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MicroRNA Profiling of Salivary Duct Carcinoma Versus Her2/Neu Overexpressing Breast Carcinoma Identify *miR-10a* as a Putative Breast Related Oncogene

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Abstract

Salivary duct carcinomas (SDC) and Her2/Neu3-overexpressing invasive breast carcinomas (HNPIBC/IBC) are histologically indistinguishable. We investigated whether common histopathologic and immunophenotypic features of SDC and IBC are mirrored by a similar microRNA (miRNA) profile. MiRNA profiling of 5 SDCs, 6 IBCs Her2/Neu3+, and 5 high-grade ductal breast carcinoma in situ (DCIS) was performed by NanoString platform. Selected miRNAs and *HOXA1* gene were validated by RT-PCR. We observed similar miRNA expression profiles between IBC and SDC with the exception of 2 miRNAs, *miR-10a* and *miR-142-3p*, which were higher in IBC tumors. DCIS tumors displayed increased expression of *miR-10a, miR-99a, miR-331-3p* and *miR-335*, and decreased expression of *miR-15a, miR-16* and *miR-19b* compared to SDC. The normal salivary gland and breast tissues also showed similar expression profiles. Interestingly, *miR-10a* was selectively increased in both IBC and normal breast tissue compared to SDC and normal salivary gland tissue. Moreover, our NanoString and RT-PCR data confirmed that *miR-10a* was upregulated in IBC and DCIS compared to SDC. Finally, we show downregulation of *HOXA1*, a *miR-10* target, in IBC tumors compared to normal breast tissue. Taken together, our data demonstrates that, based on miRNA profiling, SDC is closely related to HNPIBC. Our results also suggest that *miR-10a* is differentially expressed in IBC compared to SDC and may have potential utility as a diagnostic biomarker in synchronous or metachronous malignant epithelial malignancies involving both organs. In addition, *miR-10a* could be playing an important role as a mammary-specific oncogene, involved in breast cancer initiation (DCIS) and progression (IBC), through mechanisms that include modulation of *HOXA1* gene expression.

Keywords Salivary duct carcinoma · Her2/Neu+ breast carcinoma · MicroRNA profiling · *MiRNA-10* · Oncogene

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Introduction

Salivary duct carcinomas (SDCs) are rare, highly aggressive malignancies of salivary gland origin that are histologically similar to invasive breast cancer and account for about 2% of salivary gland malignancies [1]. They occur predominantly in the parotid and less frequently in other salivary glands, mostly in elderly men [2]. SDCs are characterized by frequent early distant metastases and a 10-year mortality rate as high as 65%. A significant percentage of patients die within 5 years of diagnosis of this disease [3]. Phenotypically, SDCs bear a close histopathologic semblance to high-grade Her2/Neu-overexpressing invasive breast carcinomas (HNPIBCs/IBCs), with both tumors frequently expressing androgen receptor (AR) and Her2/Neu by immunohistochemistry [3]. Microscopically, both of these tumors span

the same range of histopathologic patterns (cribriform, comedo, apocrine, etc.). Moreover, SDCs exhibit a similar immunohistochemical phenotype to ductal adenocarcinomas of the breast. Indeed, a third of the latter are positive for Her2/Neu, estrogen receptor/progesterone receptor (ER/PR), and AR [4]. HNPIBCs represents approximately 15–20% of breast carcinomas and are characterized by unique expression signatures, poor cellular differentiation, high rates of cellular proliferation, frequent nodal metastases, relative resistance to certain chemotherapy and overall worse prognosis [5]. Indeed, HNPIBC represent an aggressive type of breast carcinoma, which in spite of anti-Her2/Neu agents, still lead to high mortality and toxicity profile [5]. Although histopathologic, immunophenotypical and cytogenetic similarities exist between SDC and IBC [3], comparative analysis of the molecular mechanisms that drive these anatomically distinct tumors are largely unknown.

Molecular profiling has proven to be a powerful tool in the characterization of various tumors. One such method involves the characterization of microRNAs (miRNAs), which are small noncoding RNAs [6] involved in post-transcriptional regulation of gene expression [7]. They have been implicated in numerous biological processes—cell growth and differentiation [8–10]—as well as pathological processes including cancer [11, 12]. Studies on miRNA expression signatures from hematological and solid organ tumors suggest that miRNA profiles are tissue-specific, and they have a remarkable ability to discriminate between different types of cancers with a high degree of accuracy [13, 14]. MiRNA signatures are therefore potentially useful in identifying tumor type, and possible therapeutic options, when histopathologic and immunophenotypic data are unable to provide such information.

MiRNAs have been shown to play a major role in breast cancer development. Indeed, Iorio et al. demonstrated that *miR-125b, miR-145, miR-21,* and *miR-155* were deregulated in breast cancer compared to normal breast tissue [15]. Previous molecular genetic studies of SDC have focused on analysis of a limited gene panel [16, 17]. However, comparative miRNA expression profiles of SDC and HNPIBC have not been previously investigated. In this study, we analyzed the miRNA profiles of 5 cases of SDC and 6 cases of HNPIBC to determine whether the morphological similarities between these two types of cancers are mirrored by similar molecular profiles. In view of the premalignant nature of ductal carcinoma in situ (DCIS), and the potential biologic relatedness between mammary DCIS and IBC [18, 19], we included cases of high grade DCIS (n=5) in the study to ascertain if there are similar dysregulations of candidate miRNAs between DCIS and IBC. Our results demonstrate that de novo SDCs have a very similar molecular profile to that of HNPIBCs. However, *miR-10a* is highly expressed in mammary tumors (IBCs and DCIS) but not in SDCs.

