LETTER TO THE EDITOR

Atrial natriuretic peptide differentiates between primary submandibular gland squamous cell carcinoma and oral squamous cell carcinoma: preliminary data

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To the Editor,

The natriuretic peptide (NP) family is composed of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). ANP and BNP are synthetized prevalently in the heart, although their expression has also been reported in other organs and tissues, while CNP is synthetized mainly by the vascular endothelium. NPs act through specific receptors, which have been identified as NPRA, NPRB and NPRC, possessing different affinities for each NP, with NPRA showing the highest affinity for ANP.

NPs act mainly as homeostatic agents, and are secreted in response to pressure and stress on myocardial walls. They exert diuretic and vasodilatory functions regulating the water–salt balance and consequently blood volume. In addition to endocrine actions involved in blood pressure regulation, NPs can also exert local functions through paracrine and autocrine mechanisms. Indeed, several studies have pointed to the presence of non-canonical (*i.e.* extracardiac) sites for the production of ANP including the brain, gastrointestinal tract, peripheral cells of pancreatic islets, testis, macrophages (1) and, of particular interest for this study, the salivary glands (SGs) (2-4).

NP system alterations have been correlated with a wide panel of diseases including congenital heart disease, heart failure, dyspnea, sepsis, cirrhosis, leading to NPs and their receptors being used as diagnostic and prognostic markers, in particular for cardiovascular diseases. Components of the NP system have also been identified as potential targets in cancer therapy, one example being NPRA, which is documented as being involved in tumor angiogenesis (5, 6). Based on previously published data in which we recently showed that ANP is expressed in intralobular ducts of both murine and human submandibular glands (SMGs) (4), we conducted a small exploratory study to investigate expression levels of *NPPA* and *NPR1*, encoding respectively for ANP and its receptor NPRA, in primary benign pleomorphic adenoma (PA), and malignant squamous cell carcinoma (SCC) SMG

Key words: NPA; NPPA; submandibular gland; oral squamous cell carcinoma

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tumors and in primary oral squamous cell carcinoma (OSCC). Our data show that expressions of *NPPA* and *NPR1* are respectively significantly increased and abrogated in SMG SCC compared to OSCC. Primary SMG SCC, being rare and easily confused with SCC from distant sites that have spread to the SMGs via metastasis, emanating data suggest *NPPA* and *NPR1* expression could be used as diagnostic markers to help discriminate between primary and secondary SMG SCC.

MATERIALS AND METHODS

Human salivary gland tissues were obtained from patients who had undergone surgery at the Division of Surgery of the Medical School within the University of Palermo (Italy). Informed consent was obtained from all patients. The study protocol was approved by the Institutional Research Board and is in line with the ethical guidelines of the 1975 Declaration of Helsinki.

Salivary gland tissues

Human SMG tissues embedded in paraffin were selected from the archives of the Department of Pathology of the University of Palermo. Human biopsies used in this study comprised three normal submandibular salivary glands (SMGs) (median age 50 years), three SMG pleomorphic adenomas (PA, median age 34.5 years), three oral squamous cell carcinomas of non-salivary gland origin (OSCC, median age 55.5years) and three SMG SCC (median age 64.5 years). Tumor cases presented a history of variable size of primary tumors as well as variable presence of metastasis in draining lymph nodes and were reviewed by a pathologist who confirmed the initial diagnosis.

Immunohistochemical analysis

Immunohistochemistry was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometers-thick tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH 6 and pH 9 and in PT Link Dako at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H2O2 and Fc blocking by a specific protein block (Novocastra UK) the samples were incubated overnight with the primary antibodies Mouse anti-human ANP 1/50 pH 6 (Clone M622709; Abcam Cod. 2093); Rabbit Polyclonal anti-human ANP 1/100 pH 9 (Abcam Cod.76743); Rabbit Polyclonal anti-human ANP receptor A 1/50 pH 6 (Abcam Cod.70848) at 4°C. Staining was revealed by polymer detection kit (Novocastra) and AEC (3-amino-9-ethylcarbazole) substrate-chromogen. The slides were counted with Harris hematoxylin (Novocastra). All the slides were analyzed under AXIO Scope A1 optical microscope (Zeiss) and micrographs were collected using an Axiocam 503 Color digital camera (Zeiss). Negative control stainings were performed by using mouse and rabbit immune sera instead of the primary antibodies.

RNA extraction and RT-qPCR

RNA was extracted using the RNeasy FFPE kit (Qiagen) from 4 paraffin-embedded tissue sections of 5 μm each, following the manufacturer's instructions. Purified RNA was quantified using the Qubit 3.0 fluorometer (Life technology). cDNA synthesis was carried out with the Quantinova reverse transcription Kit (Qiagen) following the manufacturer's instructions. Real-time quantitative polymerase chain reactions (qPCR) were performed with the Quantinova SYBR green PCR kit (Qiagen) in triplicate on a Rotor-Gene Q system (Qiagen) following the manufacturer's instructions. QuantiTech Primer assay (Qiagen) was used with Hs_NPR1_1_SG QT00081963, Hs_NPPA_1_SG QT00203322, and Hs_ACTB_1_SG QT00095431 primers, for the quantification of human *NPR1*, *NPPA* and *ACTB* (normalizer) transcripts, respectively. Data were analyzed using the $2^{-\Delta\Delta CT}$ method using an external independent reference tissue (7).

