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RESEARCH

Breast- and Salivary Gland-Derived Adenoid Cystic Carcinomas: Potential Post-Transcriptional Divergencies. A Pilot Study Based on miRNA Expression Profiling of Four Cases and Review of the Potential Relevance of the Findings

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Abstract Adenoid cystic carcinoma (ACC) is a malignant tumor of the salivary glands but identical tumors can also arise from the breast. Despite their similar histomorphological appearance the salivary gland- and the breast-derived forms differ in their clinical features: while ACC of the salivary glands (sACC) have an agressive clinical course, the breast-derived form (bACC) shows a very favourable clinical outcome. To date no exact molecular alterations have yet been identified which would explain the diverse clinical features of the ACCs of different origin. In our pilot experiment we investigated the post-transcriptional features of ACC cases by performing microRNA-profiling on 2-2 bACC and sACC tissues and on 1-1 normal breast and salivary gland tissue. By comparing the microRNA-profiles of the investigated samples we identified microRNAs which were expressed differently in bACC and sACC cases according to their normal controls: 7 microRNAs were overexpressed in sACC cases and downexpressed in bACC tumors (let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195, miR-768-3) while 9 microRNAs were downexpressed in sACC cases and overexpressed in bACC tissues (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c, miR-768-5p, miR-1280 and miR-1826) relative to their controls. We also identified 8 microRNAs which were only expressed in sACCs and one microRNA (miR-1234) which was only absent in sACC cases. By target predictor online databases potential targets of the these microRNAs were detected to identify genes that may play central role in the diverse cinical outcome of bACC and sACC cases.

Keywords Adenoid cystic carcinoma · microRNA · Breast · Salivary gland

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Introduction

Adenoid cystic carcinoma (ACC) is a malignant tumor which can derive from different sites of the body such as salivary glands, prostate, vagina, cervix, lacrimal gland, skin or breast [1–6]. ACC of the breast (bACC) belongs to the subgroup of triple negative breast cancers (TNBCs), which express no estrogen or progesterone receptors (ER; PgR) and also lack the human epidermal growth factor receptor 2 (Her-2). Although in the past some authors handled TNBCs as a uniform entity, e.g. tumors with agressive phenotypes and poor clinical outcome [7], bACC is almost an indolent tumor with a 10 year overall survival rate of about 90 % [8]. Despite of its similar histopathologic appearance (Fig. 1), the clinical outcome of ACC differs extremely according to its origin: while bACC has a very favourable prognosis, salivary gland-

derived ACC (sACC) represents a locally agressive tumor with a high relapse rate and an overall 10-year survival rate of less than 50 % [9]. Several molecular features were found to be common in bACCs and sACCs, such as translocation between chromosome 6 and 9: t(6;9)(q22-23;p23-24), which leads to the formation of the MYB-NFIB fusion (a fusion between myeloblastosis viral oncogene homolog (avian) and nuclear factor I/B genes) [10]; overexpression of c-KIT oncogene; the loss of 6q and gains at 14q, 16q, 20p, 20q loci [11]. On the other hand, differences at the molecular level between the two tumors have also been reported, such as the mutation of PTEN (phosphatase and tensin homolog) and PIK3CA (phosphoinositide-3-kinase, catalytic, alpha polypeptide), which seem to be present only in bACC cases, while the overexpression of CCND1 (cyclin D1) and pAKT (v-akt murine thymoma viral oncogene homolog 1, also known as PKB) appear only in sACCs [11]. As none of the previously described molecular features (at gene and protein level) explain the different clinical outcome of adenoid cystic carcinomas of different origins, the question emerged if underlying posttranscriptional mechanisms could partially elucidate these diversities. In the last decades short non-coding RNA molecules (*microRNAs/miRNAs*) have been identified as posttranscriptional gene regulators which have the ability to repress mRNAs' translation into proteins [12, 13]. Although basically miRNAs are gene repressors, rarely they also can serve as activators of genes such as miR-369-3 [14] or miR-373 [15]. By the posttranscriptional regulation of genes miRNAs are involved in numerous physiological functions (embryogenesis [16, 17], glucose or lipid metabolism [18] and they also play central role in cardiovascular diseases or metabolic syndrome [19]. In the last decades, miRNAs have been identified as very important participants in different types of cancers as well [20–22] and so, they can be used as diangostic or therapeutic tools in malignant tumors [23, 24]. As the expression of miRNAs show tissue-specific expression, breast- and salivary gland-derived adenoid cystic carcinomas may express miRNAs differently which may serve as further explanation for the extreme clinical outcome of the two tumors. In our pilot experiment, we performed microRNA-profiling on breast- and salivary gland derived ACC cases to elucidate underlying differences between these tumors. After identifying miRNAs which were differently expressed in the tumors of the two organs, miRNA target prediction was performed to elucidate underlying posttranscriptional mechanisms which could potentially explain the extreme clinical outcome of adenoid cystic carcinoma of salivary glands and the breast.

Materials and Methods

ACC cases have been identified from the archives of the 2nd Department of Pathology at Semmelweis University, Budapest. Two consultant pathology departments contributed to our investigations by sending breast-derived adenoid cystic carcinoma cases for consultation purpose. Formalin-fixed, paraffin-embedded (FFPE) tissues of two sACC and two bACC cases and their normal control tissues (one case per each control) were investigated. The two bACC cases were women (64 and 67 years old) and so were the sACC cases (31 and 61 years old). One of the salivary gland-derived ACCs arose from minor salivary glands of the maxillar sinus, another from the submandibular gland. (Pathomorphological characteristics of the cases are shown in Table 1.) ER-, PgR- and Her2 and Ki67 status of the samples were determined by immunohistochemical (IHC) analysis. IHC reactions were performed by using the automated Ventana ES immunostainer system (VentanaMedical Systems Inc., Tucson, AZ, USA) according to the conditions and materials shown in Table 2. Ensvision system (K4001 from DAKO) was applied for visualisation and as chromogen 2,3-diamino-benzidine (CMD401 from CellMarque, Rocklin, CA, USA) had been used. As controls we have investigated healthy breast tissue from plastic surgery material and healthy submandibular salivary gland tissue from a woman who underwent radical neck dissection.

Representative areas of the tumors were chosen for miRNA expression profiling after pathological evaluation of the slides. Macrodissection was needed only in bACC cases (elimination of adjacent non-tumorous tissue). From each sample, ten 5 μm

