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# MiR-455-3p, miR-150 and miR- 375 are aberrantly expressed in salivary gland adenoid cystic carcinoma and polymorphous adenocarcinoma

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**Running Title: MiRNAs in salivary gland cancers**

**Key words**

**miRNA, salivary glands, adenoid cystic carcinoma, polymorphous adenocarcinoma, miR-150, miR-375, miR-455-3p**

**Abstract**

Background: Adenoid cystic carcinoma (AdCC) and polymorphous adenocarcinoma (PAC) are included among the most common salivary gland cancers. They share clinical and histological characteristics, making their diagnosis challenging in specific cases. MicroRNAs (miRNA) are short, non-coding RNA sequences of 19 to 25 nucleotides in length that are involved in post-transcriptional protein expression. They have been shown to play important roles in neoplastic and non-neoplastic processes and have been suggested as diagnostic and prognostic markers.

Methods: This study, using *quantitative RT-PCR,* investigated miR-150, miR-455-3p and miR-375 expression, in order to identify a possible molecular distinction between AdCC and PAC.

Results: miRNA-150 and miRNA-375 expression was significantly decreased in AdCC and PAC compared with salivary gland tissue controls, whilst miRNA-455-3p showed significantly increased expression in AdCC when compared to PAC, ($p < 0.05$). miR-150, miR-357 and miR-455-3p expression in AdCC, PAC and control was not associated with age, gender nor with anatomic site (major and minor salivary glands) ($p>0.05$).
Conclusion: MiR-455-3p could be used as a complimentary tool in the diagnosis of challenging AdCC cases.

## Introduction

Polymorphous adenocarcinoma (PAC), previously named as a polymorphous low grade adenocarcinoma (PLGA), a somewhat under investigated lesion, is defined as a malignant salivary gland neoplasm characterized by its cytological uniformity, diverse morphology and low metastatic potential [1] Adenoid cystic carcinoma (AdCC), a malignant neoplasm composed of epithelial and myoepithelial cells that may present as tubular, cribriform or solid morphology, is notoriously difficult to treat and is often associated with poor prognosis [2].

Whilst an inexperienced pathologist may struggle to differentiate between PAC and AdCC when analysing small incisional biopsies from the minor salivary glands, an accurate and correct diagnosis between these two lesions is paramount due to the different treatment strategies applied, specifically surgical excision vs. surgical excision with adjuvant radiotherapy for PAC and AdCC, respectively [3-5]. One should observe, however, that with recent advances in immunohistochemistry and the use of new antibodies, this diagnostic challenge has become less problematic [6,7] .

MicroRNAs (miRNA) are short, non-coding RNA sequences of 19 to 25 nucleotides in length that are involved in post-transcriptional protein expression [8] They play important roles in many neoplastic processes, and have been proposed as potential diagnostic and prognostic markers [9] The presence of altered miRNA expression in the salivary glands disorders has been described for both Sjögren's Syndrome [10] and AdCC [11]. Importantly, in AdCC, different expression patterns have been associated with poor clinical outcome.

Persson et al. (2009) [12] showed that the MYB oncogene is overexpressed when fused with the NFIB gene in AdCC. This gene fusion occurs due to a recurrent translocation observed in AdCC of both head/neck and breast. A likely activation mechanism is the expression profile of the miRNAs mapped to the translocation site. MiR-150 analysis, specifically, was found to be downregulated in AdCC when compared to normal salivary gland.

Mitani et. al. (2013) [11], in a study based on miRNA expression in AdCC and poor prognosis, reported that let-7a and miR-150 were significantly associated with tumour size, nodal involvement, and tumour stage. In addition, they described the up-regulation and down-regulation of miRNA miR-455-3p and miR-375, respectively.

Therefore, the aim of the present study was to compare miRNA expression profiles of the aforementioned miRNAs (miR-150, miR-455-3p and miR-375), in order to identify a molecular distinction between AdCC and PAC.

## Materials and Methods

*Ethics approval*

This study was approved by the Ethics in Research Committee of São Leopoldo Mandic Institute and Research Centre, Campinas, SP, Brazil (number 427.544), the Ethical Committee of the Northern Ostrobothnia Hospital District June 2005 (33/2005 NOHD) and the Institutional Research Ethics Board (Dnro 31/13/03/02/2010).

