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Down-regulation of the miRNA master regulators Drosha and Dicer is associated with specific subgroups of breast cancer

Konstantin J. Dedes [a,d], *Rachael Natrajan* [a,d], *Maryou B. Lambros* [a], *Felipe C. Geyer* [a], *Maria Angeles Lopez-Garcia* [a,b], *Kay Savage* [a], *Robin L. Jones* [c,*], *Jorge S. Reis-Filho* [a,*]

[a] *Molecular Pathology Laboratory, The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London SW3 6JB, UK*
[b] *Hospital Universitario Virgen del Rocio, Seville, Spain*
[c] *University of Washington, Department of Medicine, Seattle, WA, USA*

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ABSTRACT

Down-regulation of Drosha and Dicer has been suggested to be of prognostic value in some cancers. The aims of our study were to investigate the down-regulation of Drosha and Dicer in breast cancers and its associations with clinicopathological features, molecular subtypes and outcome. Drosha and Dicer expression was assessed with real-time RT-PCR in 245 patients with breast cancer receiving adjuvant anthracycline-based chemotherapy and compared to expression levels of normal breast tissue. Drosha down-regulation was observed in 18% of cases and was associated with high grade, high Ki-67, lack of Bcl2 expression, HER2 over-expression and gene amplification and *TOPO2A* gene amplification. Dicer down-regulation was found in 46% of cases and was associated with lack of expression of ER, PR and Bcl2 and with high grade, high Ki-67, triple-negative and basal-like phenotypes. Drosha and Dicer were concurrently down-regulated in 15% of cases and significantly associated with high grade and high Ki-67 index. No significant associations between down-regulation of Drosha and/or Dicer and outcome were observed. Our results suggest that down-regulation of Drosha and/or Dicer is not robustly associated with the outcome of breast cancer patients treated with adjuvant anthracycline-based chemotherapy but preferentially observed in distinct subgroups of breast cancer.

1. Introduction

MicroRNAs (miRNAs) are a recently discovered class of small non-coding RNA gene products that are believed to regulate the activity of messenger RNAs (mRNAs) by antisense base pairing.[1] miRNAs have sequences of only 15–30 nucleotides and have been shown to be involved in several key developmental and biological phenomena, including cell differentiation, proliferation and apoptosis. There is increasingly more coherent evidence that miRNAs play an important role in cancer development.[2,3] In fact, alterations in the expression levels of miRNAs globally, and also of specific miRNAs, have been demonstrated in several solid and haematological tumours,[4–10] some of these have been reported to be of prognostic significance.[10–14] Additionally, several miRNAs have the ability to regulate the expression of genes including oestrogen

* *Corresponding authors*: Address: Seattle Cancer Care Alliance, University of Washington, Department of Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA, USA (R.L. Jones); The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK. Fax: +44 2071535167 (J.S. Reis-Filho).
E-mail addresses: ROBIN.JONES@doctors.org.uk (R.L. Jones), Jorge.Reis-Filho@icr.ac.uk (J.S. Reis-Filho).
[d] These authors contributed equally to the present study.
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receptor alpha (*ESR1*) in breast cancer[15–17] and other miRNAs are markedly down-regulated and may have a tumour suppressor function in ovarian cancer.[9,10]

In the process of miRNA genesis, polymerase II transcribes an immature form of miRNA, which may comprise up to thousands of base pairs called pri-miRNA, which contain a 5′ cap and a (poly)A tail.[2] Following the generation of the pri-miRNAs, two enzymes play pivotal roles in the processing of these transcripts: Drosha (*RNASEN*) and Dicer (*DICER1*). Drosha, a nuclear enzyme, cuts pre-pri-mRNA segments into short double-stranded RNA precursors, known as pre-miRNA, that have an approximate length of 60–70 nucleotides.[18] The pre-miRNAs are then cleaved in the cytoplasm through Dicer into mature double-stranded miRNA fragments of approximately 15–30 nucleotides in length each.[19,20]

Given the roles played by Drosha and Dicer, it has been hypothesised that the global down-regulation of miRNA expression in tumours may be driven by alterations in the expression levels or activity of these two enzymes. In fact, down-regulation of expression levels of Dicer[21–23] and both Drosha and Dicer[24] have been shown to be associated with aggressive clinical behaviour in lung, in breast and in ovarian cancers, respectively, however, this association has been a matter of controversy. Whilst some studies have suggested a potential association between reduced levels of Drosha and Dicer and reduced survival,[24–28] others have shown the opposite (i.e. up-regulation of Dicer[29] or Drosha[30] to be associated with poor clinical outcome) or found no association with the outcome.[31] In breast cancer, down-regulation of Dicer has been associated with oestrogen receptor (ER)-negative disease and shorter metastasis-free survival, and it has consequently been proposed as a novel prognostic marker.[22] Similar associations between reduced levels of Drosha or Dicer and poor survival were found by reanalysis of publicly available gene expression array data sets.[26–28] It should be noted, however, that previous analyses included patients treated with multiple modalities of systemic therapy and that the assessment of these genes was made using microarrays.

