Video Article

Identification of OTX1 and OTX2 As Two Possible Molecular Markers for Sinonasal Carcinomas and Olfactory Neuroblastomas

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Abstract

OTX homeobox (HB) genes are expressed during embryonic morphogenesis and during the development of olfactory epithelium in adult organisms. Mutations occurring in these genes are often related to tumorigenesis in human. No data are available today regarding the possible correlation between *OTX* genes and tumors of the nasal cavity. The aim of this work is to understand if *OTX1* and *OTX2* can be considered as molecular markers in the development of nasal tumors. We selected nasal and sinonasal adenocarcinomas to investigate the expression of *OTX1* and *OTX2* genes through immunohistochemical and real-time PCR analyses.Both *OTX1* and *OTX2* were absent in all the samples of sinonasal Intestinal-Type Adenocarcinomas (ITACs). *OTX1* mRNA was identified only in Non-Intestinal Type Adenocarcinomas (NITACs) while *OTX2* mRNA was expressed only in Olfactory Neuroblastomas (ONs). We have demonstrated that the differential gene expression for both *OTX1* and *OTX2* genes might be a useful molecular marker to distinguish the different types of sinonasal tumors.

Introduction

OTX HB genes are the vertebrate homologue of the Drosophila orthodenticle genes (otd) and they encode for transcription factors which are normally expressed during embryonic morphogenesis, but they can also be expressed in the adult organism with different functions. During embryonic development they control the specification of cell identity, cell differentiation, and the positioning of the body axis[1]. The *OTX* family includes *OTX1* and *OTX2* genes which display different functions. *OTX1* is involved in brain and sensory organ development. In the adult organism, it is expressed in sensory organs and is transcribed at low levels in the anterior lobe of the pituitary gland[2]; it also plays a role in hematopoiesis, being expressed in hematopoietic pluripotent and progenitor cells[3]. *OTX2* is involved in the development of the rostral head and its translated protein acts as a morphogen because it generates a gradient through which other genes are activated or repressed in a spatio-temporal manner, thus contributing to cell proliferation and differentiation. In the adult organism, *OTX2* is found exclusively in the choroid plexus and pineal gland[4].

Mutations in *OTX* genes are often related to the appearance of human congenital, somatic, or metabolic defects. Gain or loss mutations in *OTX* genes could promote tumorigenesis if they are not able to properly control cellular growth and/or differentiation[5]. In leukemias and lymphomas as well as in many solid tumors (*e.g.,* medulloblastomas[6], aggressive non-Hodgkin lymphomas[2], breast carcinomas[7], colorectal cancers[8], and retinoblastoma[9]), the deregulated expression of *OTX* HB genes is well documented[10]. In addition, *OTX2* mutations have been demonstrated in cases of anophthalmia and microphtalmia[11] due to the crucial role for this gene in the control of eye development.

In the context of solid neoplasms, the discovery of molecular and phenotypic markers is an important challenge for the diagnosis, classification, and treatment of several types of tumor[11], including those that originate in the nasal cavity and paranasal sinuses. In fact, despite that these areas occupy only a modest anatomical space, mucosal epithelium, glands, soft tissues, bone, cartilage or neural/neuroectodermal, and hematolymphoid cells can be often the site for the origin of complex and histologically different groups of tumors. Different types of neoplasms involving the sinonasal tract present a variety of features that overcome what is usually seen in the upper aerodigestive tract or even throughout most parts of the body[12].Sinonasal malignancies are rare and present an annual incidence of 1:100,000 inhabitants worldwide, and so this prevents studies regarding the pathways involved in the tumorigenesis and the testing of alternative treatment strategies.Despite this, the advances in imaging techniques, surgical approaches, and radiotherapy have improved the clinical management of sinonasal cancer.Moreover, the development of cell lines as well as animal models and cancer genetic profiling currently constitute the basis for the future targeted anticancer therapies[13]. To date, there are no reports regarding *OTX1* and/or *OTX2* expression in neoplasms of the nasal cavity, paranasal sinuses, and nasopharynx. Since we have previously observed that *OTX1* and *OTX2* are involved in breast cancer[7], we wondered if these genes could be present not only in the normal nasal mucosa but also in tumors of the nasal cavity. To reach this goal we obtained from the Department of Pathology of the "Ospedale di Circolo" in Varese samples of normal mucosa, and nasal and sinonasal adenocarcinomas collected from 1985 to 2012 and classified according to the World Health Organization (WHO) classification of Head and Neck Tumors. We choose to analyze

them through real-time PCR and immunohistochemistry analyses and we evaluated *OTX1* and *OTX2* expression to determine if they can be considered molecular markers for these types of tumors.

