward primer 5'-AACCTTGTGCCTTGGAAATG-3' and reverse primer 5'-CCCTATGTTTCCACCCCTTT-3' at 60°C to amplify a 246 bp fragment. For C/EBP-beta (GI, 18314512): forward primer 5'-GACAAGCACAGCGACGAGTA-3' and reverse primer 5'-AGCTGCTCCACCTTCTTCTG-3', were used at 59°C to provide 158 bp of complementary DNA. KRT10 (GI 623408): forward 5'-GGCTGCGGTAGGTTTGAATTT-3', reverse 5'-GCAAGGACCTGACTACAGAA-3' at 59°C to provide a 326 bp fragment. KRT15 (GI, 60655738): forward 5'-TGTTGGAGGCCACCTCTTTGTTCA-3' and reverse 5'-TCATCCTGGAGATCGACAATGCCA-3' at 59°C to produce 407 bp fragment. GAPDH (standard control, accession No. J02642): forward primer P3: 5'-GACCCCTTCATTGACCTCAA CTAC-3' and reverse primer P4: 5' AAGTTGTCATGG ATGACCTTGGCC-3'; at 60°C to produce a 401 bp fragment. All PCR were performed in a Perkin Elmer GeneAmp PCR System 2400, MA, USA. The PCR products were analyzed by standard agarose gel (2% wt/vol) electrophoresis, with ethidium bromide staining.

Statistical analysis

Contingency tables and Fisher's exact probability tests were used to evaluate the statistical significance of the differences between groups. Spearman's correlation coefficient r was determined and P values <0.05 were considered significant.

Results

C/EBP-alpha immunoreexpression

C/EBP-alpha protein was detected in areas of stratified columnar and squamous epithelia. The reaction was mostly in epithelial cell nuclei, although the cytoplasm was sometimes stained. Both basal and suprabasal cells were stained. The normal looking areas at the borders of the tumors remained unstained in all cases. Only one of the eight polyps displayed weak

positive staining (Table 2). By contrast, 10 of the 26 SIP lesions were stained. Six of these stained tumors were from patients with known recurrences (Table 3). The number of cells labeled was significantly higher in recurrent SIP than in non recurrent tumors ($P=0.014$). Interestingly, the follow-up period to recurrence was shorter in cases expressing the protein (1.66 years) than that in negative cases (4.5 years). The immunoreexpression of C/EBP-alpha was not statistically associated with patient age, anatomic localization or gender, although the protein was less often detectable in female patients (22%) than in males (47%).

Investigations of the epithelial differentiation marker, K10, and of the cell proliferation marker, cyclin-D1, provided further insight into the importance of C/EBP-alpha expression. K10 was detected in six cases out of 26 SIP (2 with recurrences and 4 with no recurrences) and in none of the 8 polyposis cases. The keratin was detected in the cytoplasm of suprabasal cells with different morphologies. Both polygonal and tall cells were labeled (Fig. 1d), but staining was more frequent for polygonal than for tall cells. C/EBP-alpha and K10 immunoreexpressions were strongly correlated ($r=0.593$; $P=0.001$). The two proteins were colocalized in individual cells, with C/EBP-alpha in the nucleus and K10 in the cytoplasm. K10 immunoreexpressions were not correlated with other clinicopathological data.

Cyclin-D1 was detected in 25 cases: 18 out of 26 SIP and 7 out of 8 polyps. Basal and adjacent parabasal cells were stained. The protein was not detectable in normal looking respiratory epithelium and appeared patchy in the stratified epithelia. Staining intensity was higher for SIP than for polyps and there was a statistically significant correlation between C/EBP-alpha and cyclin-D1 immunoreexpressions ($r=0.524$, $P=0.006$). *In situ*, the two proteins were often found in the same

Table 2. Immunolocalization and grading of the reaction in tissues.