*Case selection*

Haematoxylin and Eosin (H&E) stained slides were obtained from the Departments of Oral Pathology, São Leopoldo Mandic Research Centre and Oulu and Helsinki University Hospitals, Finland. Two pathologists reviewed five, fifteen and five cases of normal salivary gland tissue (control), AdCC and PAC, respectively, all with sufficient material to perform the study. Cases suspicious of cribriform adenocarcinoma were excluded. Normal salivary gland tissue (control) was obtained from the periphery of lesions diagnosed as mucoceles, having confirmed the absence of inflammation, and granulation tissue, hence considered normal salivary gland. Table 1 shows the clinical information related to each case used.

*RNA extraction*

Ten μm sections were taken from the formalin-fixed paraffin embedded (FFPE) biopsy of each aforementioned case. Each section was compared with its H&E stained counterpart, under a light microscope, in order to identify the areas containing tumour. The tumour was manually dissected from the slide, using a scalpel blade (no. 15), and

placed in labelled 2 ml Eppendorf tubes. In order to perform RNA extraction, the AdCC and PAC specimens required a minimum of 70% tumour cells.

Total RNA was extracted using the miRNeasy FFPE kit (Qiagen®, Hilden, Germany), as per the manufacturer's instructions, and quantified using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States)

*Quantitative RT-PCR of miRNA*

Two hundred ng of the total RNA was used for each sample for cDNA synthesis, using the miScript II RT Kit (Qiagen®), as per the manufacturer's instructions. Three ng from each cDNA sample was then amplified via RT-PCR using the miScript SYBR® Green PCR kit (Qiagen®), according to the manufacturer's instructions. The universal primer provided with the miScript SYBR® Green PCR kit (Qiagen®) was used alongside primers for miR-150, miR-375 and miR-455-3p (Metabion, Steinkirchen, Germany). The primers were designed using the BLAST software [13]. Mean CT was derived from triplicate reactions, with the expression of each miRNA determined by the $2^{-\Delta\Delta Ct}$ method [14], where RNU6-2, provided by Qiagen, was used as an endogenous control. Primers sequences are presented in Table 2.

*Statistical Analysis*

Statistical analysis was carried out using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Owing to a small number of samples, data were analysed using non-parametric tests [15], namely Kruskal-Wallis one-way ANOVA. A significant difference was considered when $p < 0.05$.

## Results

Both miRNA-150 and miRNA-375 expressions were significantly decreased in AdCC and PAC compared with the control salivary glands tissue (Fig 1, A and B). miR-455-3p was highly expressed in AdCC when compared to PAC, ($p < 0.05$), although some AdCC samples did show low expression similar to PAC samples. There was no statistical difference between AdCC and PAC miR-455-3p expression when compared to the control (Fig 1, C). miR-150, miR-357 and miR-455-3p expression in AdCC, PAC and control was not associated with age, gender nor with anatomic site (major and minor salivary glands) ($p>0.05$).

## Discussion

This study evaluated the expression of the microRNAs miR-150, miR-375 and miR-455-3p in malignant neoplasms of the salivary glands, in comparison to normal small salivary gland tissue as controls. The initial aim was to find possible aberrantly expressed miRNA candidates that could help to distinguish, on a molecular basis, AdCC from PAC, since they may represent a diagnostic challenge for pathologists. After analysis, miRNA 455-3p was observed as a possible candidate to be used as a complementary tool in distinguishing the tumours studied. Additionally, it is likely that miRNAs 150, 375 and 455-3p are involved in their pathogenesis, since their expression was aberrant when compared to normal salivary glands.

The analysis of miR-150 expression levels were based on studies by Persson et al. (2009) [12] and Mitani et al. (2013) [11], in which they described its decreased expression in AdCC. In the present study, however, a reduced expression of this microRNA was observed in both AdCC and PAC compared to normal salivary gland tissue. The fact that both malignant tumours revealed significantly decreased expression levels, without difference between them ($p > 0.05$), suggests that miR-150 is not a viable option to apply as a stand-alone microRNA to differentiate between AdCC and PAC. Nevertheless, the finding indicates that it may be involved in the pathogenesis of these salivary gland tumours. MMP14 has been described as a target for miR-150-5p. This membrane-type MMP activates MMP2 and is involved in tumour invasion in epithelial ovarian carcinoma [16] Although AdCC and PAC do not metastasize as frequently as other adenocarcinomas, both shares marked tissue invasion capability characterized by frequent perineural invasion [1]. Morbidity related to these tumours occurs often due to local relapse and may be related to enhanced MMP activity.

