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Abstract	Abstract

Introduction: Breast cancer outcomes vary across different ethnic groups. MicroRNAs (miRs) are small non-coding RNA molecules that regulate gene expression across a range of pathologies, including breast cancer. The aim of this study was to evaluate the presence and expression of miRs in breast cancer samples from different ethnic groups. **Materials and Methods:** Breast cancer tissue from 4 ethnic groups, i.e., British Caucasian, British Black, Nigerian, and Indian, were identified and matched for patients' age, tumour grade/type, and 10 × 10 µm sections taken. Tumour areas were macrodissected, total RNA was extracted, and cDNA was synthesised. cDNA was applied to human miScript PCR arrays allowing the quantification of 84 of the most abundantly expressed/best-characterised miRs. **Results:** Differential expression of 9 miRs was seen across the 4 groups. Significantly higher levels of

miR-140-5p, miR-194 and miR-423-5p (the last of which harbours the single-nucleotide polymorphism rs6505162) were seen in the breast tumours of Nigerian patients when compared with other ethnic groups (all $p < 0.0001$). miR-101 was overexpressed in breast cancers in the Indian patients. An in silico analysis of miR-423-5p showed that the AC genotype is mainly associated with Europeans (57%), while Asians display mostly CC (approx. 60%), and Africans mainly AA (approx. 60%). **Conclusions:** This study shows divergence in miR expression in breast cancers from different ethnic groups, and suggests that specific genetic variants in miR genes may affect breast cancer risk in these groups. Predicted targets of these miRs may uncover useful biomarkers that could have clinical value in breast cancers in different ethnic groups.

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Introduction

Differences in clinical presentation and outcome exist among breast cancer patients of different ethnic groups. Accumulating evidence suggests that the molecular profile of breast cancer differs between ethnic groups, which, in turn, can influence their behaviour and response

to therapy [1, 2]. Modern molecular pathology techniques have revealed 4 core taxonomic groups of breast cancer; luminal A, luminal B, basal, and HER2+, which can now be assessed using immunohistochemistry as a surrogate [3, 4]. Most published information is derived from studies on tumours from individual ethnic groups rather than direct comparative investigations of tumours from multiple backgrounds within a single programme. Published evidence implies that there are indeed some distinct differences, such as the preponderance of triple-negative breast cancer in African Americans [5, 6], and better overall survival in Japanese versus Western populations [7]; interrogation of the SEER database has shown racial differences in tumour size and the number of positive lymph nodes in stage-matched women with breast cancer [8]. The identification of specific molecular profiles would aid clinicians in treatment planning and assist the search for potential idiosyncratic causative factors in breast cancer from different genetic backgrounds.

MicroRNAs (miRs) are small, noncoding RNAs that have emerged over the last decade as key regulatory molecules capable of influencing gene expression across a range of pathologies, including breast cancer. Numerous studies are beginning to elucidate their potential as diagnostic, prognostic, and predictive biomarkers [9, 10]. Evidence is building to show that polymorphisms in some miRs are associated with breast cancer susceptibility [11]. More recently, elevated plasma levels of miR-21 and miR-146a were identified in women with breast cancer [12]. However, the expression of miRs in breast cancer of various ethnic origins is yet to be elucidated.

The aim of this study, therefore, was to evaluate the presence and expression of miRs in breast cancers obtained from 4 different ethnic groups; British Caucasian, British Black (including triple-negative [TN] and non-TN), Nigerian, and Indian.

Materials and Methods

Sample Cohorts and Ethical Approval

Formalin-fixed paraffin-embedded (FFPE) samples were obtained from all 4 ethnic groups. All were ductal “no special type” breast carcinomas, lymph node-positive, in patients with mean ages of 72, 46, 59, and 57 years in the British Caucasian, British Black, Nigerian, and Indian groups, respectively. With the TN cohort being the exception, all cancers were ERα-positive, grade 2; the TN cohort comprised 3 grade 3 cancers and 1 grade 2 cancer. For the British Caucasian and British Black patients, ethical approval was obtained from Leeds East (06/Q1206/180) Research Ethics Committees. Institutional Review Board approval was obtained for the Indian (Kidwai Memorial Institute of Oncology Medical Ethics Committee) and Nigerian (local departmental approval) cases. Tissue samples were pseudo-anonymised and data was analysed anonymously. Patients’ identities were not disclosed to the research team, so specific informed consent was not required.