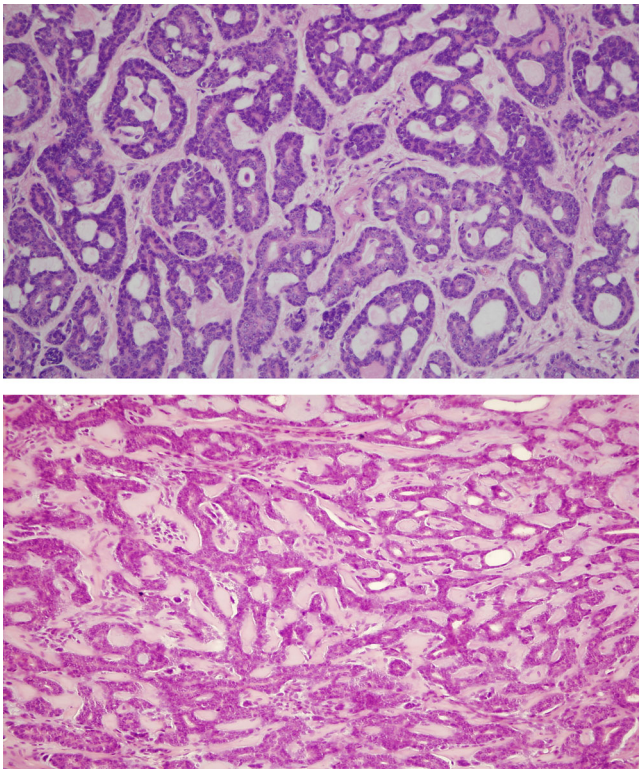

Fig. 1 Microscopic images of breast-derived (*above*) and salivary gland-derived (*below*) adenoid cystic carcinoma (Hematoxylin-Eosin; 20× magnification)

Table 1 Clinical and pathological features of the investigated cases

Tissue type	Age	Sex	Grade	LN	Primary (P)/Recurrent (R)	Recurrence(s)
NB*	42	F	–	–	–	–
bACC1	67	F	III	unknown	P	unknown
bACC2	64	F	I	unknown	P	unknown
NS**	56	M	–	–	–	–
sACC1	61	F	II	x	R (P: 14 years before)	numerous
sACC2	31	F	II	unknown	R (P: 12 years before)	numerous

* *NB* normal breast tissue, ** *NS* normal salivary gland tissue

thick sections were used for total RNA extraction by Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Quality control was performed by Agilent Bioanalyzer Pico6000 chip kit (Agilent, Palo Alto, CA, USA) and RNA was quantifyed by Qubit fluorometric RNA assay (Life Technologies, Gand Island, NY, USA). By applying Genisphere HSP labeling kit (Genisphere, Hatfield, PA, USA) 1 μg of total RNA from each sample was labeled with biotin according to the instructions of the manufacturer. The labelled RNA-isolates were hybridized with their complementary strands on Affymetrix miRNA arrays (Affymetrix, Santa Clara, CA, USA). The expression level of the miRNAs correlated with the grade of hybridization of each sample. Hybridization rate was made detectable by the presence of streptavidin-phycoerythrin complex which was bound to the biotin-labelled RNA. The photointensity of each reaction correlated with the quantity of miRNAs in each sample, which was evaluated by miRNA QC Tool software (Affymetrix, Santa Clara, CA, USA).

Hierarchial clustering was performed and heatmap was displayed using Genesis 1.7.6. Software (Techn. Univ. Graz, Inst. for Genomics and Bioinformatics, Graz, Austria) software on those miRNAs that were present in all of the investigated samples. The averaged photointensity (binary logarithm values) of miRNAs in tumorous tissues was normalized to the photointensity in their normal control.

Multistep target identification was carried out by Ingenuity Pathway Analysis® (IPA) Software (Ingenuity Systems, Inc., Redwood City, CA, USA) and online target predictor databases. IPA® analysis was performed on those 57 microRNAs which were present in each investigated sample, while online target prediction was only accomplished on those microRNAs which differed in breast- and salivary gland-derived tissues and on those which were only present or only absent in sACC cases. For online target prediction we used the miRecords database [25] which integrates the results of 10 different target predictor websites (DIANA-microT, MicroInspector, miRanda, MirTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargetScanS) and enables the identification of already validated miRNA targets, not only the predictions. If no validated target of a special miRNA was reported in this database, potential targets of the miRNAs were identified by Tarbase software [26] and in some cases TargetScan [27–30] and miRDB [31, 32] were also used.

Results

Immunohistochemistry

None of the investigated tumorous tissues expressed ER or PgR and all of them had negative Her2-status. Ki67-index of one of the the bACC cases was 50 %, while the other <1 % proliferative activity. One of the sACC cases also showed <1 % Ki67-positivity, while the proliferative activity of the other case was 7 %.

Affymetrix® GeneChip

Out of the investigated 847 human microRNAs on Affymetrix GeneChip 275 human miRNAs were present in at least one

Table 2 Conditions of the applied immunohistochemical analysis

Antibody	Manifacturer	ID No.	Antigen retrieval	Dilution of the PA*
ER	Novocastra	6003537	MW** 90 min	1:100
PgR	Novocastra	6010350	MW** 90 min	1:200
Her2/4B5	Ventana	8002996	MW** 30 min	ready to use
Ki67/MIB1	Dako	M7240	MW** 30 min	1:100

* *PA* primary antibody, ** *MW* microwave

Target Retrieval Solution: S1699 from DAKO, Carpinteria, CA, USA

investigated sample from which 57 miRNAs were expressed in each investigated tissue. To define which of these 57 microRNAs differed consistently between breast- and salivary gland-derived tissues Genesis hierarchial clustering analysis was performed. According to the results of this analysis the level of 7 miRNAs (let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195, and miR-768-3p) decreased in both bACC cases, while increased in sACCs in comparison with their control tissues. These microRNAs were enrolled in microRNA-subgroup "A". In contrast the expression of another 9 miRNAs (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c, miR-768-5p, miR-1280 and miR-1826) increased in breast-derived ACCs and decreased in sACCs compared to their control tissues and were listed in microRNA-subgroup "B". (Fig. 2)

According to the Affymetrix GeneChip analysis nine miRNAs had specific distribution: 8 of these were detectable only in sACC cases (miR-181a-2*, miR-125a-3p, miR-1275, miR-134, miR-17*, miR-206, miR-379, miR-382), while one microRNA (miR-1234) was only absent in the sACCs.

Target Prediction

Ingenuity Pathway Analysis® (IPA)

Based on the results of the Affymetrix® GeneChip we performed IPA® analysis on those 57 miRNAs which were present in each investigated sample (Table 3). IPA® is a web-based, comprehensive database that integrates all published data of genes, proteins, chemicals and drugs. According to this type of analysis the diverse expression of the 57 miRNAs in breast- and salivary gland-derived cases may potentially affect different intracellular pathways. While in bACCs miRNAs were principally connected to TP53 (tumor protein p53), DGCR8 (DiGeorge syndrome critical region gene 8), LAMTOR3 (late endosomal/lysosomal adaptor, MAPK and MTOR activator 3), AKT (the PDGF-activated serine-threonine-kinase) and PRIM1 (primase, DNA, polypeptide 1) genes, in salivary glands PTEN, ESR1 (estrogen receptor-1), IGF1R (insulin-like growth factor-1) and the transcription factor FOXO1 (forkhead box O1) seem to be the most affected targets (see the appendix 1 and 2). The flowsheet of our whole procedure is depicted in Fig. 3.