The same authors reported a significant under-expression of miR-375 in salivary AdCC, which was also demonstrated in the current study. MiR-375 was originally characterised as a pancreatic islet cell-specific molecule whose function was to regulate glucose-induced insulin secretion [17]. Following genome-wide miRNA expression profiling of various tissues, miR-375 has been described as being significantly reduced in different carcinomas, including gastric and head and neck carcinoma, amongst others [18,19] , where it acts as a tumour suppressor by targeting important oncogenes (*AEG-1, PDK1, ATG7, IGF1R, JAK2, 14-3-3Z, YAP1* and *SP1*), as well as being involved in cell proliferation, apoptosis, invasion and migration [20]. Interestingly, miR-375 is downregulated in renal cancer cells where it targets PKD1 causing reduction of cell proliferation, migration and invasion [21]. KRAS-induced activation of the canonical NF-$\kappa$

B pathway has been described as one mechanism that causes increased PRKD1 expression in pancreatic cancer [22]. Hot spot point mutations (E710D) of the *PRKD1* gene are considered the most important driver mutations in PACs, although the oncogenic mechanism is still not well understood [23,24]. The current study allows one to hypothesise that reduced miR-375 expression could allow PKD1 overexpression, hence generating oncogenic advantages for PAC cells. Although PRKD1 aberrations have not been described in AdCC, its overexpression due to miR-375 downregulation could contribute to anti-apoptotic events, which would also lead to biological advantages for AdCC cells.

In this study, miR-455-3p showed a significantly increased expression in AdCC when compared to PAC, which corroborates the findings by Mitani et al. (2013) [11]. miR-455-3p has been reported as overexpressed in triple negative breast carcinomas, where it targets the *EI24* (*autophagy associated transmembrane gene*) gene, a supposed tumour suppressor gene downstream of TP53 presenting pro-apoptotic activity [25,26]. These anti-apoptotic events have been shown to occur frequently in AdCC and are correlated with prognosis [27].

Several authors have reported the difficulties faced by pathologists in the diagnosis of salivary gland tumours from incisional biopsies, specifically AdCC and PAC, an important challenge owing to the difference in biological behaviour of these tumours. In recent years, publications have focused on the importance of immunohistochemistry in order to differentiate them [6,28-30].

Recently, the discovery of hot spot point mutations (E710D) in the *PRKD1* gene in approximately 70% cases of PAC has provided the possibility of the development of new diagnostic markers for this tumour [23,24]. It is important to mention, however, that the identification of such mutations requires the use of dideoxynucleotide sequencing

which may preclude its use as a diagnostic tool in several laboratories [24]. On the other hand, the analysis of miRNA 455-3p expression can be performed using a cheap and less sophisticated technique (quantitative PCR) and could be used as a complementary tool, combined with immunohistochemistry, to aid in diagnosis of more challenging cases.

Studies based on global microRNA expression in salivary gland neoplasms could reveal microRNAs that are important in the pathogenesis of these lesions, facilitating their diagnosis while improving their prognosis. MiR-455-3p could be used as a complimentary tool to diagnose challenging cases of AdCC.

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Conflict of Interest

The authors declare no potential conflict of interests.