MiR Identification

Four to five cases were identified from each ethnic group and matched according to the age of the patient and the tumour grade/type. Ten 10-µm full-face sections were cut and enriched for tumours by macrodissection. This was done by macroscopically selecting cellular tumour areas of each section. Subsequently, total RNA was extracted (RNeasy FFPE kit) and cDNA synthesised (RT^2 miRNA first strand kit). cDNA was applied to the RT^2 miRNA PCR array human miFinder, which allows quantification of 84 of the most abundantly expressed/best-characterised miRs using SYBR Green PCR (an asymmetrical cyanine dye used as a nucleic acid stain). All reagents were from Qiagen and experimental procedures were followed according to the manufacturer’s instructions. Data was analysed using the miScript miRNA PCR array data analysis tool (Qiagen).

Data Mining and Statistical Analyses

TargetScan, microRNA.org, and DIANA-TarBase platforms were used for data mining. The HapMap data was accessed through NCBI.

Statistical analyses (two-tailed) of PCR data were performed using the statistical software package GraphPad Prism. All comparisons were double-sided. A p value of <0.05 was considered significant.

Results

A total of 17 matched breast carcinomas were analysed. These included five British Caucasian tumours and 4 cancers from each of the other 3 ethnic groups. Differential expression of 9 of the total 84 miRs examined were seen across the 4 ethnic groups, specifically: miR-181a, miR-19b, miR-140-5p, miR-130a, miR-101, miR-302a, miR-194, miR-423-5p and miR-302c (Fig. 1). In general, these were significantly higher in the breast tumours in Nigerian and Indian women than in British Caucasian and British Black women. In particular, significantly higher levels of miR-140-5p, miR-194 and miR-423-5p were seen in the breast cancers of Nigerian women than in the other ethnic groups. miR-101 was overexpressed in the breast cancers of Indian patients.

The predicted top 10 targets, derived from 3 databases, for each of these miRs are shown in Table 1. miR-140-5p had 3 gene targets, SEPT2, HDAC4, and VEGFA, which were identified in >1 database while miR-194-5p had 1 (EP300).

No common gene targets were identified for miR-423-5p, located on chromosome 17; it was of interest, however, as it harbours the single-nucleotide polymorphism (SNP) rs6505162, an A>C polymorphism found in the pre-miR region. This phenotype has recently been shown to be associated with increased familial breast cancer risk in South American patients with a family history of breast cancer [13]. As shown in Table 2, an in silico analysis showed that this SNP was differentially associated with ethnicity, with European patients mainly A/C (57%), Asians

mostly C/C (approx. 60%) and Africans mainly A/A (approx. 60%). An overview of the relation of miR SNPs and breast cancer risk in previous cohorts is presented in Table 3.

Discussion

Since their discovery, there has been considerable interest in using miRs as either biomarkers of disease or of therapy response in various malignant tumours. We present evidence that miRs are differentially expressed in the breast cancers of different ethnic groups with some (miR-140-5p, miR-194, and miR-423-5p) being highly expressed in breast tumours of Nigerian patients, and 1 (miR-101) in those of Indian patients, when compared with tumours in the British Black and British Caucasian groups. This analysis was performed on macrodissected FFPE blocks, which permitted the use of relatively small amounts of tumour tissue. In several studies, miRs have been shown to be successfully isolated from FFPE tissue and the results are comparable to those obtained from frozen tissue [14]. Due to their small size, miRs are minimally affected by formalin fixation and can even be analysed in fixed liver carcinoma samples that are up to 30 years old [15].

Population studies have shown that breast cancers from different ethnic groups often enrich for a particular molecular subtype. For example, there is preponderance towards basal breast cancer in African/Americans [6, 16], hormone receptor-negative breast cancer in American Indian/Pakistani women, SEER data [17], luminal A tumours in Chinese and Japanese [18], and HER2+ cancers in Asian/Pacific Islanders [19].

We have previously shown, using tissue microarrays, that breast cancer in Nigerian patients is enriched for the TN phenotype [20]. There is limited, predominantly epidemiological, data on ethnic breast cancer within the UK population that points to similar variation in the biology and outcome of the disease across different backgrounds [21, 22].