Statistical analysis

One-tailed Mann-Whitney test was carried out to analyze the RT-qPCR data with *P< 0.05 considered as statistically significant.

RESULTS

This preliminary study investigated whether levels of *NPPA* expression in the SMG could be subjected to significant changes in neoplasia. Quantitative PCR was used to compare *NPPA* expression levels

in SMG tissue resected from patients diagnosed with SMG primary benign PA and malignant SCC tumors and either normal SMG tissues or malignant tumors of the buccal mucosa OSCC. PA is the most common benign tumor affecting the SMG, representing 40 to 60% of all SMG neoplasia. While SMG primary SCCs constitute a rare occurrence, OSCC, which can spread to the SMG, is a common cancer in humans that represents 90% of oral cancers and constitutes an important global health concern.

In this exploratory study we analyzed *NPPA* expression levels in three biopsies obtained from three independent patients for each type of neoplasms. The *ACTB* gene was used to normalize mRNA levels between samples. *NPPA* expression levels were not statistically different between normal SMG, PA and OSCC (Fig. 1A). In contrast, all SMG primary SCC studied exhibited an increase in *NPPA* expression, which was significant when compared to OSCC *NPPA* levels (Fig. 1A), suggesting SMG SCC have specific molecular characteristics compared to OSCC, and increased *NPPA* expression represents one of these features.

We complemented this study by analyzing expression levels of *NPR1* encoding the major ANP receptor in the nine cases of neoplasms studied. We found that *NPR1* expression was below the level of detection in all cases of primary SMG SCC studied, while *NPR1* was expressed at similar levels in SMG PA and OSCC (Fig. 1B), further illustrating primary SMG SCC and OSCC are molecularly distinct entities. Histochemical analysis of NPRA, confirmed

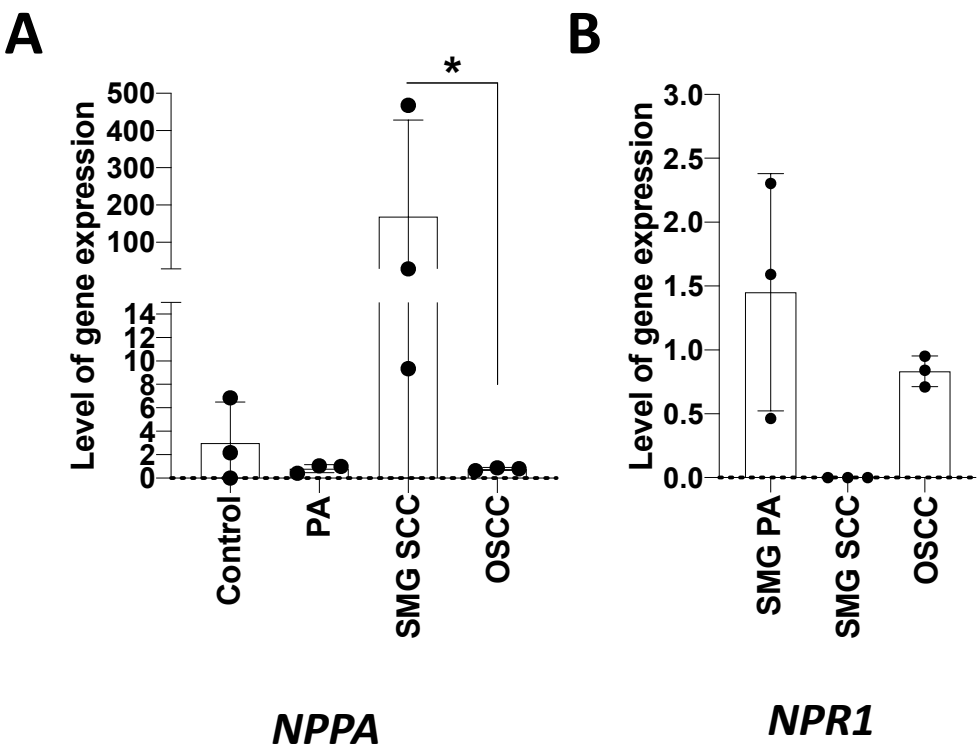

Fig. 1. *Expression of NPPA (**A**) and NPR1 (**B**) in normal salivary submandibular glands (SMGs) (Control), SMG pleomorphic adenomas (SMG PAs), SMG squamous cell carcinomas (SMG SCCs), and oral squamous cell carcinomas (OSCCs) of non-salivary gland origin originating in the buccal mucosa (OSCCs). NPPA and NPR1 expression are significantly increased and absent, respectively, in SMG SCCs in comparison with OSCCs. One-tailed Mann-Whitney test was carried out with *P< 0.05 considered as statistically significant.*

the PCR data by showing an elevated expression of this receptor in the SMG-SCC compared to the other categories (Fig. 2).