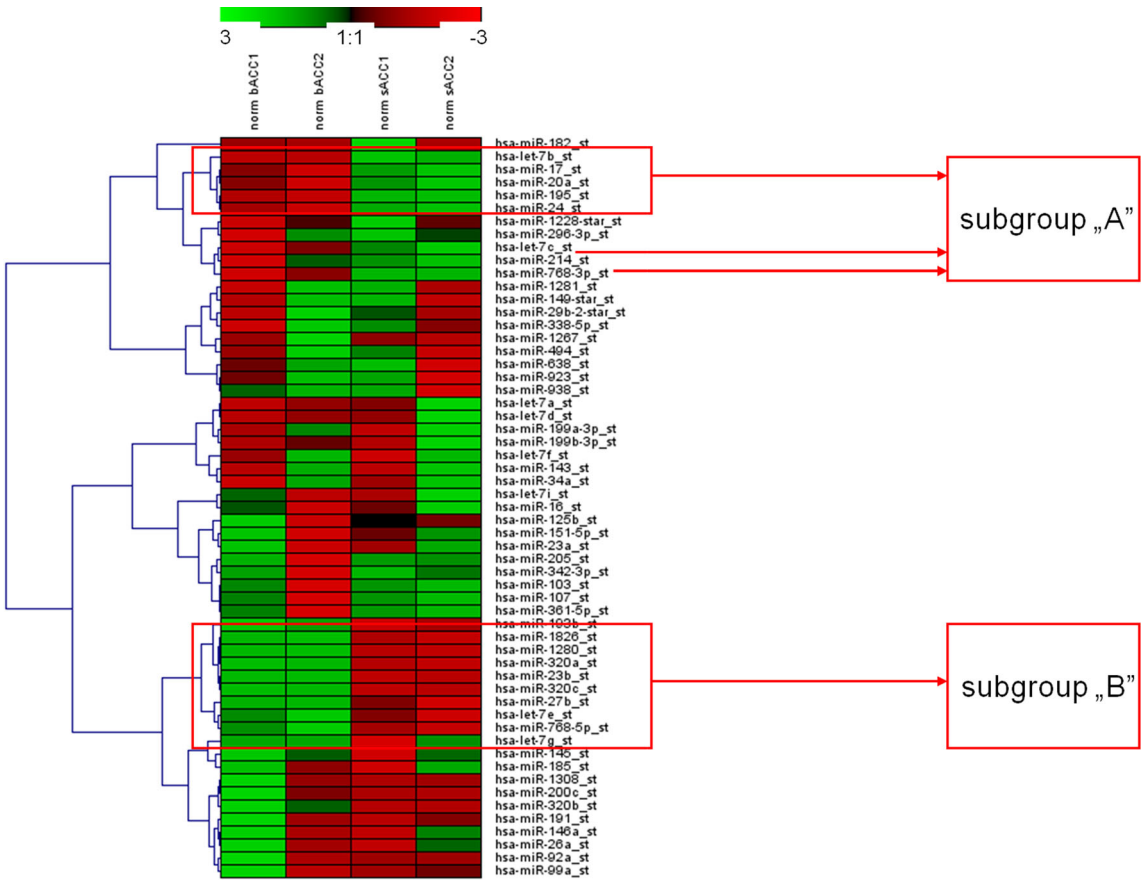

Fig. 2 Genesis Heat Map analysis of the expression of those 57 miRNAs, which were expressed in all of the investigated cases. *Green*: overexpressed accoring to its normal control. *Red*: downexpressed according to its control

Table 3 The list of the 57 miRNAs that were present in each investigated sample

hsa-let-7e_st	hsa-miR-182_st	hsa-miR-320a_st
hsa-let-7f_st	hsa-miR-1826_st	hsa-miR-320b_st
hsa-let-7g_st	hsa-miR-185_st	hsa-miR-320c_st
hsa-let-7i_st	hsa-miR-191_st	hsa-miR-338-5p_st
hsa-miR-103_st	hsa-miR-193b_st	hsa-miR-342-3p_st
hsa-miR-107_st	hsa-miR-195_st	hsa-miR-34a_st
hsa-miR-1228-star_st	hsa-miR-199a-3p_st	hsa-miR-361-5p_st
hsa-miR-125b_st	hsa-miR-199b-3p_st	hsa-miR-494_st
hsa-miR-1267_st	hsa-miR-200c_st	hsa-miR-638_st
hsa-miR-1280_st	hsa-miR-205_st	hsa-miR-768-3p_st
hsa-miR-1281_st	hsa-miR-20a_st	hsa-miR-768-5p_st
hsa-miR-1308_st	hsa-miR-214_st	hsa-miR-923_st
hsa-miR-143_st	hsa-miR-23a_st	hsa-miR-92a_st
hsa-miR-145_st	hsa-miR-23b_st	hsa-miR-938_st
hsa-miR-146a_st	hsa-miR-24_st	hsa-miR-99a_st

Target Prediction by Online Databases

For target prediction by online databases 25 miRNAs were chosen: members of subgroup "A" (let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195, and miR-768-3p), members of subgroup "B" (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c, miR-768- 5p, miR-1280 and miR-1826), miR-1234 which was expressed in every investigated tissues but sACC cases and those miRNAs which were only present in sACCs (miR-181a-2*, miR-125a-3p, miR-1275, miR-134, miR-17*, miR-206, miR-379, miR-382).

Target Prediction of miRNAs with no Expression in sACC Cases: miR-1234 According to the results of miRecords no validated targets of miR-1234 have been identified, but predictions of TargetScan refer to its regulatory role in PAK6 (p21 protein-activated kinase 6) and DAB2IP (DAB2 interacting protein).

Target Prediction of miRNAs Expressed only in sACC Cases: miR-181a-2, miR-1275, miR-125a-3p, miR-17*, miR-206, miR-379, miR-134 and miR-382* As they were only present in sACCs, miR-181a-2*, miR-1275, miR-125a-3p, miR-17*, miR-206, miR-379, miR-134 and miR-382 may carry essential information about the differences between salivary gland- and breast-derived adenoid cystic carcinomas. Out of this 8 miRNAs we found already validated target genes only in the case of miR-181a-2*, miR-206, miR-379 and miR-382. This database mention miR-181a-2* (an immature form of miR-181a) as a validated regulator of BCL2 (B-cell CLL/lymphoma 2), ESR1, HOXA11 (homeobox A11), CDKN1B (cyclin-dependent kinase inhibitor 1B/p27/KIP1), PLAG1 (pleiomorphic adenoma gene 1), CDX2 (caudal type homeobox 2), GATA6 (GATA binding protein 6) and NLK (nemo-like kinase) genes. Some experiments have reported miR-206 as regulator of ESR1, MET (met proto-oncogene), TAC1 (tachykinin, precursor 1) and GJA1 (gap junction protein, alpha 1). According to miRecords miR-379 has only one validated target: re2737 which is an SNP variant of the IC53 gene. NC_001802 is a sequence which is interestingly identical with a part of the HIV1 (human immunodeficiency virus 1) and is reported as a potential target of miR-382. Interestingly only ESR1 has more than one potential regulators from this group: miR-181a-2* and miR-206. In the case of miR-1275, miR-125a-3p, miR-134 and miR-17* no validated targets were represent in the database of miRecords, so we needed to lean on the predictions of other databases. According to TargetScan miR-1275 was presented as a potential regulator of IGF1 (insulin-like growth factor 1/somatomedin C), GDF6 (growth differentiation factor 6) and IGFBP5 (insulin-like growth factor binding protein 5) Sequence-based predictions suggest that MAPK1 and BRCA1 (breast cancer1) may be potential targets of miR-125a-3p, while the immature form of miR-17 (miR-17*) may regulate the apoptosis facilitator BCL2L11/BCL2-like 11 according to TargetScan. Interesingly, TargetScan reports miR-134 as a potential regulator of KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene.