Patients (n)	K15	K10	Cyclin-D1	C/EBP-alpha	C/EBP-beta
SIP (26)	1/4/13/8	20/4/2/0	8/10/8/0	16/3/3/4	3/7/7/9
Polyposis (8)	3/3/2/0	8/0/0/0	1/7/0/0	7/1/0/0	5/2/0/1
Total (34)	4/7/15/8	28/4/2/0	9/17/8/0	23/4/3/4	8/9/7/10

Protein levels were graded as negative/weak/moderate/strong reaction. C/EBP-alpha levels were significantly correlated with K10 levels, $r=0.593$, $P=0.001$ and with cyclin-D1 levels, $r=0.524$, $P=0.006$. C/EBP-beta levels were correlated K15 levels, $r=0.426$, Contingency table and Fisher's exact probability test, $P=0.030$.

Table 3. Immunolocalization in recurrent and non recurrent SIP.

Tumors (n)	K15	K10	Cyclin-D1	C/EBP-alpha	C/EBP-beta
Recurrence (10)	0/2/4/4	8/0/2/0	1/4/5/0	4/2/0/4	0/2/4/4
No recurrence (16)	1/2/9/4	12/4/0/0	7/6/3/0	12/1/3/0	3/5/3/5
Total (26)	1/4/13/8	20/4/2/0	8/10/8/0	16/3/3/4	3/7/7/9

Protein expression was graded negative/weak/moderate/strong reaction. With contingency table and Fisher's exact probability test, $P=0.014$.

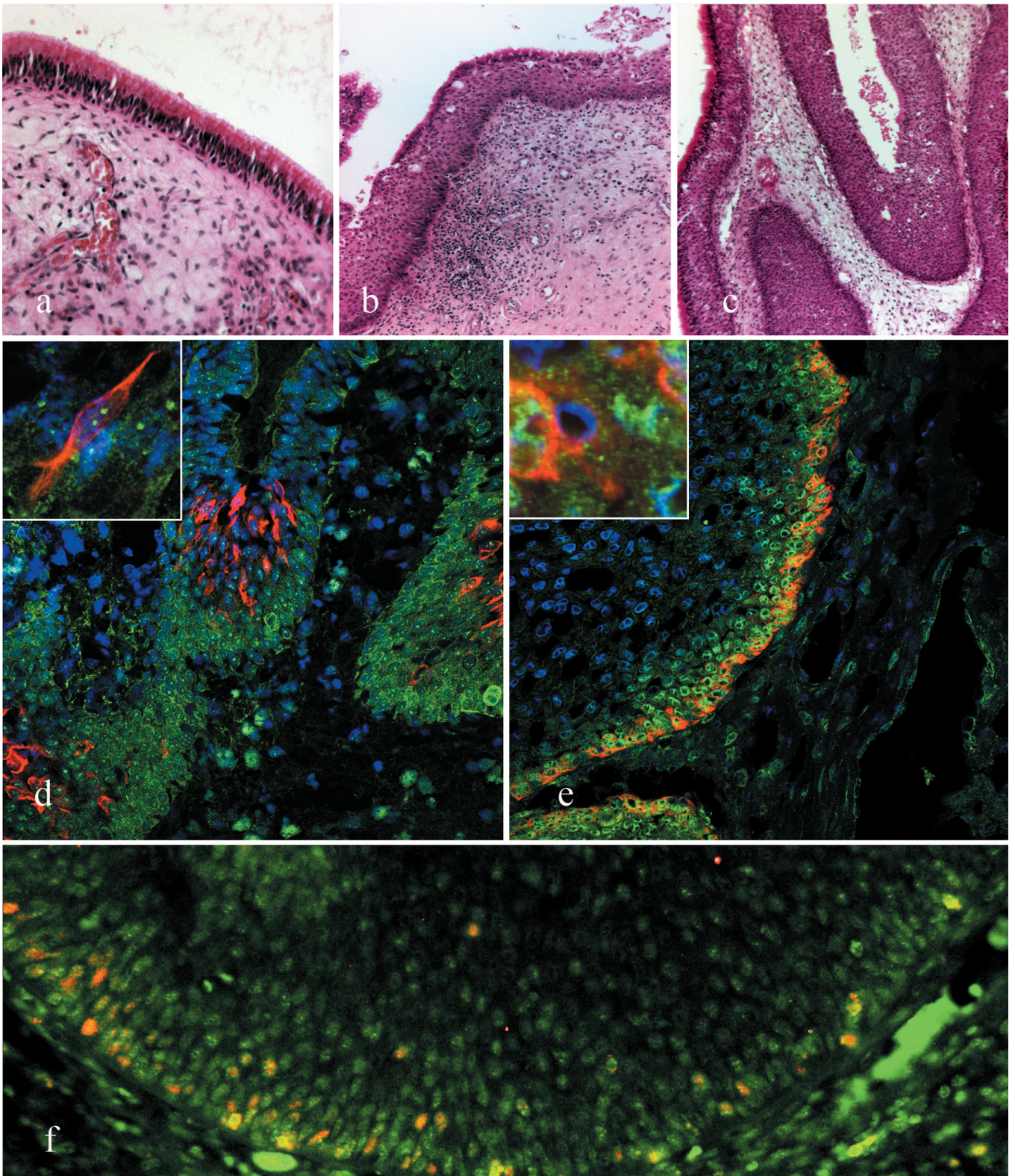
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Fig. 1. Histological aspects of normal respiratory mucosa (a), polyposis (b) and SIP (c). Colocalization of C/EBP-alpha and K10 in SIP (d), colocalization of C/EBP-beta and K15 in SIP (e) colocalization of C/EBP-alpha and cyclin-D1 in SIP (f). The transcription factors are labeled in green and the keratins and cyclin-D1 are labeled in red. C/EBP-alpha colocalized with K10 in differentiating cells (see a squamous cell in the inset). C/EBP-beta colocalizes with K15 in undifferentiated cells; the inset shows labeled basal cells of the tumor epithelium. The keratins were typically found in the cytoplasm. Note the nuclear colocalization of the transcription factor C/EBP-alpha and cyclin-D1, yellow to orange color in f. a, d-f, x 25; b, c, x 10; insets in d, e, x 65