Thus, we suggest that *miR-10a* may be a useful biomarker for discriminating HNPIBC from SDC. Furthermore, overexpression of *miR-10a* leads to downregulation of *HOXA1* in breast cancer, which might contribute to the initiation and progression of mammary carcinogenesis.

Materials and Methods

Tumor Characterization and Sampling

SDCs (n=5), IBCs (n=6) and DCIS (n=5) formalin-fixed, paraffin-embedded (FFPE) cases were selected from The Ohio State University Wexner Medical Center archive after approval through the Institutional Review Board of The Ohio State University, Department of Pathology (Institutional Review Board-approved protocol number 2002H0089). All cases were re-reviewed to confirm the diagnosis. In view of the morphologic overlap between the two entities, none of the patients with de novo SDC had concurrent IBC. The diagnosis of SDC was based on established criteria [3]. All IBC cases were Her2/Neu amplified by both immunohistochemistry (3+ positivity) and fluorescence in situ hybridization (two of which were also positive for ER and PR). All DCIS cases were morphologically high-grade and all but 1 case were negative for ER and PR.

The relative stability of miRNAs in FFPE archival tissue has made them an attractive resource for miRNA profiling studies, and indeed such tissue has been shown to be comparable to fresh frozen specimens [20].

Against this background, we had optimized a technique in our recent work using FFPE material in which we used the patient's non-tumoral tissue as a means to minimize the variability introduced by using normal tissue controls from different patients [21]. We used a tissue microarrayer to core tumor and non-tumoral areas that had been mapped on hematoxylin and eosin-stained (H&E) sections so that the tumor samples were matched with paired controls from the same patient. In this regard, representative H&E-stained slides were identified for each case, and the tumoral and non-tumoral ("normal") appearing areas were marked and used as a guide for the tissue microarrayer to simultaneously obtain both tumor tissue and normal tissue for each patient as duplicate (paired) samples. Both areas (tumor and non-tumoral) were appropriately marked as a guide for the tissue microarrayer to obtain multiple 1.75 mm cores simultaneously from the corresponding areas on the FFPE blocks.

For *HOXA1* validation experiments, a separate cohort of snap-frozen Her2/Neu3+ positive IBC and normal breast specimens (n=3 per group) were obtained through the Tissue Procurement Service of the Department of Pathology at The Ohio State University Wexner Medical Center.

RNA Extraction

Paraffin-embedded tissue cores from blocks were deparaffinized in xylene before protease digestion. RNAs were extracted by using the Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Invitrogen, AM1975) according to the manufacturer's protocol. Samples were eluted in water and cleaned up by glycogen-Na acetate precipitation procedure. Briefly, 1/10 vol of 3 M sodium acetate (pH 5.2), 1 µg glycogen, and 3.5 volumes of ice-cold 95% ethanol was added to each sample's tube. Samples were incubated on ice for at least 30 min, centrifuged at top speed for 20 min, washed twice with cold 70% ethanol, and finally re-suspended in RNase-free water.

Frozen tissues were extracted by using TRIzol solution (Invitrogen). Tissues were frozen immediately after collection and maintained at −80 °C until extraction. Each sample was homogenized in 1 ml of TRIzol and extracted following the manufacturer's protocol.

NanoString nCounter Platform Profiling

The NanoString nCounter Human miRNA Expression Assay Kit (http://www.NanoString.com/) was used to profile human and human-associated viral miRNAs in all samples. 100 ng of total RNA was used as input for nCounter miRNA sample preparation reactions. All sample preparation and hybridization reactions were performed according to manufacturer's instructions (NanoString Technologies). Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed.

Real Time Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase and real-time PCR (RT-PCR) reactions were performed according to the manufacturer's protocols. All RT-PCR reactions were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems).

Mature *miR-10a* (Applied Biosystems, assay ID: 000387), *miR-21* (Applied Biosystems, assay ID: 000397) and *miR-148* (Applied Biosystems, assay ID: 000470) expression were analyzed by Taqman micro-RNA assay and normalized to RNU44 (endogenous control, assay ID: 001094) according to the manufacturer's protocol. 10 ng of total RNA from all samples was reverse transcribed for each target and endogenous control. Samples were analyzed in triplicate, and the expression of *miR-10a, miR-21 and miR-148* was determined using the comparative Ct method ($2^{-\Delta Ct}$).

Expression of the target gene *HOXA1* was also tested by RT-PCR, using *GAPDH* and *OAZ1* as housekeeping genes for normalization. Targets were assayed using Taqman probes (*HOXA1* assay ID: Hs00939046_m1, cat#: 4331182, *GAPDH* assay ID: Hs02758991_g1, cat# 4351370, *OAZ1* assay ID: Hs00427923_m, cat# 4351370) on total cDNA obtained from the same RNA samples previously checked for miRNA expression. Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems).