Target Prediction of miRNAs with Different Distribution in bACC and sACC Cases Target prediction was also performed in the cases of miRNAs belonging to subgroup "A" (let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195, miR-768-3) and subrgoup "B" (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c, miR-768-5p, miR-1280 and miR-1826). Based on the data of miRBase database four miRNAs have been excluded from further investigation: the mature and immature form of hsa-miR-768 (-3p and -5p) because of overlapping with an snoRNA (HBII-239), hsa-miR-1826 which is a fragment of the 5.8S rRNA and miR-1280, as it has been reported as a fragment of a tRNA. Analysing the database of miRecords we have found several already validated target genes that seem to be regulated by subgroup "A" and "B" members. Table 4 summarizes those genes that were present as the targets of more than one microRNA, and in Table 5 those genes were collected that were presented as the targets of one single miRNA. Among these we could identify important elements of the cell cycle (CCNA2, CDK4, CDKN1A and CDKN1B), known transcription factors such as E2F1, E2F2, E2F3 and also genes that are necessary in the mechanism of the programmed cell death (BCL7A, p21). ADORA2B was also identified as potential target of miR-24 and AURKB seems to be regulated by miR-27b.

Sixteen (16) genes were found as validated targets of more miRNAs of subgroup "A". One of these genes was CCND1: according to miRecords let-7b, miR-17, miR-20a and miR-

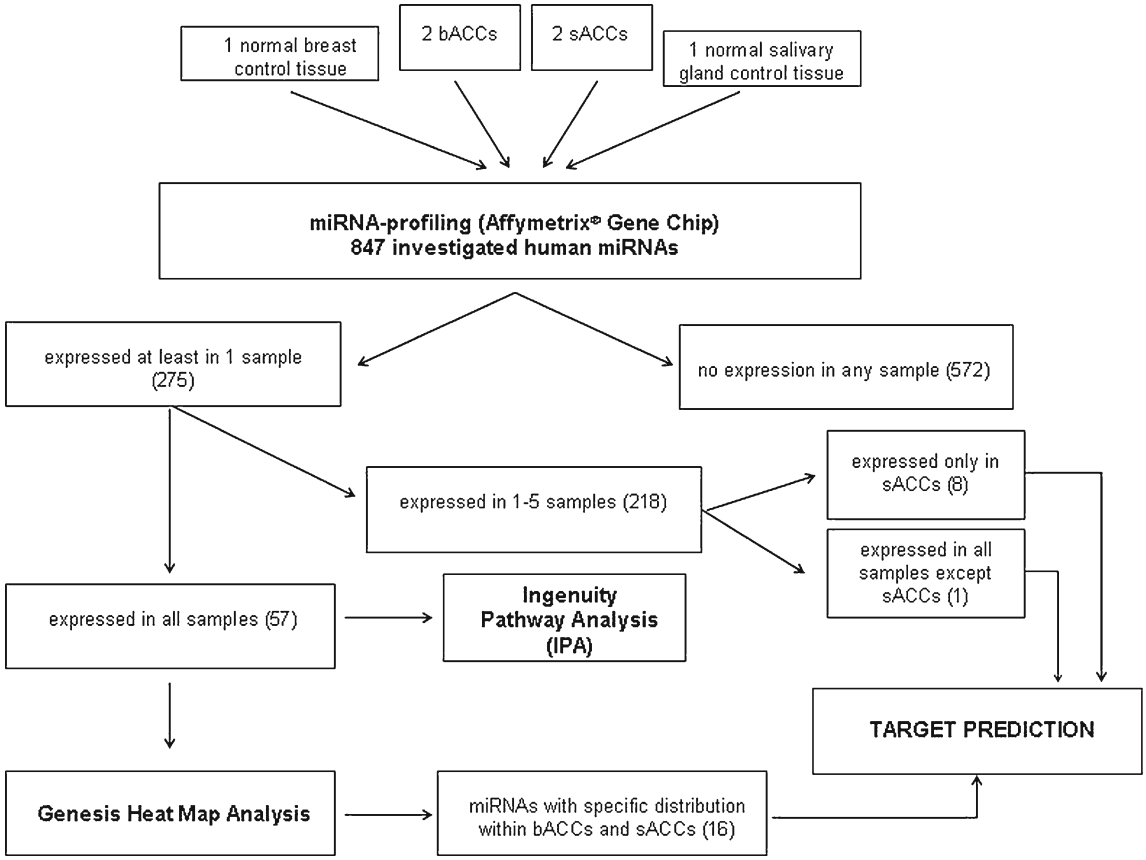

Fig. 3 The flowsheet of the procedure: miRNA-profliling and target prediction on bACC and sACC cases

Table 4 Validated targets of members of subgroup "A" and "B": multiple-regulated genes ("+": interaction has been reported; "–": no interaction has been reported)

	Subgroup "A"								Subgroup "B"			
	GENE NAME	let-7b	let-7c	miR-17	miR-20a	miR-24	miR-195	let-7e	miR-23b	miR-27b	miR-193b	miR-320a
Regulated by members of subgroup A	BCL2	–	–	+	+	–	–	–	–	–	–	–
	BIM	–	–	+	+	–	–	–	–	–	–	–
	BMPR2	–	–	+	+	–	–	–	–	–	–	–
	CCND1	+	–	+	+	–	+	–	–	–	–	–
	CDC25A	+	–	–	–	+	–	–	–	–	–	–
	CDK6	+	–	–	–	–	+	–	–	–	–	–
	IL-8	–	–	+	+	–	–	–	–	–	–	–
	JAK1	–	–	+	+	–	–	–	–	–	–	–
	MAP3K12	–	–	+	+	–	–	–	–	–	–	–
	MEF2D	–	–	+	+	–	–	–	–	–	–	–
	MYC	–	+	–	–	+	–	–	–	–	–	–
	RUNX1	–	–	+	+	–	–	–	–	–	–	–
	VEGFA	–	–	+	+	–	–	–	–	–	–	–
*	NOTCH1	–	–	–	–	+	–	–	+	+	–	–
	HMGA2	+	+	–	–	–	–	+	–	–	–	–
**	PLAU	–	–	–	–	–	–	–	–	+	+	–

Table 5 Validated targets of members of subgroup "A" and "B": genes regulated only by one miRNA ("+": interaction has been reported; "–": no interaction has been reported)