In summary, primary SMG SCC are characterized by increased expression of *NPPA*, the coding gene for ANP, and absence of expression of *NPR1*, encoding ANP major receptor, compared to OSCC.

DISCUSSION

Major salivary gland malignancies are rare, accounting for 0.2% of all malignant neoplasms and 9% of head and neck cancers. While SCC is the most common type of malignant neoplasm in other head and neck locations, it is a rare type of primary SMG malignancy. As the SMG is anatomically close to head and neck structures, such as the skin and oral mucosa that exhibit a higher incidence of SCC, SCC of the skin or the oral mucosa can easily spread to the SMG by direct invasion. Furthermore, SCC originating from distant sites can also spread to the salivary glands via metastasis. Hence, a proportion of malignant tumors found in the SMG are not primary tumors that have arisen in the SMG. Combined with the rarity of SMG primary SCC, these considerations illustrate the complexity of diagnosing SMG SCC. While it is currently recommended that primary SMG SCC are diagnosed by exclusion of other metastatic cancers, it is undeniable that molecular markers specific to SMG SCC would help diagnose this pathology. We have shown here that *NPPA* and *NPR1* expression levels may help discriminate between SMG primary SCC and OSCC. Indeed, *NPPA* is significantly increased in SMG SCC compared to OSCC. Furthermore, *NPR1* is expressed in OSCC, while *NPR1* expression is undetectable in SMG SCC. Despite the small number of primary SMG SCC analyzed, mostly due to the rarity of this malignant neoplasm, the preliminary results of this study warrant further investigations using a larger sample set.

The combined increase in *NPPA* expression and absence of *NPR1* expression identified in SMG SCC raises the question of the significance of a potential increase in ANP production in SMGs developing primary SCC. *NPR1* encodes NPRA, characterized as the main receptor for ANP. ANP has also been shown to bind - albeit with a lower affinity - to the NPRB receptor, which is structurally related to NPRA and exhibits similar guanylyl cyclase activity to NPRA following ligand binding (8). ANP binds as well with high affinity to the NPRC receptor, which is believed to function as a clearance receptor, by removing natriuretic peptides from the extracellular compartment (9). The lack of *NPR1* expression, in all SMG SCC analyzed, strongly suggests abnormal ANP production does not have a local role in neoplastic SMG. Future studies should investigate whether there is increased ANP concentration in the saliva of SMG SCC patients, in which case salivary ANP could also be used as a diagnostic tool to discriminate between primary SMG SCC and OSCC. Furthermore, increased ANP production in SMG SCC could be associated to increased plasma ANP concentration and a potential risk of hyponatremia (low blood sodium),

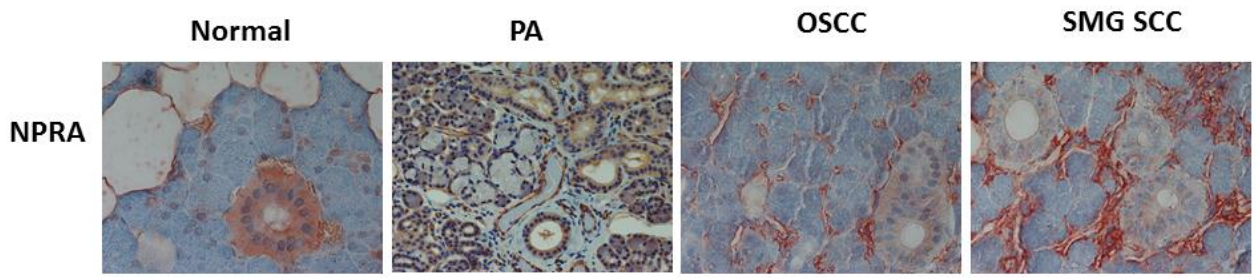

Fig. 2. *NPRA expression in a subset of human submandibular glands from patients with normal SMGs, Pleomorphic adenoma (PA), Oral squamous cell carcinoma (OSCC) and SMG squamous cell carcinoma (SMG-SCC). Note the high expression of NPRA in the periacinar regions of SMG-SCC compared to other categories of tissues.*

an important clinical problem with symptoms ranging from vomiting to confusion, seizures and coma. While a number of small cell lung cancers have been shown to ectopically produce ANP (10, 11) and exhibit hyponatremia, such clinical manifestation has not yet been reported for cases of SMG SSC.

Characterizing the cells expressing *NPPA* and *NPR1* in SMG SCC and OSCC respectively, should help understand the differences between these SCC of distinct origin at the molecular level. Modulation of the NP system in SMG SCC versus OSCC could take place within any of the cell types present within the tumor: neoplastic or non-neoplastic resident cells, endothelial cells or infiltrated immune cells. Macrophages, which are key players in malignant tumors, may be good candidates for *NPPA* upregulation in SMG SCC. Indeed, murine macrophages secrete ANP, and ANP expression in mouse macrophages has been shown to be regulated by compounds affecting macrophage function (1) - could this be happening also in humans? More work is needed in this direction before arriving at a final conclusion.

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