biopsy specimen and sometimes in the same cells (Fig. 1f).

C/EBP-beta immunoexpression

C/EBP-beta was detected in 26 cases (3 polyps and 23 SIP) at different levels (Table 2). It was found mostly in the nucleus of suprabasal cells, although some basal cells were also stained. Staining was more intense in SIP than in polyps, although one of the three positive polyps also displayed intense staining. No significant correlation was found between C/EBP-beta levels and the clinicopathological data for papilloma patients. By contrast, C/EBP-beta levels were significantly correlated with K15 levels ($P=0.03$). This polypeptide was detected in the cytoplasm of basal and parabasal cells in 25 of the 26 SIP studied. It was present in larger amounts in areas of squamous metaplasia. C/EBP-beta and K15 were often colocalized in the same cells, with C/EBP-beta in the nucleus and K15 in the cytoplasm (Fig. 1e). C/EBP-beta levels were not significantly correlated with either K10 or cyclin-D1 levels although individual cells coexpressing C/EBP-beta and cyclin-D1 were identified.

Colocalization of C/EBP-alpha and C/EBP-beta was studied using rabbit antibody for the former, and mouse antibody for the latter. Only eleven cases double expressed the proteins, one was polyp and the other 10 were SIP cases. The two proteins were occasionally detected in the same cells, though the C/EBP-alpha was rather cytoplasmic and C/EBP-beta being nuclear. On the statistical level, the correlation between alpha and beta was weak and statistically not significant. However, double localization of C/EBP-alpha and C/EBP-beta

was significantly associated with recurrence in SIP. Thus, the two proteins were found in 6 out of 10 (60%) recurrent tumors and only in 3 out of 16 (19%) non recurrent tumors $P<0.05$. Double localization occurred once in polyps and the reaction was graded weak for both proteins.

RT-PCR

Fresh tissues from 15 patients (11 SIP and 4 polyposis) were investigated for the expression of the mRNA using RT-PCR technique. The results further confirmed the expression of the C/EBPs, the keratins and the cyclin-D1 genes in SIP. Thus C/EBP-alpha was detected in 9 of 11 SIP and in one polyp. The bands were rather weak compared with those of the control marker GAPDH (Fig. 2). C/EBP-beta was expressed in 10 out of 11 SIP and in 2 of the 4 polyps. The bands were more important in the SIP than in the polyps. K10 was expressed in 8 SIP and only very weakly in one polyp. In SIP the bands were generally moderate in size though, as seen in Fig. 2, some cases of SIP expressed important quantities. K15 mRNA was detected in all SIP cases and in one polyp. The bands were important in SIP compared with the control GAPDH. Finally, cyclin-D1 was detected in 8 SIP but not in the polyps. The levels of expression were rather moderate and comparable with those of the GAPDH expression levels.