	Subgroup A						Subgroup B				
GENE NAME	let-7b	let-7c	miR-17	miR-20a	miR-24	miR-195	let-7e	miR-23b	miR-27b	miR-193b	miR-320a
ACVR1B	–	–	–	–	+	–	–	–	–	–	–
AURKB		–	–	–	+	–	–	–	–	–	–
BCL7A	+	–	–	–	–	–	–	–	–	–	–
BRCA1	–	–	–	–	+	–	–	–	–	–	–
CCNA2	–	–	–	–	+	–	–	–	–	–	–
CDK4	–	–	–	–	+	–	–	–	–	–	–
CDKN1A	–	–	+	–	–	–	–	–	–	–	–
CDKN2A	–	–	–	–	+	–	–	–	–	–	–
DHFR	–	–	–	–	+	–	–	–	–	–	–
E2F1	–	–	–	+	–	–	–	–	–	–	–
E2F2	–	–	–	–	+	–	–	–	–	–	–
E2F3	–	–	–	–	–	+	–	–	–	–	–
EDG1	–	–	+	–	–	–	–	–	–	–	–
EIF3S1	–	+	–	–	–	–	–	–	–	–	–
FEN1	–	–	–	–	+	–	–	–	–	–	–
KRT	+	–	–	–	–	–	–	–	–	–	–
LIN28	+	–	–	–	–	–	–	–	–	–	–
MAPK14	–	–	–	–	+	–	–	–	–	–	–
MED28	–	+	–	–	–	–	–	–	–	–	–
MKK4	–	–	–	–	+	–	–	–	–	–	–
NCOA3	–	–	+	–	–	–	–	–	–	–	–
p21	–	–	+	–	–	–	–	–	–	–	–
RTCD1	–	+	–	–	–	–	–	–	–	–	–
TGFBR1	–	+	–	–	–	–	–	–	–	–	–
TRIM71	–	+	–	–	–	–	–	–	–	–	–
VIM	–	–	+	–	–	–	–	–	–	–	–
ADORA2B	–	–	–	–	–	–	–	–	+	–	–
CYP1B1	–	–	–	–	–	–	–	–	+	–	–
DAD1	–	–	–	–	–	–	+	–	–	–	–
ESR1	–	–	–	–	–	–	–	–	–	+	–
MCL1	–	–	–	–	–	–	–	–	–	–	+
MET	–	–	–	–	–	–	–	+	–	–	–
MMP13	–	–	–	–	–	–	–	–	+	–	–
PPARG	–	–	–	–	–	–	–	–	+	–	–
RABGAP1L	–	–	–	–	–	–	–	+	–	–	–
SMC1A	–	–	–	–	–	–	+	–	–	–	–
ST14	–	–	–	–	–	–	–	–	+	–	–
WNT1	–	–	–	–	–	–	+	–	–	–	–

195 are already validated regulators of this gene. Interestingly while cyclin D1 may be regulated by 4 members of subgroup "A", none of the subgroup "B" members are reported as its regulators. Further 12 genes (BCL2, BIM, BMPR2, CDC25A, CDK6, IL-8, JAK1, MAP3K12, MEF2D, MYC, RUNX1 and VEGFA) seem to be regulated only by subgroup "A" members. Most of these belong to the targets of miR17/92 miRNA cluster, a special miRNA subgroup. PLAU was the only gene which seem to be regulated only by subgroup "B" members and additionally we identified two genes which seem to be regulated by both "A" and "B" subgroups: HMGA2 which may be the target of let-7b, let-7c (subgroup "A") and let-7e (subgroup "B") and NOTCH1 which may be regulated by miR-24 (subgroup "A"), miR-23b and miR-27b

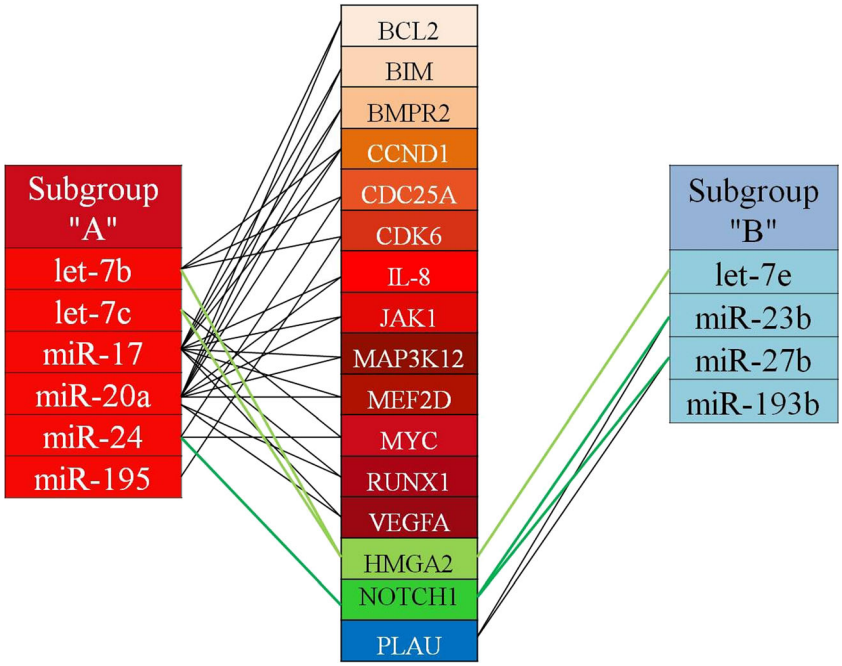

Fig. 4 Multiply regulated target genes and their potential connections with regulating miRNAs

(subgroup "B"). Multiply regulated target genes and their regulator miRNAs are depicted in Fig. 4. (The analized microRNAs and their potential targets genes are summarized in Tables 6 and 7, where the already published information of the potential targets and there genes are also indicated.)

Discussion

Although adenoid cystic carcinoma of the breast is histomorphologically identical with the ACC of salivary glands, the clinical outcome of these tumors differ. Despite of the already reported molecular differences between bACCs and sACCs [11, 33–35], no mechanisms have yet been identified, which could completely explain the diverse clinical course of these tumors. As miRNAs may serve as tumor suppressors or oncogenes in many types of human cancers, we assumed, that they may play important role in the diverse outcome of breast- and salivary gland-derived adenoid cystic carcinomas. In our present pilot study we compared two bACC tissues to one normal breast control tissue and two sACC cases to one normal salivary gland tissue and comparatively analysed the ACC cases. According to our results on this small number of cases we found some differences between breast- and salivary gland-derived tissues: there were microRNAs that were present only in sACC samples (miR-181a-2*, miR-1275, miR-125a-3p, miR-17*, miR-206, miR-379, miR-134 and miR-382), while one of the investigated microRNAs was absent only in sACC tissues (miR-1234). The expression of let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195 was higher in salivary gland-derived tumors than their normal control, while another 6 microRNAs (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c) overexpressed in breast-derived ACCs according to their control tissue. For identifying the potential targets of these miRNAs we used two different ways to evaluate our data: IPA® analysis and target identification by online databases.