Statistical Analysis

Raw NanoString data, which are proportional to copy number, were log-transformed and normalized by the quantile method after application of a manufacturer-supplied correction factor for several miRNAs. Data were filtered to exclude relatively invariant features (IQR 0.5) and features below the detection threshold (defined for each sample by a cutoff corresponding to approximately twice standard deviation of negative control probes plus the mean of them) in at least half of the samples. Using R/Bioconductor and the filtered dataset, linear models for microarray data analysis [22] were employed with a contrast matrix in order to identify deregulated miRNAs. P values were used to rank miRNAs of interest, and correction for multiple comparisons was done by the Benjamini Hocheberg method [23]. Raw data that were above background, as well as the corresponding quantile-normalized data, were also imported into MultiExperiment Viewer [24] to create heat maps. For the RT-PCR data, Student's *t* test was employed to determine the statistical significance of values obtained.

Results

SDCs Closely Resemble IBCs Histopathologically

A histopathologic review of five cases of SDC, six cases of IBC and five cases of DCIS showed a striking microscopic similarity (Fig. 1). All three tumor types exhibited ductal proliferation with central necrosis (comedocarcinoma) and high-grade cellular and nuclear pleomorphism. In all three tumors, neoplastic cells were cuboidal to columnar, with nuclei showing prominent nucleoli. SDC and IBC showed such prominent morphologic phenotypic mimicry that they were difficult (if not impossible) to distinguish from each other. Our data highlighted the value of identifying molecular biomarkers to differentiate between these histologically and embryologically similar cancer types.
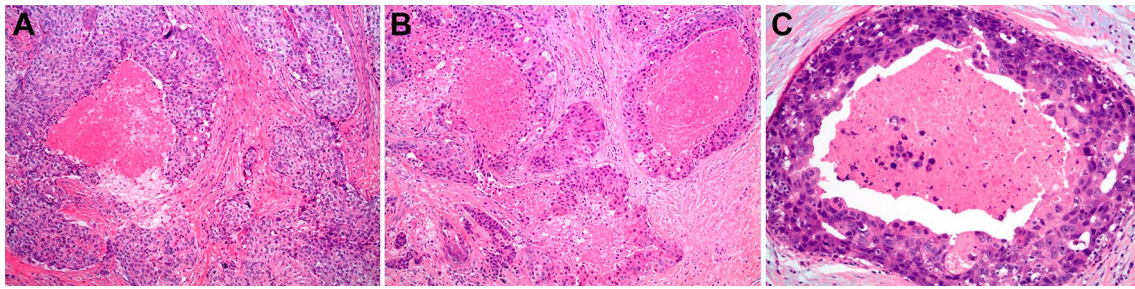
Fig. 1 Histopathological photomicrographs of **a** invasive breast carcinoma (IBC), **b** salivary duct carcinoma (SDC) and **c** ductal carcinoma in situ (DCIS) of breast, showing striking histopathologic similarity, rendering IBC and SDC morphologically indistinguishable. The central comedonecrosis and marked cellular and nuclear atypia are prototypic features of all three entities [H&E, × 200]

MiRNA Profiling Shows SDC to be More Closely Related to IBC than DCIS

In order to identify distinguishing molecular characteristics of SDS, IBC and DCIS, we performed miRNA profiling of tumors and surrounding normal tissues from the three tumor types (Fig. 2a). We observed an increased expression of *miR-21* and *miR-155*, and a decreased expression of *miR-10b* and *miR-145* in IBCs compared to surrounding normal breast tissue. In DCIS, we observed an increased expression of *miR-21* compared to surrounding breast tissue (Table 1). We also found an increased expression of *miR-21* and decreased expression of *miR-148* in SDC compared to normal salivary tissue (Fig. 2b). Detailed miRNA profiles are provided in Supplementary Tables 1 and 2. Our data suggest that, although there are morphological similarities between these tumors, distinct molecular pathways drive their respective carcinogenic processes.

Further comparative analysis of miRNA profiles between SDC, IBC and DCIS identified a distinct miRNA signature for SDC as shown by the hierarchical clustering on the heat map (Fig. 3a). Interestingly, comparisons between SDC and IBC revealed only two miRNAs which were differentially expressed in these two cancers, *miR-10a* and *miR-142-3p*, found to be more expressed in IBC than in SDC (Fig. 3b). On the other hand, SDC versus DCIS comparisons showed seven differentially expressed miRNAs: *miR-10a, miR-99a, miR-331-3p, miR-335* up-regulated; and *miR-15a, miR-16, miR-19b*, down-regulated in DCIS compared to SDC. Finally, IBC vs DCIS miRNA profile comparisons show differential miRNA expression in five miRNAs: *miR-15a, miR-16, miR-19b, miR-222* and *miR-374* which were down-regulated in DCIS compared to IBC. These data are summarized in Table 2. It should be noted that these results were based on analysis of NanoString data alone. Taken together, our data seems to suggest that miRNA expression profiles show SDC to be more closely related to IBC than DCIS.