Protocol

All the studies were performed according the Declaration of Helsinki (1975) with written informed consent and approved by the Ethical Committee of the University of Insubria in Varese.

1. Collection of the Samples

1. Collect and divide all the human Formalin-Fixed Paraffin-Embedded (FFPE) samples into different subgroups according the WHO classification of Head and Neck Tumors[14].
   NOTE: Here we used the following samples: Normal sinonasal mucosa as control (10 samples); Inverted Papilloma (IP, ICD-O code 8121/1); Sinonasal ITAC (ICD-O code 8144/3, 32 samples); NITAC (ICD-O code 8140/3, 12 samples); Adenoid Cystic Carcinoma (ACC, ICD-O code 8200/3); Pleomorphic Adenoma (PA, ICD-O code 8941/3); ON (ICD-O code 9522/3, 13 samples); Poorly Differentiated Neuroendocrine Carcinoma (PDNEC, ICD-O code 8041/3, 19 samples); Neuroendocrine Tumor (NET, ICD-O code 8041/3).

2. Immunochemistry

1. **Deparaffinization and rehydration of sections**
   1. Heat the sample slides in an oven at 60 °C for 30 min and rehydrate 3 µm thick FFPE sections using an alcohol series to water.
   2. Briefly wash the slides in xylene for 10 min and repeat this step; wash the slides for 5 min in 100% alcohol and repeat this step using 95%, 85%, and 75% alcohol serially. Rinse the slides for 5 min in distilled water.
2. **Blocking the endogenous activity**
   1. Block the endogenous activity by placing the slides in 3% aqueous hydrogen peroxide for 12 min.
3. **Antigen retrieval**
   1. Perform antigen retrieval by treating with 10 mM Citrate Buffer (pH 6) for 10 min in a microwave treatment.
4. **Incubation with primary antibody**
   1. Wash the sections in TBS buffer (pH 7.4), add 0.2% of Triton X, and incubate overnight at 4 °C with goat anti-human OTX2 antibody diluted 1:100.
5. **Incubation with secondary antibody**
   1. Incubate the sections for 1 h at room temperature with biotinylated rabbit anti-goat secondary antibody diluted 1:200 followed by ABC-peroxidase complex (see **Tables of Materials**).
6. **Developing the immunoreaction**
   1. Develop the immunoreaction using 3,3'-diaminobenzidine tetrahydrochloride and counterstain the nuclei with Harris Hematoxylin.
7. **Mounting and imaging**
   1. Dehydrate the sections using a crescent alcohol-scale and clarify them using a clearing substance of terpene origin (see **Table of Materials**). Embed sections in mounting medium, place the sections on a microscope slide, and observe the sections through an optical microscope.

3. RNA Extraction and Reverse-transcription

1. **RNA extraction**
   1. Extract RNA from the sections by performing the first step of immunoprecipitation protocol (see section 2.1) and keep the slides in distilled water. Overlap the unstained sections with the corresponding hematoxylin-eosin stained sections in order to identify fragments of interest.
   2. Perform the RNA extraction using a commercial RNA extraction kit for FFPE samples (see **Table of Materials**) and following manufacturer's instructions.
2. **RNA reverse-transcription**
   1. Use a commercial kit to retro-transcribe RNA into cDNA (see **Table of Materials**) following the protocol. Retro-transcribe at least 1,000 ng of total RNA to perform several analyses.

4. Real-time PCR and Data Analysis

1. **Real-time PCR**
   1. Perform quantitative real-time PCR analyses (qRT-PCR) with probe-based technology (see **Table of Materials**) and a thermal cycler.
2. **Preparation of the PCR reaction mix**

1. Prepare the PCR reaction mix using 12.5 µL of probe-based master mix, 1.25 µL each of OTX1, OTX2, and ACTB probes (see **Table of Materials**), 50 ng of cDNA, and nuclease-free water up to 25 µL of total volume.
2. Perform all the reactions in triplicate using the ACTB gene as the endogenous control to normalize gene expression levels.
3. Centrifuge the plate at 1,109 x g for 3 min and store the plate protected from light at 4 °C until the experiment.