The comprehensive miRNA-profiling by IPA® analysis identified some potential differences between adenoid cystic carcinomas of different origin. In breast-derived tissues DGCR8 (an essential element in miRNA biogenesis [36]) was identified as a potential target of miRNAs with specific expression in the breast-derived samples. Although no reports of DGCR8 in adenoid cystic carcinomas have been published yet, this gene has already been connected with cell invasion in breast cancer cell lines [37] and it also seems to be affected in the development of pleomorphic adenomas of the salivary glands [38]. TP53, the well known tumor suppressor gene was also predicted as a potential target of microRNAs showing specific distribution in *bACC* tissues. Mutation status of TP53 has been investigated in benign and malignant salivary gland-derived tissues, and skin-derived tumors. Although one report suggests that TP53 may play role in the progression of ACC cases [39], the applicability of TP53 is controversial in differentiating benign and malignant salivary gland tumors: TP53 mutations are infrequent in these tumor types [40–42]. One publication has reported the potential role of AKT in adenoid cystic carcinoma cases [43], while no literature data was found about the role of LAMTOR3 and PRIM1 in adenoid cystic carcinoma of any origin before. According to the IPA® analysis, in *sACC* cases important cancer-related genes may be regulated by microRNAs found in our system: ESR1, IGF1R, PTEN and FOXO1 were identified

Table 6 Potential target genes of microRNAs identified in our study and previous reports of those in adenoid cystic carcinoma cases I

Gene name	Previous description in ACC cases*	Predictions by our analysis			
		+/–	Tissue type	Method	Potential regulator(s)
DGCR8	no data found	+	1	IPA	let-7c
					miR-195
TP53	39-42	+	1	IPA	miR-195
					miR-320
					miR-17
LAMTOR3	no data found	+	1	IPA	miR-195
AKT	43	+	1	IPA	miR-185
PRIM1	no data found	+	1	IPA	miR-195
					let-7c
PTEN	39	+	2	IPA	miR-17
					miR-20a
IGF1R	no data found	+	2	IPA	miR-145
					miR-99a
FOXO1	no data found	+	2	IPA	miR-145
					miR-182
DAB2IP	no data found	prediction	5	TargetScan	miR-1234
PAK6	no data found	prediction	5		miR-1234
PLAG1	55	+	2	miRecords	miR-181a-2*
ESR1	no data found	+	2	IPA	miR-193b
					miR-17
			2	miRecords	miR-181a-2*
			2		miR-206
			4		miR-193b
HOXA11	no data found	+	2	miRecords	miR-181a-2*
GATA6	no data found	+			
CDX2	no data found	+			
CDKN1B/KIP1	62-64	+			
BCL2	65	+			
NLK	no data found	+			
MET	71	+	2	miRecords	miR-206
TAC1	no data found	+	2		miR-206
GJA1	no data found	+	2		miR-206
IGF1	no data found	prediction	2	TargetScan	miR-1275
IGFBP5	no data found	prediction	2		miR-1275
GDF6	no data found	prediction	2		miR-1275
MAPK1	78, 79	prediction	2		miR-125a-3p
BRCA1	76, 77	prediction	2		
BCL2L11	80	prediction	2		miR-17*
KRAS	no data found	prediction	2		miR-134

in this type of analysis. Although ESR1, IGF1R and FOXO1 are frequently investigated genes in different types of breast cancers [44–47], no reports were found about these in breast- or salivary gland-derived adenoid cystic carcinomas. Molecular features of PTEN were investigated in a low number of cases of adenoid cystic carcinoma [39], but the exact role of this gene in ACC cases is still unexplored. According to the results of IPA® analysis we may hypothesize that the differences among the microRNA-profiles of bACC and sACC tissues may lead to devergencies in the gene expression-profiles of these tissues.

The potential targets of the microRNAs in of our system were also identified by target predictor online databases. Depending on the data of miRecords and TarBase we collected those target genes which were listed as already validated

Table 7 Potential target genes of microRNAs identified in our study and previous reports of those in adenoid cystic carcinoma cases II

CCND1	96	+	3	miRecords	miR-17
					miR-20a
					miR-195
					let-7b
BCL2	65	+	3	miRecords	miR-17
BIM	100	+	3		miR-20a
BMPR2	no data found	+	3		
IL-8	99	+	3		
JAK1	no data found	+	3		
MAP3K12	no data found	+	3		
MEF2D	no data found	+	3		
RUNX1	no data found	+	3		
VEGFA	106, 107	+	3		
CDC25A	no data found	+	3	miRecords	let-7b
					miR-24
CDK6	108	+	3		let-7b
					miR-195
MYC	103	+	3		let-7c
					miR-24
PLAU	no data found	+	4		miR-27b
					miR-193b
NOTCH1	no data found	+	3, 4		miR-24
					miR-23b
					miR-27b
HMGA2	no data found	+	3, 4		let-7b
					let-7c
					let-7e

targets of microRNAs expressed only in sACC samples (miR-181a-2*, miR-1275, miR-125a-3p, miR-17*, miR-206, miR-379, miR-134 and miR-382), absent only in sACC samples (miR-1234) and also of microRNAs of subgroup "A" (let-7b, let-7c, miR-17, miR-20a, miR-24, miR-195) and subgroup "B" (let-7e, miR-23b, miR-27b, miR-193b, miR-320a, miR-320c).

According to TargetScan DAB2IP and PAK6 were predicted as potential targets of miR-1234. Although no previous data refers to the role of the tumor suppressor DAB2IP in adenoid cystic carcinomas, it was previously reported as being inactivated in breast, prostate and lung cancers through methylation [48–50]. The decrease of DAB2IP gene was described in pancreatic cancer cases with wild-type KRAS status [51]. PAK6 was investigated in cancerous prostate tissues before but its potential functions were also reported in neuronal development and PAK6 probably may contribute to the crosstalk between estrogen and androgen receptors in various tissues as well [52–54]. According to our analysis miR-181a-2* may have many potential targets, such as PLAG1. PLAG1 was reported before as being overexpressed in pleomorphic adenomas of salivary glands as a result of a recurrent translocation: t(5;8)(p13;q12) [55]. Although PLAG1 is investigated widely in benign tumors of salivary glands and the thyroid gland, we only could find a few reports about this gene in adenoid cystic carcinoma cases. Despite its proven tumorigenic feature in benign salivary gland-derived tumors [56, 57], the tumorigenic role of PLAG1 in sACC cases could not be proven yet [58]. ESR1 was also reported as a validated target of miR-181a-2*, but this gene may also be regulated by other microRNAs investigated by us in this study: miR-206 and miR-193b. Despite the obvious role of ESR1 in many breast-derived malignant tumors, its function is not at all clear in salivary gland malignancies and we have no data about any potential connection between sACCs and ESR1. Further potential targets of miR-181a-2*, HOXA11, GATA6 and CDX2 take important part in embryogenesis [59–62]. Although no literature data has been found about any potential role of HOXA11 and GATA6 genes in adenoid cystic carcinoma cases, a case report investigates its potential role in skin-derived ACC [63]. CDX2 expression play role in the differentiation of tumors arising from the sinonasal-region [64]. Interestingly, many reports refer to the role of the cell cycle regulator CDKN1B/KIP1 in the development of sACC cases. According to these data downexpression of KIP1 may be in correlation with the development and progression of salivary gland-derived adenoid cystic carcinoma cases [65–67]. As KIP1 seems to be influenced by miR-181a-2* in our sACC cases, this microRNA may play central role in the agressiveness and spreading of sACC tumors. Besides miR-181a-2* the antiapoptotic BCL2 may also be regulated by miR-17, miR-20a (members of "subgroup "A"). As BCL2 may play role in the progression of adenoid cystic carcinoma cases, its regulation by miRNAs may also be an important epigenetic mechanism [68]. No literature data refers to the role of NLK in adenoid cystic carcinomas of any organ. Although according to the inspected databases miR-1275 and miR-125a-3p have no validated targets yet, these miRNAs have already been reported as important elements in tumors: miR-1275 was presented as a potential therapeutic target in glioblastomas [69] and miR-125a-3p was reported as an independent prognostic factor in gastric cancer [70]. miR-125a-3p may also possibly regulate invasion and migration in NSCLCs [71] while miR-17* may be a targetable tumor suppressor in prostate cancer [72]. miR-206 is one of the most frequently downregulated miRNAs in human cancers [73]. In our system this microRNA was present only in salivary gland-derived ACCs. Beyond the previously discussed ESR1, potential targets of miR-206 are MET, TAC1 and GJA1 genes. While no report has been published about potential roles of GJA1 and TAC1 genes in ACC cases, c-MET was reported as a central component of invasiveness in salivary gland-derived ACC tumors [74]. IC53 was reported as being upregulated in colon adenocarcinoma and causing invasivity of colorectal cancer. As its SNP variant (rs2737) has already been described as a potential protector

against proliferation caused by IC53 [75], its inhibition by miR-379 may highlight an important mechanisms in sACC cases.