MiR-10a Is Selectively Expressed in Normal Breast Compared to Salivary Gland Tissue

Real-time PCR validation of SDC, IBC and DCIS tumors showed a significantly increased expression of *miR-10a* in IBC and DCIS compared to SDC (Fig. 3d). This led us to further investigate whether *miR-10a* upregulation in IBC and DCIS tumors compared to SDC parallels the same pattern in the surrounding non-tumoral ("normal") mammary and salivary gland tissue respectively. Our analysis of adjacent non-tumoral tissues from IBC and SDC cases revealed an increased expression of *miR-10a* in breast tissue compared to salivary tissue (Fig. 3c, e). Our data suggests that, in contrast to SDC, *miR-10a* could be playing an important role in breast tumors, possibly acting as a mammary specific oncogene, the activation of which might function as an initiator of tumorigenesis.

HOXA1, a Target of *miR-10a* Is Down-Regulated in IBC

Given the differential expression levels of *miR-10a* observed between SDC and IBC, we next investigated whether putative targets of *miR-10a* were differentially regulated in these cancers. One of the many putative *miR-10a* targets as determined by in silico experiments using Target Scan and PicTar is *HOXA1*, a gene known to be implicated in a number of cancers [25–27]. *HOXA1* regulation by *miR-10a* has been reported in some studies [28, 29]. We therefore evaluated gene expression levels of *HOXA1* in snap-frozen tissues of normal breast and IBC cases from new patients using RT-PCR. Our results showed a significant decrease in *HOXA1* expression in IBC compared to normal breast tissue (Fig. 3f). These results correlate with the increased *miR-10a* expression levels in IBC compared to IDC, supporting the hypothesis that over-expression of *miR-10* in IBC leads to downregulation of *HOXA1* which might contribute to the initiation and progression of mammary cancers.
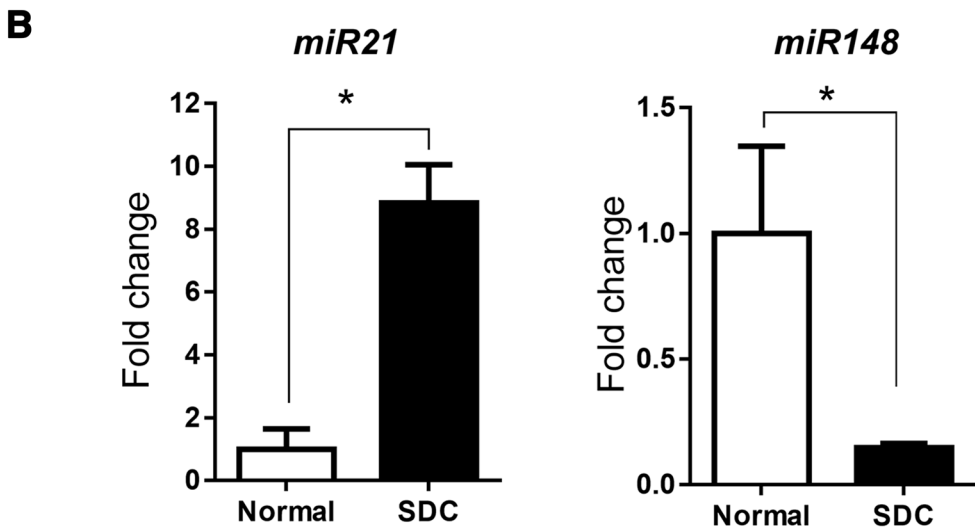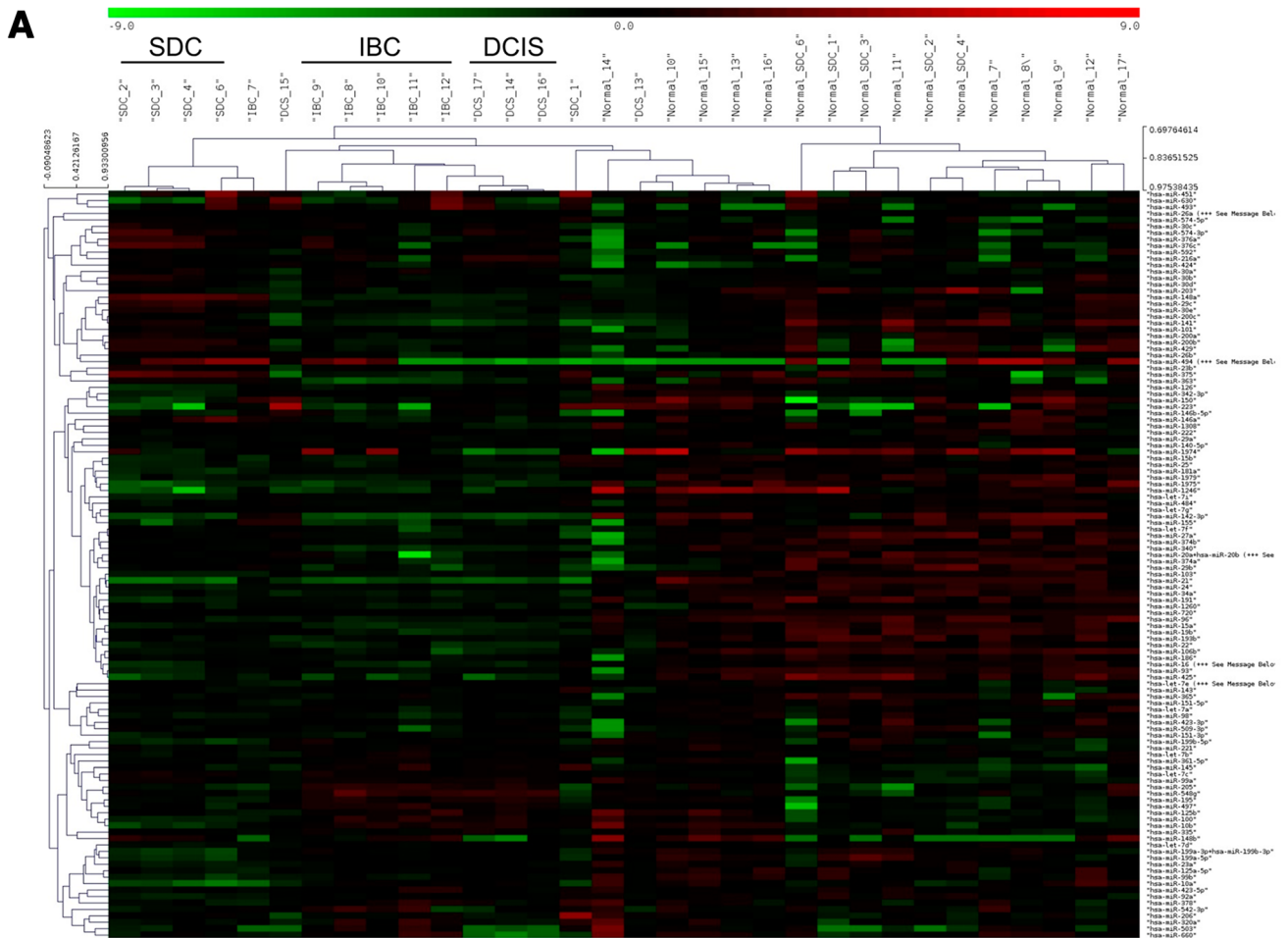
Fig. 2 a Global miRNA profiling of normal breast, normal salivary tissues, IBC, DCIS and SDC as determined by NanoString analysis. Heat maps show the distinct microRNA signature of SDC, IBC and DCIS tumors. b RT-PCR validation of *miR-21* and *miR-148* expression in SDC tumors compared to normal salivary tissue, *$p < 0.05$