Discussion

In this study, we investigated C/EBP-alpha and C/EBP-beta RNA and protein levels in SIP and non tumor controls. These RNAs and proteins were found almost exclusively in the tumor cells. These findings demonstrate that these transcription factors are commonly produced in SIP. These two highly-related proteins are produced independently in SIP, reflecting differences in their roles in these tumors. Furthermore, a study of target genes demonstrated the production of two keratin polypeptides, K10 and K15, which have not previously been considered in SIP. These findings implicate C/EBP-alpha and C/EBP-beta in the pathogenesis of SIP and in the regulation of tumor cell proliferation and differentiation.

In normal keratinocytes, C/EBP-beta plays a critical role in mediating keratinocyte proliferation and survival, reviewed in (Ewing et al., 2008). Several tumors also had high C/EBP-beta levels, which have been reported to be associated with greater invasiveness in human colorectal cancer (Rask et al., 2000). It has also recently been shown that cyclin-D1 can interact with C/EBP-beta to alter gene expression and that C/EBP-beta is important for the unique patterns of altered gene expression observed in human cancers displaying cyclin-D1 overproduction (Lamb et al., 2003). It was recently shown that C/EBP family members may cooperate with the nuclear factor kappa B to induce anti-apoptotic factors independently of, or in synergy with, the

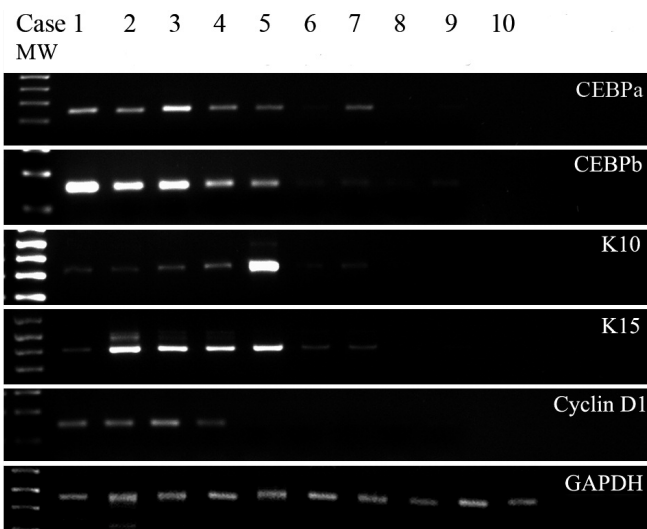


Fig. 2. Detection of mRNA by RT-PCR in representative specimens of SIP and polyposis. The cDNA for the various transcription factors and markers of proliferation and differentiation were variably detected in the tissue extracts. The reference gene GAPDH was invariably detected in all specimens. Cases 1 to 6 represent SIP and 7 to 10 represent polyposis tissues.

canonical activation pathway (Dooher et al., 2011). Interestingly, C/EBP-alpha was as effective as C/EBP-beta in the activation of the anti apoptotic gene targets.

In this study, K15, a poorly characterized cytokeratin, was detected in most SIP. It has been shown that K15 is preferentially produced in slowly cycling basal cells of the interfollicular epidermis and in the bulge region of hair follicle, suggesting that this protein could possibly be an epidermal stem cell marker. However, its widespread distribution in different stratified epithelia makes it a reliable marker of epithelial stratification. In this study, a significant statistical correlation was found between K15 and C/EBP-beta levels. This correlation was demonstrated by immunocolocalization, in both SIP and polyposis. These results are consistent with the proposed role of C/EBP-beta in the activation of K15 gene transcription (Radoja et al., 2004).