As the adenoid cystic carcinoma constitues a special subgroup of triple-negative breast cancers, the role of BRCA1 was already investigated in bACC tissues before [76, 77]. The exact connection between this gene and miR-125a-3p in bACC cases still remains unanswered, as to date no previous literature data refers to their connection. MAPK1/ERK which is another gene possibly being regulated by miR-125a-3p may influence the proliferation and the development of distant metastases in ACC cases [78, 79]. Hypermethylation of BCL2L11 was previously described in ACC cases before [80]. According to our analysis BCL2L11 seems to be regulated by miR-17*, a microRNA present only in sACC cases.

Among the miRNAs of subgroup "A" and "B" we could identify members of already described human miRNA clusters. miRNAs belonging to the same cluster are mostly encoded at the same loci and as their nucleotide sequence is mostly very similar, they usually have many target genes in common. The *miR-17/92 cluster* is one of the most studied subgroup of human microRNAs containing miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-a [81]. miRNAs belonging to miR-17/92 cluster are usually activated by Myc [82] and play central role in tumorigenesis, proliferation, migration and metastasis. They are encoded by genes located at locus 13q31.3. Interestingly this region may be affected in many type of malignancies such as colorectal cancer [83], rhabdomyosarcoma [84], by LOH in breast cancer [85] and hepatocellular carcinoma [86]. Members of this miRNA-cluster were first reported as oncogenes in a B-cell lymphoma model in 2005 [87] and since then they have already been described as oncogenes in various types of cancers such as colon cancer [88], lung cancer [89], osteosarcoma [90], angiosarcoma [91], malignant lymphoma [92, 93] and rhabdomyosarcoma [94]. The role of miR-17 is contradictory in breast carcinomas: it has already been described both as tumor suppressor and as oncogene in human breast cancer [95]. In uterine cervix carcinomas miR-20a functions as stimulator of invasion [96] of cancer cells, while in endothelial cells the miR-17-20 cluster has been described as an inhibitor of angiogenesis [97]. We could identify two members of cluster-17/92 in our cases: miR-17 and miR-20a. Their expression changed differently in bACC and sACC cases compared to their normal control tissues: while they were overexpressed in sACC cases, their expression decreased in bACC tissues (subgroup "A"). Overexpression of miR-17 has been reported in many types of human cancers [90, 98, 99] and it potentially regulates the E2F1 gene which encodes a known transcription factor [100]. According to our analysis, E2F1 was reported as the potential target of miR-20a. Expression of E2F1 was investigated in adenoid cystic carcinoma cases, but only showed low expression rate (3 %) [101]. An important cell cycle regulator, CDKN1A/p21 was found to be regulated by miR-17, while CCND1 seem to be regulated by more subgroup "A" members: miR-17, miR-20a, let-7b, miR-195. Interestingly no members of subgroup "B" seem to regulate any of these genes. The exact role of p21 protein in adenoid cystic carcinoma cases was investigated before but its exact role in this type of tumor is still unknown [43, 102]. Amplified CCND1 status was described in adenoid cystic carcinomas which points on its potential oncogenic role in these tumors [103], so its potential regulation by microRNAs may be an important finding of our investigations. Potential target genes of miR-17/92 cluster members include RUNX1, IL-8, JAK1, BIM, MEF2D, MAP3K12, BMPR2 and VEGFA. Although most of these genes (RUNX1, JAK1, MEF2D, MAP3K12, and BMPR2) have not been associated before with adenoid cystic carcinoma cases, significant elevation of serum IL-8 levels has been detected before in salivary gland-derived adenoid cystic carcinomas [104]. Interestingly, the other target of the miR-17/92 cluster, BIM has already been reported as being targeted by Flavokawain B, which induces the apoptosis of adenoid cystic cancer cells through the elevation of BIM-expression [105]. As in our cases miR-17 and miR-20a were upregulated compared to their normal control tissue, their elevated expression may lead to the downexpression of BIM. If our hypothesis is correct, the regulatory effect of these microRNAs may play central role in the downregulation of BIM and so the inhibition of apoptosis in ACC cases. VEGFA also appeared in previous reports about adenoid cystic carcinoma cases, and correlated with their invasivity and metastasis-forming [106, 107], so its post-transcriptional regulation may be important in this respect. Deeper understanding of the exact functions of these genes may potentially bring us closer to the understanding of the clinical features of breast-and salivary gland-derived adenoid cystic carcinomas. The post-transcriptional regulation of CDK6 was also investigated before in adenoid cystic carcinoma cases but its exact role in ACCs is still unknown [108].

miR-24, miR-23b and miR-27b were identified as members of another miRNA subgroup, the miR-23b/27b cluster. The miRNAs belonging to this subgroup have been studed widely in prostate cancers and they are usually reported as being downregulated in the cancerous tissues compared to normal tissue controls [109, 110]. According to the most recent reports, miR-23b and miR-27b play role in the suppression of the metastasis process in human prostate cancer [111]. Interestingly, in our system miR-24 belongs to subgroup "A" while miR-23b and miR-27b are members of subgroup "B". Nevertheless, the role of miR-23b/27b cluster may differ in bACC and sACC cases as their members regulate different genes. miR-193b may also play essential role in bACC and sACC cases as its function as tumor suppressor was already reported in NSCLCs, melanoma and prostate cancer [112–114]. According to our analysis, ADORA2B may be potentially regulated by miR-24 and miR-27b. As this gene was already

reported as a suppressor of metastasis formation of breast cancer [115], it may have a partial role in the diverse clinical outcome of salivary gland and breast-derived tissues.

Conclusion

In this pilot study differences of the complex miRNA regulation mechanisms of sACC and bACC cases, as well as of normal and tumor tissues were found. We beleive that our findings are promising in respect to the understanding of the biological differences between ACCs of different organs.