3. **Setting the thermal cycler**
   1. Set the thermal cycler profile with an initial hot start cycle at 50 °C for 2 min and 90 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and a final cycle at 60 °C for 1 min.

4. **Gene expression level analyses**
   1. Normalize the gene expression levels through the comparative cycle threshold (ΔCt) method using the ACTB gene as the endogenous control.
   2. Evaluate gene expression levels using the $2^{-\Delta Ct}$ method and plot the results.

5. **Statistical analyses**
   1. Perform statistical analyses using Student's *t*-Test, considering statistically significant results with $p < 0.05$.

Representative Results

In the normal mucosa we observed strong and homogenous nuclear reactivity for OTX genes both in the ciliated pseudostratified respiratory-type epithelium and in the submucosal glandular cells (**Figure 1A**). We found nuclear expression for OTX1 in all NITACs samples (**Figure 1B**), while little or absent immunoreactivity was highlighted in ITACs (**Figure 1C**). Intense immunoreactivity was present in all ONs (**Figure 1D**); among PDNECs, OTX expression varied in intensity and percentage of positive cells (**Figure 1E**). Immunohistochemical statistical analysis performed with Chi square and/or Fisher's exact test demonstrated that the absence of OTX genes in ITAC samples was statistically significant (n = 23, $p < 0.01$).

Real-time PCR analyses confirmed the expression of both OTX1 and OTX2 genes in control samples (**Figure 2A-B**); OTX1 but not OTX2 was expressed in NITAC samples and both genes were completely downregulated in ITACs (**Figure 2A-B**). In ON samples we found only the expression of OTX2 gene while OTX1 was downregulated, and among the PDNEC samples the expression of both genes varied (**Figure 2A-B**).

Student's *t* test performed on real-time PCR confirmed statistically significant data for *OTX1* in controls vs. ITACs (n = 9, $p < 0.05$), controls vs. ONs (n = 6, $p < 0.05$), controls vs. PDNECs (n = 8, $p < 0.05$), and for *OTX2* in control vs. NITACs (n = 5, $p < 0.05$), controls vs. ITACs (n = 9, $p < 0.05$), controls vs. ONs (n = 6, $p < 0.05$), and controls vs. PDNECs (n = 8, $p < 0.05$).

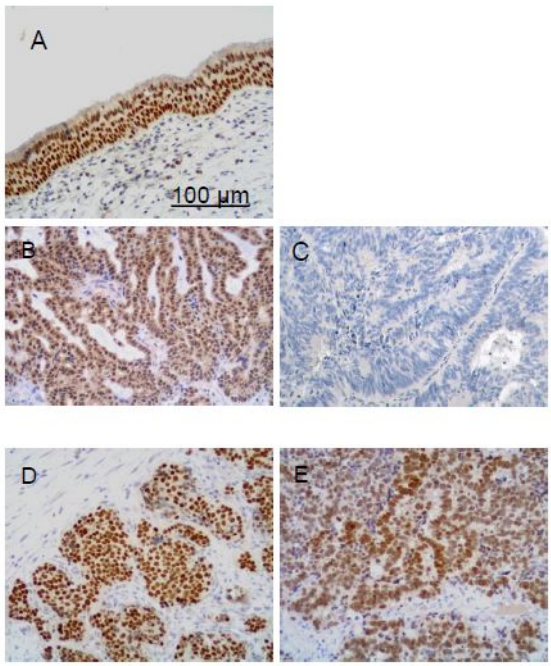

Figure 1: Representative images of *OTX* immunohistochemical expression in control and neoplastic tissues. The reaction developed by the peroxidase-conjugated secondary antibody revealed the signal (dark brown) was visible in 5 control FFPE sections of normal sinonasal mucosa (**A**), 7 cases of Non-intestinal-type adenocarcinomas (NITACs) (**B**), 7 cases Olfactory neuroblastomas (ONs) (**D**), 11 cases Poorly differentiated neuroendocrine carcinomas (PDNECs) (**E**), while no immunoreactivity is detected in the 23 cases of Intestinal-type adenocarcinomas (ITACs) analyzed (**C**). DAB-Hematoxylin; original magnification: 200X, scale bar = 100 µm.