It was less straightforward to study the correlation between C/EBP-beta and cyclin-D1. No statistical association was found, but these two proteins were often colocalized in the nuclei of some basal and parabasal tumor cells.

By contrast, the loss of C/EBP-alpha is thought to be permissive for tumor growth. C/EBP-alpha levels are low in human hepatocarcinoma (Tseng et al., 2009), lung cancer (Costa et al., 2007) and head and neck carcinomas (Bennett et al., 2007). Collectively, these studies support the notion that the loss of C/EBP-alpha may be permissive for cancer cell growth. C/EBP-alpha is thought to downregulate the cell cycle by binding to CDK4 and CDK2 and preventing the formation of complexes with cyclin-D and cyclin-E, respectively. C/EBP-alpha can also inhibit E2F transcription activity and increase the activity of the CDK inhibitor p21 (Lopez et al., 2009). Consistently with this, we found that C/EBP-alpha levels were significantly associated with cyclin-D1 levels, although these two proteins were not colocalized.

The finding that C/EBP-alpha levels are high in SIP is consistent with the proposed tumor suppressor function for the protein, because none of the tumors underwent malignant transformation during the follow-up period. C/EBP-alpha production was associated with the transactivation of several differentiation-related genes. Thus, unlike a previous study on keratin levels in SIP (Schwerer et al., 2001) we demonstrated the production of K10 through detection of the protein with two monoclonal antibodies and detection of the corresponding mRNA.

A pathogenic role has been reported for C/EBPs, based on studies in transgenic mice transfected with a dominant negative construct inhibiting DNA binding by C/EBP family members. The expression of this construct during a two-step skin carcinogenesis protocol inhibited papilloma development and its expression after skin papilloma development resulted in tumor regression (Oh et al., 2007). Our results suggest that C/EBP-alpha and C/EBP-beta modulate the proliferation and

differentiation of SIP tumor cells.

In normal stratified epithelia, C/EBP-alpha promotes cell differentiation and arrests cell cycle progression. Our results, on the contrary, present associations between this transcriptional factor production and cell proliferation and tumor recurrence. This contradiction may be explained in the light of recent findings on the effect of viral infection in epithelial cells. Thus, the early viral protein E7 of HPV-16 (not uncommon in SIP) overrides the C/EBP-alpha mediated cell cycle withdrawal without compromising its transcriptional activity and its ability to promote differentiation (Muller et al., 1999). Furthermore, both C/EBP-alpha and C/EBP-beta were implicated in the reproductive life cycle of various HPV types (Wooldridge and Laimins, 2008). Interestingly, a correlation between the high risk viruses and recurrence in SIP was recently established (Lawson et al., 2008). Further studies will be needed in order to establish the interactions between C/EBPs and HPV types in SIP and their consequences.

Recurrence is thought to occur as a result of incomplete tumor resection or through persistent/hidden tumor foci (Depondt et al., 2008), and the production of C/EBPs could participate by promoting cell survival. A recent paper has shown that C/EBP-alpha and C/EBP-beta cooperate with nuclear factor NF-kappa-B p50 to activate anti apoptotic targets, including Bcl2 (Dooher et al., 2011). C/EBPs may form homo- or hetero-dimers in order to activate target genes with different consequences. In the present paper, colocalization was useful to study protein associations *in situ*. However, it is not sufficient to study dimerization, protein-protein interactions and protein complex formation. It is therefore beyond the limits of the present study to conclude or exclude the presence of hetero-dimers between C/EBP-alpha and C/EBP-beta. Whilst C/EBP-alpha appeared in the present study as a candidate marker for recurrence in SIP, the results also showed that the double production of both transcription factors significantly associate with recurrence. We cannot therefore exclude a role of C/EBP-beta in the pathogenesis of SIP and in recurrence. Further studies using inhibitors and/or activators of the C/EBPs would provide further insight into the role of these transcriptional factors in the pathogenesis of SIP.

In conclusion, the present results describe C/EBP-alpha and C/EBP-beta expression in SIP and their possible implication in the regulation of tumor growth and differentiation. Finally, C/EBP-alpha production may prove useful in terms of a prognostic marker for the recurrence in sinonasal inverted papilloma.

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