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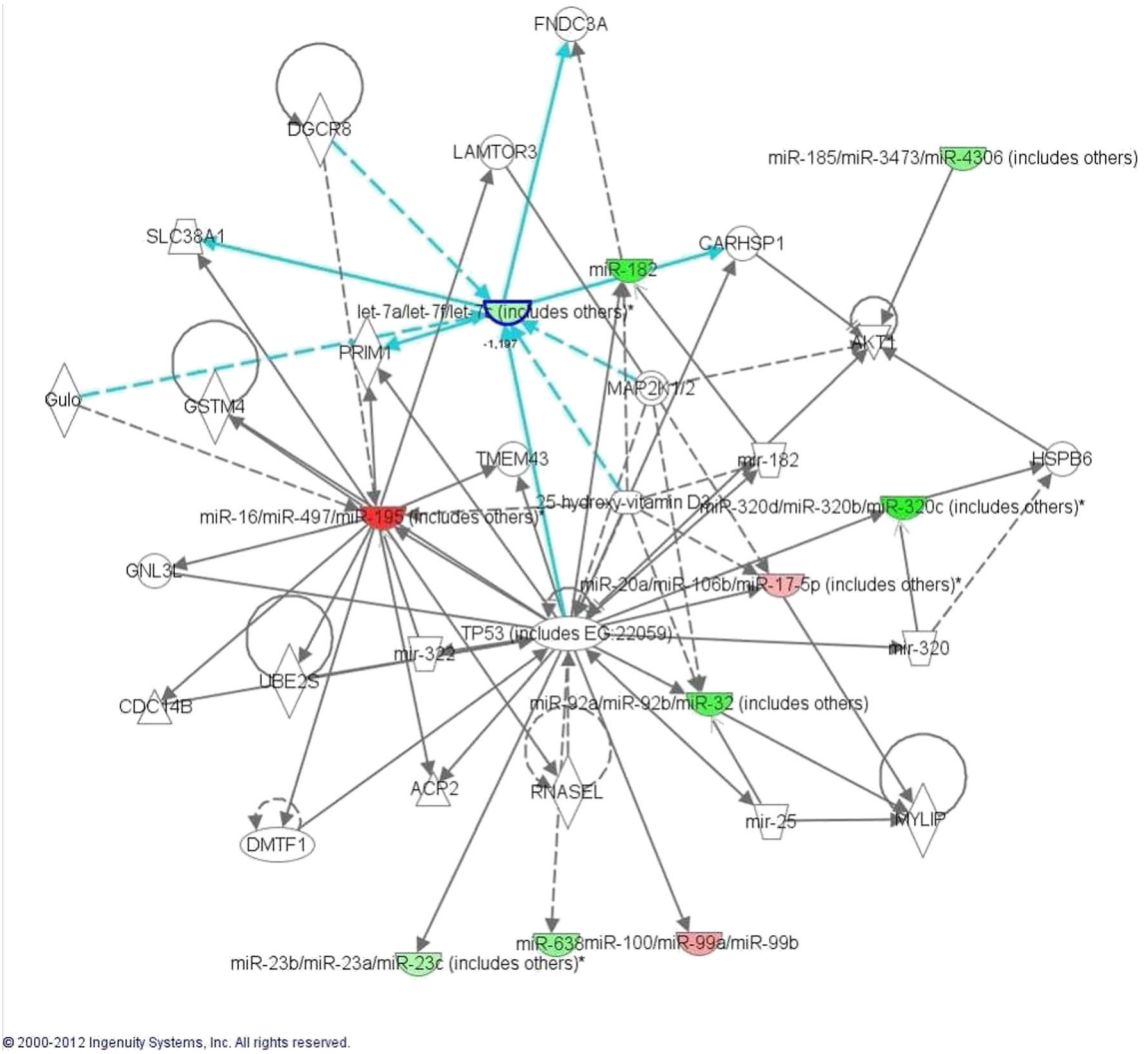
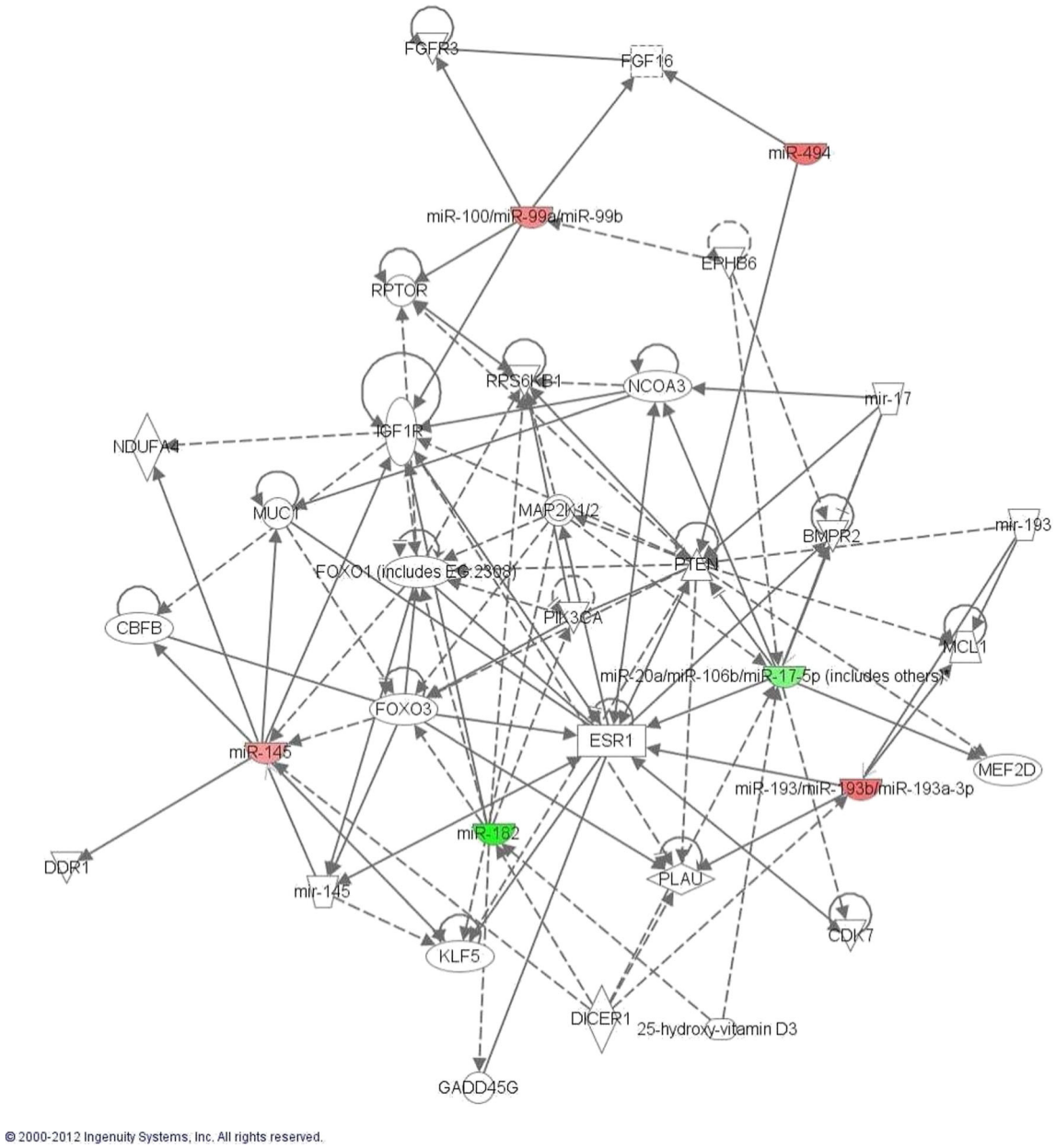
Appendixes

App. 1 Ingenuity Pathway Analysis (IPA®): intracellular pathways and their connections to miRNAs of our system; network in bACCs.



App. 2 Ingenuity Pathway Analysis (IPA®): intracellular pathways and their connections to miRNAs of our system; network in sACCs



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