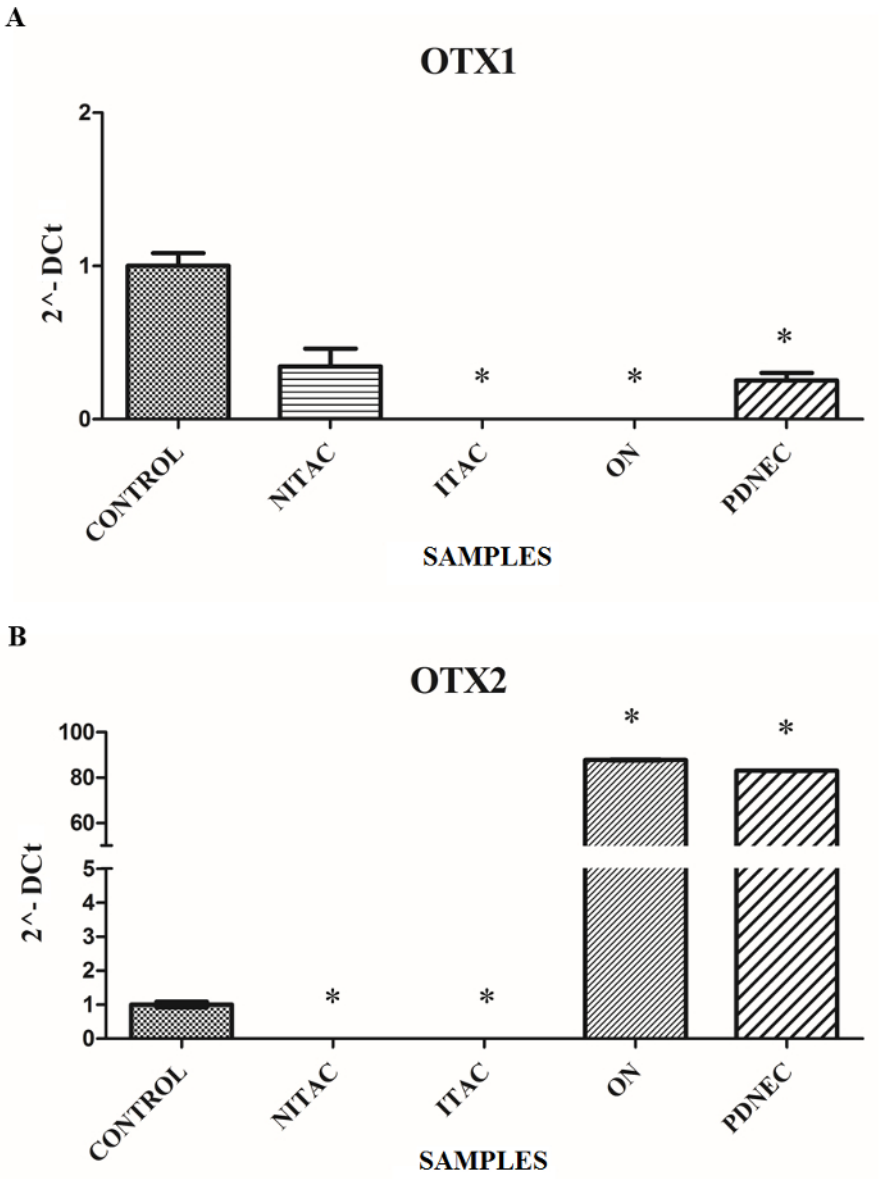

Figure 2: Real-time PCR analysis of *OTX1* (A) and *OTX2* (B) mRNA expression in normal and neoplastic nasal tissues. For real-time PCR analyses, the following samples were used: 5 FFPE sections of control normal mucosa, 5 cases of Non-intestinal-type adenocarcinomas (NITACs), 9 cases of Intestinal-type adenocarcinomas (ITACs), 6 cases of olfactory neuroblastomas (ONs), 8 cases of Poorly differentiated neuroendocrine carcinomas (PDNEC). On the X axis the type of sample is reported, while the Y axis represents the $2^{-\Delta Ct}$ values. Statistically significant data ($p < 0.05$) between control and tumors were obtained by Student's *t*-test and are highlighted with asterisks. Please click here to view a larger version of this figure.