Table 1 miRNA expression comparisons between normal breast tissue, IBC and DCIS tumors from patients determined by NanoString analysis

microRNA	Mean			Fold change		p-values	
	Normal	IBC	DCIS	Log twofold change	Linear fold change	Raw p-values	Adj p-values
hsa-miR-21	10.87	14.84		3.97	15.70	1.49E−07	5.09E−06
hsa-miR-155	5.52	7.65		2.13	4.37	9.15E−04	3.00E−03
hsa-miR-145	11.31	9.42		−1.90	0.27	4.59E−04	1.64E−03
hsa-miR-10b	6.75	6.00		−0.75	0.59	1.89E−02	3.78E−02
hsa-miR-21	10.87		13.75	2.88	7.39	8.12E−05	5.99E−04

Discussion

SDCs are highly lethal salivary gland malignancies characterized by poor prognosis in spite of multimodality treatment, consisting of surgical resection, with or without adjuvant chemoradiation therapy. Despite their frequent expression of Her2/Neu and AR, response to targeted Her2/Neu inhibition and androgen deprivation therapies, respectively, has been disappointingly inconsistent [1, 30, 31]. There is thus an urgent need for a basic understanding of the pathogenesis of these rare tumors, with a view to developing novel therapeutic targets.

Moreover, given the striking histopathologic and immunophenotypical similarities between SDC and HNPIBC, in those exceptionally rare clinical circumstances where synchronous or metachronous tumors occur, distinguishing between the two malignancies may be exceptionally challenging. Because there may be differing implications for staging, prognosis and therapy, the discovery of a specific molecular signature that would discriminate these two tumors could facilitate more accurate diagnostic and prognostic assessments, and provide insights into possible therapeutic strategies.

As regulators of gene expression, miRNAs have increasingly become the focus of extensive research in cancer development as well as in the discrimination of various cancer types. In this study, we used miRNA profiling to determine whether the similarities that exist among SDC, HNPIBC, and high-grade DCIS are reflected in the molecular landscape of these cancers. The goal was to identify specific molecular signatures that discriminate among these histopathologically similar malignancies in order to characterize pathways that potentially could represent new targets for specific therapy. Our results demonstrate that there are striking similarities in the miRNA expression profiles of SDC and IBC. This finding is in accord with previous studies that have used different platforms to compare the two entities [1, 32]. It should be noted that, without RT-PCR validation, miRNA expression as determined by nanostring analysis should be interpreted with caution. Although beyond the scope of our current study, a more extensive validation of the differential miRNA profiles observed between SDC, DCIS and IBC will be required.