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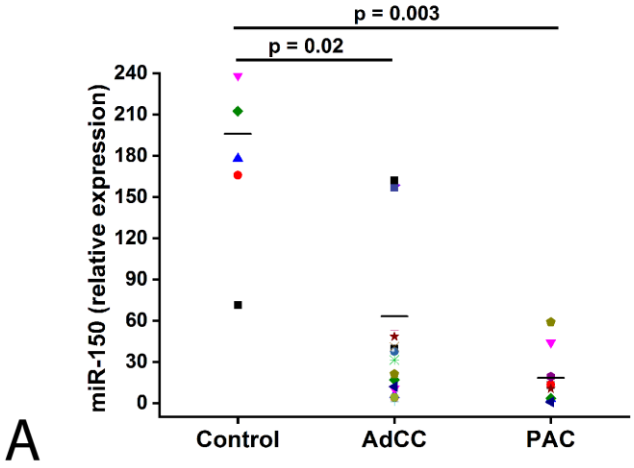
| Sample diagnosis | Age in years | Sex | Location of the salivary gland/tumor |
|---|---|---|---|
| Normal | 34 | Male | Lower lip |
| Normal | 26 | Male | Floor of the mouth |
| Normal | 30 | Male | Lower lip |
| Normal | 13 | Male | Lower lip |
| Normal | 65 | Female | Lower lip |
| ACC | 77 | Female | Parotid |
| ACC | 60 | Male | Submandibular |
| ACC | 68 | Female | Parotid |
| ACC | 54 | Female | Submandibular |
| ACC | 79 | Female | Parotid |
| ACC | 82 | Female | Parotid |
| ACC | 80 | Female | Palate |
| ACC | 72 | Female | Palate |
| ACC | No Information | Female | Palate |
| ACC | 46 | Female | Floor of the mouth |
| ACC | No Information | No Information | Parotid |
| ACC | No Information | No Information | No Information |
| ACC | 57 | Female | Floor of the mouth |
| ACC | 48 | Female | Hard palate |
| ACC | 19 | Male | Hard/soft palate |
| ACC | 55 | Male | Right maxilla |
| ACC | 62 | Male | Maxilla |
| ACC | 38 | Female | Hard palate |
| ACC | 68 | Male | Metastasis lymph node |
| PAC | 62 | Female | Right buccal mucosa |
| PAC | 59 | Male | Tongue |
| PAC | 50 | Female | Upper lip |
| PAC | 70 | Female | Hard palate |
| PAC | 68 | Male | Retromolar region |
| PAC | 63 | Male | Palate |

| | | | |
|---|---|---|---|
| PAC | 72 | Female | Palate |
| PAC | 64 | Male | Palate |
| PAC | 70 | Male | Palate |

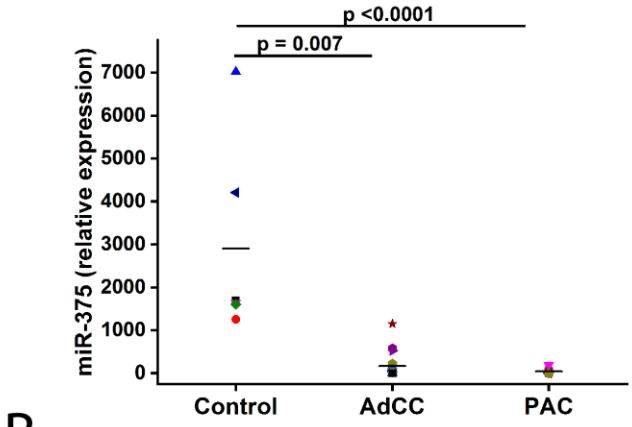
**Table 1:** Clinical data obtained for the samples used in this study.

| Primers | Primer sequence (Forward) |
|---|---|
| miR-455 | 5-GGC AGT CCA TGG GCA T-3 |
| miR-375 | 5-AGT TTG TTC GTT CGG CTC-3 |
| miR-150 | 5-GTC TCC CAA CCC TTG TAC-3 |

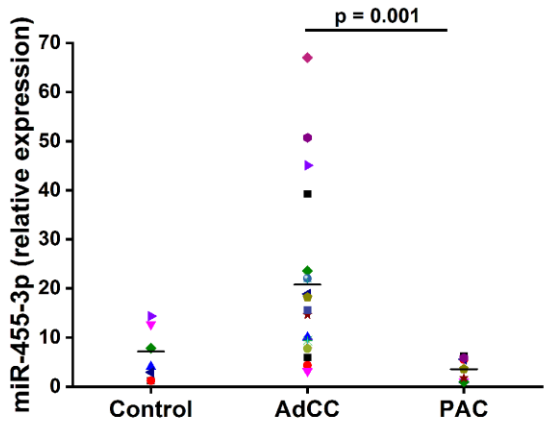
Table 2. Primers sequence used in this study




A




B

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