Discussion

This study shows for the first time that, based on mRNA levels, the HB genes OTX1 and OTX2 are expressed in normal sinonasal mucosa and submucosal glands, inflammatory polyps, sinonasal Schneiderian papillomas, and in the different epithelial and neuroectodermal neoplasms, including squamous carcinomas, non-intestinal type sinonasal adenocarcinomas, salivary gland-type tumors, neuroendocrine neoplasms, and ONs.

Modifications and Troubleshooting:

To avoid RNA degradation, we performed the deparaffinization protocol in the Laboratory of Pathology. After we scratched the slides, all the samples were rapidly cooled and stored in dry ice until further use. We performed all the subsequent analyses in a laminar-flow safety hood using a dedicated set of pipettes with sterile tips to maintain aseptic experimental conditions and to avoid RNA contaminations.

Limitations of the Technique:

The principal limitation of the technique relies on the availability of samples since tumors of sinonasal cavity are often rare. The amount of extracted RNA is another important issue because RNA extraction from FFPE samples results in fragmented RNA which is difficult to analyze.

Significance with Respect to Existing Methods:

The diagnosis of tumors of the nasal cavity usually consists of X-rays analyses, endoscopic exam of the nasal cavity, CT scan, magnetic resonance (RMN), and biopsy. During the recent years, the advances of surgical instrumentation and the progress of imaging techniques had led endoscopic surgery as the preferred strategy for sinonasal tumors. Notably, this technique has been reported as feasible for different benign or malignant types of sinonasal tumors[15]. Our technique based on qRT-PCR allows the identification of molecular biomarkers, which can differentially discriminate samples of different tumors: in fact, we provide evidence that the absence of both the HB genes in ITACs can be used as molecular markers for this type of tumor as well as the absence of OTX2 in NITACs. Moreover, this technique can be applied immediately after the biopsy and can give definitive results in less than 6 hours with respect to the days or weeks necessary for the report from the hospital.

Future Applications:

Our future studies involve analyzing gene and protein expression levels of OTX1 and OTX2 HB genes using real time PCR analyses, Western blot, and immunofluorescence with specific antibodies to detect not only OTX1 and OTX2, but also p53 family expression levels. Since we previously demonstrated that OTX1 expression is regulated by p53 in breast cancer[7], it would be interesting to understand if such a regulation exists also in nasal cavity tumors. An immunofluorescence assay and a Chromatin Immunoprecipitation (ChiP) assay can reveal, respectively, a protein-protein and a DNA-protein interaction between p53 and OTXs. If such a connection is found, the targeted silencing of p53 (and the family members, p63 and p73), will reveal if the over-expression or down-regulation of OTX genes is dependent on p53.

p53 loss of function, through mutation in p53 itself or perturbations in pathways signaling to p53, is a common feature in the majority of human cancers[16]. Most of the p53 mutations cluster within the DNA-binding domain; therefore, DNA-binding activity is the critical function that is altered, suggesting that changes in transcriptional genes could be the key to mutant p53 activity[16]. p53 mutations have been reported as a common feature of sinonasal cancer, with an overall frequency of 77%, and they show association to adenocarcinoma and wood-dust exposure[17]. Thus, in our samples, it would be interesting to evaluate mRNA and protein levels (by qR-PCR and Western Blot analysis) and to perform the sequencing of this gene, in order to identify or exclude the presence of activating/inactivating mutations. Finally, if a tumor-type specific recurrent mutation is found, it would be noteworthy to produce engineered cells and mice (with one mutant allele) that harbor this mutation, and examine if the mutation can recapitulate the disease.

Critical Steps:

Critical steps of this study include the obtainment of well-stored samples, either as biopsies stored in RNA protective buffer or FFPE samples of at least 8 µm thick, and the extraction of at least 200 ng of RNA to perform qRT-PCR analyses.

Disclosures

The authors have nothing to disclose.

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