Although the role of specific miRNAs in the pathogenesis of SDC is yet to be fully characterized, evidence suggests that they are significant contributors to salivary gland epithelial malignancies. For example, an examination of mucoepidermoid carcinoma (MEC) of the upper aerodigestive tract by Chiosea et al. revealed a significant correlation between the DICER protein, which is involved in miRNA maturation, and advanced stages/high grades of MEC as determined by immunohistochemistry [33]. We found *miR-21* to be significantly up-regulated in SDC compared to normal salivary gland and *miR-148* to be down-regulated in SDC compared to surrounding normal salivary gland, suggesting that these miRNAs may be functioning as oncomir and tumor suppressor miRNA, respectively, in SDC.

miR-21 is located on chromosome 17q23.2 in humans and has been shown to be the most commonly upregulated miRNA in both solid and hematological malignancies [12, 34]. The mechanism of oncogenesis is varied amongst the different tumor types. For example, in colorectal carcinomas, it has been shown that *miR-21* induces "stemness" by down-regulating transforming growth factor receptor beta while it stimulates invasion and metastasis by suppressing PDCD4 [35, 36]. In non-small cell lung cancer, *miR-21* enhances oncogenic K-ras activation and modulates tumorigenesis by targeting *SPRY2, BTG2*, and *PDCD4* [37]. *MiR-148* is down-regulated in many solid organ malignancies, including breast, colorectal, pancreatic, hepatocellular, endometrial and non-small cell lung cancers [38]. It has been shown that increased expression of *miR-148a* inhibits both tumor growth and metastasis in a hepatocellular carcinoma tumor stem cell-line by controlling the ACVR1/BMP/Wnt signaling pathway [39]. In the context of our current study, differential expression of *miR-21* and *miR-148* between normal salivary gland and SDC, suggests these miRNAs may represent potential tumor biomarkers and possible therapeutic targets. Additional studies will be needed to determine the biologic roles of specific miRNAs in SDC pathogenesis.

The differential expression of *miR-10a* between SDCs and IBCs illustrates the potential utility of miRNAs to

distinguish between these histologically similar cancer types. This will be particularly useful in resolving clinical dilemmas, such as one in which an IBC develops synchronously with a secondary nodule in the parotid gland, the latter of which could either represent a metastasis from the breast primary or a de novo SDC. The impact on staging and treatment would be significant, depending on which scenario is correct.

Our miRNA analysis of breast tumors relative to surrounding non-involved normal breast tissue is in agreement with a previous study by Iorio et al. [15], which showed five miRNAs that were dysregulated (*miR-10b, miR-125b*, and *miR-145* down-regulated; and *miR-21* and *miR-155* up-regulated). In our study, we found four of these miRNAs (*miR-10b, miR-145, miR-21* and *miR-155*) to be dysregulated. Interestingly, *miR-10b*, which was down-regulated in IBC compared to normal breast tissue (in both Iorio et al. and our study), has been shown to be highly expressed in metastatic breast cancer cells [40]. Indeed, therapeutic targeting of *miR-10b* inhibits breast cancer metastasis but does not inhibit primary mammary tumor growth in a mouse breast cancer model [41]. This highlights the potential value of miRNA targeting in cancer therapy.

A significant finding in our study was the selectively increased expression of *miR-10a*, which followed a tissue-specific expression pattern. First, *miR-10a* displayed increased expression in normal breast tissue compared to the normal salivary gland tissue. Second, *miR-10a* expression was increased in HNPIBC compared to SDC. These results suggest that *miR-10a* could be biologically relevant in mammary oncogenesis, at least in a subset of breast carcinomas. This is not surprising, as previous studies have reported an amplification of the *miR-10* gene locus in breast cancer patients [42, 43]. Thus, it is plausible that *miR-10a* could act as mammary tissue oncogene, and its overexpression may be an initiation step in mammary carcinogenesis. Indeed, *miR-10a* up-regulation might facilitate malignant transformation of normal glandular breast epithelium, and could trigger uncontrolled growth that initially leads to DCIS and then to IBC. Previous research has linked *miR-10a* to increased chemo-resistance of breast cancer cell-line MCF7 to cisplatin [44]. Hence, *miR-10a* might not only promote tumorigenesis but may also be involved in chemoresistance, suggesting that this microRNA could be a potential therapeutic target in some breast cancer types. Further studies are warranted to determine the correlation between *miR-10a* expression and pre-neoplastic ductal breast lesions such as usual ductal hyperplasia (UDH) and atypical ductal hyperplasia (ADH), including columnar cell hyperplasia and flat epithelial atypia. Given these results, it is possible that *miR-10a* plays a tissue-specific role in breast carcinogenesis compared to the histopathologically similar SDC.

To further define the involvement of *miR-10a* in breast carcinogenesis, we investigated the expression of the putative *miR-10a* target *HOXA1. HOXA1* is a transcription factor that is part of the homeobox gene cluster A, involved in gene expression, cell differentiation, and temporo-spatial body development [25]. It is believed that the *HOX* gene family is an important regulator of tumor invasion and metastasis. *MiR-10a* and *miR-10b* are both located close to the homeobox genes and in silico studies (Target Scan) show that *miR-10a* targets *HOXA1*, which has also been confirmed by luciferase assay experiments [29]. *MiR-10a* has also been shown to repress transcriptional expression of another member of the HOX gene family, *HOXD4*, in breast cancer cells [45]. In line with these studies, our analysis of breast and salivary gland tumors showed decreased expression of *HOXA1* in IBC compared to SDC, which is consistent with the enhanced *miR-10a* expression observed in IBC tumors.