In this study, miR-423-5p emerged as being highly expressed in the African tumour samples. The in silico analysis identified a significantly high expression of the SNP rs6505162 A/A sequence in 60% of the breast cancers in Nigerian patients compared with 30 and 10% in the European and Asian patients, respectively (Table 2). From the HapMap data, individuals of ancestry from northern or western Europe are mainly AC (58%), while those with ancestry from sub-Saharan (Nigeria) or east (Kenya) Africa are mainly AA (62 and 66%, respectively), and those who are ethnic Gujarati Indians overwhelmingly have at least 1 C allele (41% AC and 38% CC). The African populations stand out as having a low representation of the C allele.

Farazzi et al. [23], using deep sequencing, showed that, in general, there is low expression of miR-423 in breast carcinoma but that both mature forms of miR-423 are highly expressed in invasive carcinomas that later develop breast cancer metastasis. Conversely, in a matched case control series of Australian women, a C>C polymorphism located in the pre-miR region of miR-423 conferred a reduced breast cancer risk [24]. Recently, functional studies of miR-432 in breast cancer cell lines showed 2/5 cell lines with a somatic mutation of rs6505162 SNP, and this correlated with proliferating cell nuclear antigen (PCNA) and mutant TP53. miR-423 has also been identified as 1 of 7 population-differentiated miRs between African and non-African tumours [25]. The pre-miR-423-12C SNP blocked the endogenous processing of pri-miR-423 to its 2 mature miRs [26]. This data suggests that miR-432 may have important prognostic significance in breast cancer. It should be noted, however, that another miR, miR-3184, is co-located with the miR-423 locus. The miR-3184 pre-miRNA is slightly smaller than that of miR-423 (by 12 base pairs 3′ and 7 bases 5′), but perfectly overlaps it [27]. This would place rs6505162 in the pri-miR-3184, and it is possible that some of the effects for this SNP may be mediated through miR-3184 instead of miR-423, hence future research should simultaneously examine both miRs.

In this study, we showed that miR-101 is most highly expressed in tumours of Indian patients. This miR has been shown to stimulate MCF 7 growth and proliferation in an estradiol-

deprived environment via activating phosphorylated ALK. miR-101 promoted estrogen independency and tamoxifen resistance in an in vivo-selection system [28].

SNPs are germline variations interspersed in the human genome. Since they are often located within non-coding regions, their functional significance is not always clear. The discovery of miRs and their ability to bind the 3′-UTR of target genes, is providing a functional explanation of how some SNPs, positioned in non-coding regions, could contribute to cancer susceptibility, with data emerging that miR SNPs are associated with many cancers such as breast cancer [29], gynaecological malignancy [30], lung cancer [31], laryngeal cancer [32], and others. Such genetic variants may alter miR processing and/or target selection, likely contributing to cancer susceptibility and disease progression. Certain SNPs in miRs were shown to modify breast and ovarian cancer risk in a Jewish [33] and an Australian cohort [24]; a summary of the available data on miR SNPs and breast cancer risk is provided in Table 3. For example, CC homozygosity at rs6505162, which could be seen predominantly in our Indian breast cancer patients, was shown to be associated with increased ovarian cancer risk in a high-risk Jewish population (RR 2.77; p = 0.028; 95% CI, 1.11--6.9) [33].

The latest release of miRBase (release 21, June 2014, http://www.mirbase.org), contains 2,588 mature human miRs, so we acknowledge the limitations of our study in examining only 84 miRs. We also acknowledge the relatively small number of breast cancer cases included in this study. Nevertheless, this study proof-of-principle study emphasises the divergent roles of miRs in breast cancer, suggesting that specific genetic variants of miR genes may affect breast cancer risk in different ethnic groups. This opens up some interesting avenues for use in a larger study in the future. For example, miR gene targets could allow the modification of risk/outcome within a particular ethnic group(s). miR-140-5p, for instance, has been shown to act as a tumour suppressor gene by regulating VEGFA and modifying the outcome in Caucasian colorectal cancer [34]. Next-generation sequencing offers a platform to study and identify novel miRs [35]. Further comprehensive investigation of miRs in breast cancer is

warranted, in order to verify their role and also the potential diagnostic and therapeutic implications for women of various ethnic backgrounds.

Acknowledgements

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Appendix after References (Editorial Comments)

Legend(s)

Fig. 1. Differential expression of miRs across the 4 ethnic groups. The y axis shows miR fold change. Hash symbols and asterisks represent significant and highly significant differences, respectively. NG, Nigerian; IN, Indian; GB, British Caucasian; GBB, British Black.