MiR-10a expression and concomitant repression of *HOXA1* has been shown to be an important regulatory pathway in a number of cancers, including acute myeloid leukemia [46] and pancreatic cancer [28]. In pancreatic cancer, *miR-10a* over-expression decreases *HOXA1* levels, which trigger increased invasiveness of malignant epithelial cells [28]. In another study, retinoic acid receptor antagonists, which inhibit *miR-10a* expression, prevented the metastatic behavior of pancreatic cancer [47]. Therefore, it is possible that down-regulation of *HOXA1* in IBC tumors might prevent the differentiation of ductal epithelial cells and increase their invasiveness, characteristic of high-grade carcinomas. Furthermore, it seems that *HOXA1* might be up-regulated in certain types of breast cancers (basal-like, Her2Neu-, ER-) but decreased in others (Her2Neu+, ER+), and its down-regulation might be influenced by *miR-10a* via 3′UTR direct targeting, or via enhanced *HOXA1* promoter methylation. It should be noted that our data did not show a significant difference in the expression of *miR-10a* between normal breast tissue and IBC. While additional experiments will be required to explain this apparent discrepancy, other studies using in silico and in vitro reporter assays demonstrate that *HOXA1* is targeted by other miRNAs including *miR-30b, miR-30c, miR-30e, miR-99a miR-100, miR-181c* and *miR-203* [48–54]. Interestingly, we observed a moderate increase in *miR-30e* expression in IBCs compared to normal breast tissue (Supplementary Table 2). It is therefore possible that downregulation of *HOXA1* is mediated by one or more miRNAs during breast oncogenesis.

In conclusion, our studies reveal similar miRNA expression patterns between SDC and IBC, but with differential expression of *miR-10a* in IBC compared to SDC. We also suggest that *miR-10a* could be useful in resolving difficult clinical scenarios of patients with synchronous HNPIBC and SDC, and serve as a potential biomarker in the early
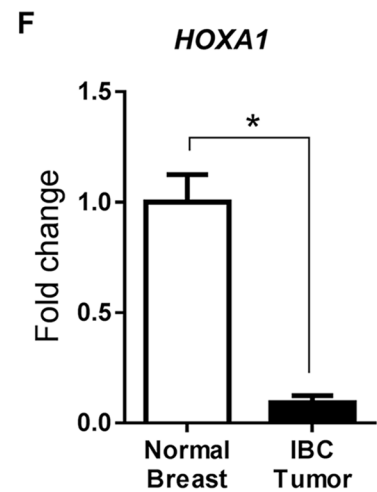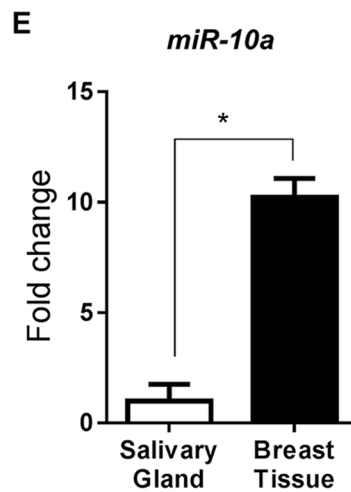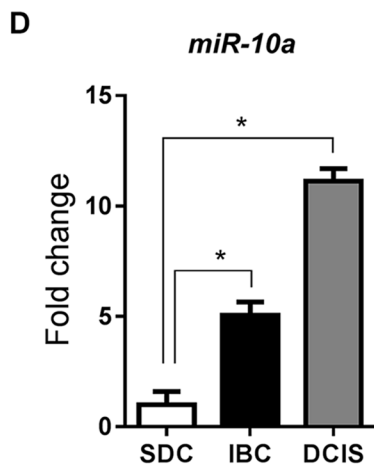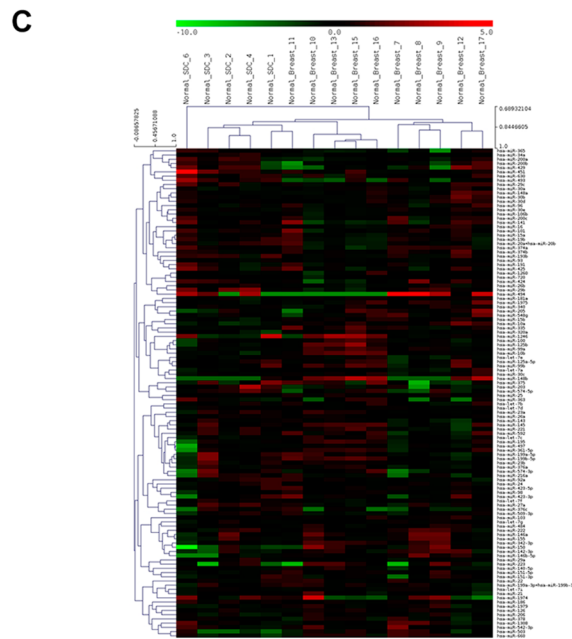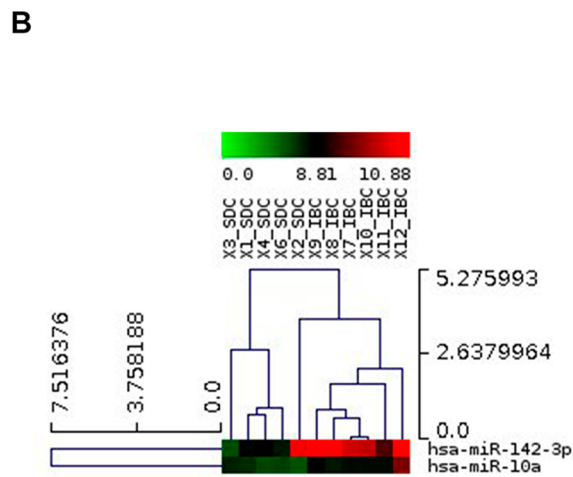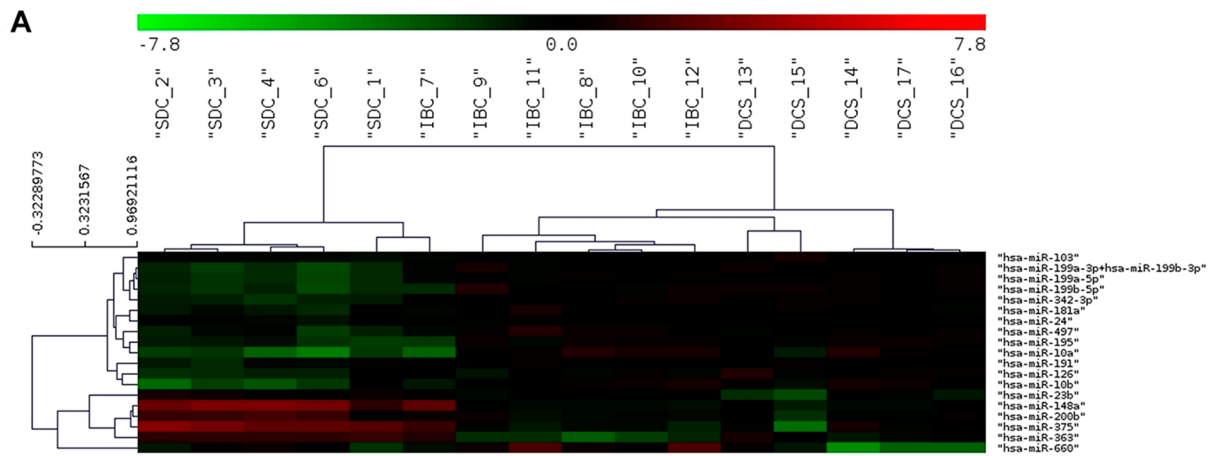
<**Fig. 3** **a** Heat map showing the NanoString gene expression profiles of SDC, IBC and DCIS. **b** Heat map comparison between SDC and IBC showing expression of *miR-142-3p* and *miR-10a*. **c** Heat map showing miRNA profiles of normal salivary and normal breast tissues as determined by NanoString analysis. **d** Gene expression of *miR-10a* in SDC, IBC and DCIS as determined by RT-PCR. **e** Gene expression of *miR-10a* in normal salivary and normal breast tissue as determined by RT-PCR. **f** *HOXA1* gene expression in IBC and normal breast tissue as determined by RT-PCR analysis, $*p < 0.05$

Table 2 Selected miRNA expression comparisons between tumors from SDC, IBC and DCIS patients determined by NanoString analysis

microRNA	Mean			Log fold change	p-values
	SDC	IBC	DCIS		
hsa-miR-142-3p	8.314	10.417		2.1027	0.0375
hsa-miR-10a	6.718	8.432		1.7137	0.0016
hsa-miR-99a	8.974		10.128	1.1535	0.0167
hsa-miR-10a	6.718		8.293	1.5745	0.0099
hsa-miR-335	5.374		6.680	1.306	0.0100
hsa-miR-331-3p	5.642		6.800	1.158	0.0002
hsa-miR-16	12.796		12.078	−0.7185	0.0040
hsa-miR-19b	9.336		7.890	−1.446	0.0060
hsa-miR-15a	10.312		9.305	−1.007	0.0078
hsa-miR-374a		7.903	6.348	−1.5558	0.0176
hsa-miR-19b		9.288	7.890	−1.3983	0.0038
hsa-miR-15a		10.432	9.305	−1.1267	0.0068
hsa-miR-222		8.935	7.913	−1.0225	0.0148
hsa-miR-16		12.968	12.078	−0.8908	0.0025

detection of a subset of breast cancer. We propose a new tumorigenic mechanism in breast cancers, represented by the over-expression of a breast specific miRNA, *miR-10a*, that down-regulates *HOXA1* and leads to proliferation of less differentiated ductal epithelial cells with increased invasiveness. Conceivably, down-regulation of *miR-10a* through antisense compounds might prove to be a potentially effective therapy for these types of cancer.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This study was approved by the Institutional Review Board of The Ohio State University, Department of Pathology (Institutional Review Board-approved Protocol No. 2002H0089).

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