Table(s)

Footnote(s)

Table 1. Predicted top 10 targets for miR-140-5p, miR–194, and miR–423-5p using the TargetScan, microRNA.org, and DIANA-TarBase platforms

Database									
TargetScan	microRNA.org	DIANA	TargetScan	microRNA.org	DIANA		TargetScan	microRNA.org	DIANA
miR-140-5p			miR-194		miR-194-3p	miR-194-5p	miR-423-5p		
ZNF800	TBC107	**HDAC4**[a]	MTMR2	TBC1D7	CIMP	**EP300**[a]	NRSN2	AFG3L1	WIZ
YOD1	KAT2B	**VEGFA**[a]	KIAA1210	FLJ43390	LIMA1	MEIS2	C20orf27	PK1A	C22orf42
FGF9	ANKRD42	BCL2L11	LRRFIP1	EPC2	COMT	BAZZA	FOXP4	CD1C	MICALL1
SEPT2	PRDM1	SNX27	CHORDC1	PTPN20A	S100A4	AKAP6	SOX12	PLCB1	IRGQ
MMD	GALK2	UBE3A	TUSC3	PTPN20B	EXD3	YAP1	SUFU	CNTLN	C17orf70
C15orf29	C14orf101	CHD7	XPNPEP3	NEUROD1	LRRC42	SRCAP	NACC1	ZBT9	DDX
SNX16	JAG1	ZNF7	MAP2	CHORDC1	KIF1A	SLC5A24	TRPM2	LOC100128340	TNP02
CMTM6	**SEPT2**	ENTPD1	DISC1	SLK	SEZ6L2	GPR156	SYP	GPATCH8	PANX2
SNX2	ECT2	DAG1	C13orf27	HBEGF	COMMD1	ITSN1	CPLX2	ODF3L1	KIAA1671
TSSK2	GCA	NDUFC1	ANGPTL1	FMR1	TPD52	SNX1	CCDC64	SCAF1	UBE2J2

Entries for miR-194 on DIANA are only listed in –5p/–3p nomenclature. Database versions used were: TargetScan (release 6.2, June 2012); microRNA.org (August 2010 release); DIANA-TarBase (v7.0). Bold type denotes genes predicted in >1 database.

[a] Genes predicted in >1 database and also matched and experimentally validated using miRanda.

Table 2. Frequency of miR-423-5p SNP rs6505162 amino acid change across ethnic groups by in silico analysis

Ethnic group	Frequency of genotype, % rs6505162		
	A/A	A/C	C/C
European	30	57	10
Asian	10	20	60
African	60	20	10

Table 3. Summary of previous studies of miR SNPs and breast cancer risk in different ethnic cohorts

SNP ID	miR (allele)	Genotype	Genotyping method	Ethnicity	OR (95% CI)	Reference
rs6505162	miR-423-CA	AA AC CC	MassARRAY	Jewish	ref. 2.84 (1.17–6.85) 2.77 (1.11–6.90)	[33]
rs11614913	miR-196a-2 (T/C)	CC CT TT	MassARRAY	USA	ref. 0.84 (0.63–1.12) 0.44 (0.28–0.70)	[36]
		CC CT TT	MassARRAY		ref. 0.73 (0.56–0.96), adjusted 0.54 (0.38–0.75), adjusted	[37]
rs3746444	miR-499 (A/G)	AA AG GG AG/GG	PCR-RFLP	Asian	ref. 1.19 (0.97–1.46) 1.75 (1.07–2.85) 1.25 (1.02–1.51	[38]
		AA AG GG		Asian	ref. 1.41 (1.06–1.87), adjusted non-significant	[37]
rs895819	miR-27a	AA AG GG	sequencing	German	ref. 0.77 (0.66–0.91) 0.88 (0.68–1.15)	[39]
		CT TT	MassARRAY	German	ref. 1.96 (1.16–3.33)	[33]
rs4919510	miR-608	GG GC CC	sequencing	Asian	 non-significant non-significant	[37]
rs2292832	miR-149	CC CT TT	PCR-RFLP	Asian	non-significant	[40]
rs2910164	miR-146a	GG GC CC	sequencing TaqMan MassARRAY	Caucasian Asian	 non-significant	[41] [42]
rs3746444	miR-499	TT TC CC	PCR-RFLP sequencing	Asian Caucasian	non-significant 	[38] [43]
rs6505162	pre-miR-423	CA	TaqMan genotyping assay	South American	increases the risk of familial BC in families with a strong history of BC	[13]

miR, microRNA; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; RFLP, restriction fragment length polymorphism; ref., reference; BC, breast cancer**.**

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