The aims of this study were to investigate the prevalence of Drosha and Dicer down-regulation in primary breast cancer samples and their associations with clinicopathological features and molecular subtype of the tumours. In addition, we tested whether Drosha and Dicer expression levels would be associated with the outcome of breast cancer patients uniformly treated with anthracycline-based chemotherapy ± endocrine therapy.

2. Material and methods

2.1. Sample cohort

A tissue microarray (TMA) was constructed with primary breast cancer samples with replicate 0.6 mm cores of 245 invasive breast cancers (185 invasive ductal carcinomas, 27 invasive lobular carcinomas, 25 invasive mixed carcinomas and 8 invasive breast carcinomas of other special types). Patients were selected on the basis that they were diagnosed and managed at the Royal Marsden Hospital, London, UK, between 1994 and 2000. Consequently, all patients were managed uniformly according to the Royal Marsden Breast Unit guidelines, with therapeutic surgery (69 mastectomy and 156 wide local excision) followed by anthracycline-based chemotherapy. The majority of patients ($n = 219$) received a regimen containing epirubicin [mainly 5-fluorouracil, epirubicin (60 mg/m^2) and cyclophosphamide (FEC)] and 26 patients received a doxorubicin-containing regimen [mainly 5-fluorouracil, doxorubicin and cyclophosphamide (FAC)]. Tamoxifen was prescribed as an adjuvant endocrine therapy in 96.4% of patients with ER-positive tumours. During this time period (1994–2000) there was no published evidence reporting the significant survival benefit of anti-HER2 therapy in the adjuvant setting and, therefore, none of these women were treated with either trastuzumab or lapatinib. Complete follow-up was available for 244 patients, ranging from 0.5 to 125 months (median = 67 months, mean = 67 months). Tumours were graded according to a modified Bloom–Richardson scoring system[32] and the size was categorised according to the TNM staging.[33] Additionally, 10 normal breast samples from reduction-mammoplasties were retrieved from the hospital database. The study was approved by the Royal Marsden Hospital Ethics Committee and the results are reported according to the Reporting Recommen dations for Tumour Marker Prognostic Studies (REMARK) guidelines.[34]

2.2. Quantitative real-time PCR (qRT-PCR)

RNA was extracted from representative tumour sections containing >50% of tumour cells and from sections of normal breast samples enriched for terminal duct–lobular units (>50% of the surface area). All tumour samples were formalin-fixed, and paraffin-embedded. The assessment of the composition of tumour and normal samples was made independently by two pathologists (F.C.G. and J.S.R.-F.). Briefly, RNA was extracted using the RNeasy FFPE RNA Isolation Kit (Qiagen) followed by an additional DNase treatment and stored at –80 °C. RNA quantification was performed using the Ribogreen Quant-iT reagent (Invitrogen, UK) and reverse transcription was performed with the Superscript III (Invitrogen, UK) using random hexamers as previously described.[35] Four hundred nanograms of RNA per reaction was used with triplicate reactions performed for each sample. Quantitative real time PCR was performed using TaqMan® chemistry on the ABI Prism 7900HT (Applied Biosystems), using the standard curve method.[35] Assays were purchased from Applied Biosystems. In addition, two reference genes (TFRC and MRPL19) were used, having been previously selected as effectively normalising for degradation of RNA.[35–37] Target gene expression levels were normalised to the geometric mean of the two reference genes and normalised to a pool of 10 normal breast samples. (Assay on demand ID: Hs00174609_m1-TFRC, Hs00608522_g1-MRPL19, Hs00998583_g1-DICER, and Hs00203008_m1-DROSHA).[35] mRNA levels obtained from qRT-PCR were then converted into ratios of decreased expression ($\leqslant 1$) or increased expression (>1) relative to the levels of Dicer and Drosha mRNA levels of the 10 normal breast tissue samples as previously described for the analysis of Drosha and Dicer expression in ovarian cancers using qRT-PCR.[24]

2.3. *Immunohistochemistry and chromogenic in situ hybridisation*

The details of the immunohistochemical methods and scoring systems for ER, progesterone receptor (PR), HER2, epidermal growth factor receptor (EGFR), cytokeratin (Ck) 14, Ck 5/6 and Ck 17, Ki-67, p53, topoisomerase II alpha, caveolin-1 (CAV1) and caveolin-2 (CAV2), FOXA1, E-cadherin, CD44, Bcl2, nestin and cyclin D1 detection are described elsewhere.[38–42] Based upon the expression of HER2, ER, Ck 5/6 and EGFR, tumours were classified into basal, HER2 and luminal according to the immunohistochemical panel proposed by Nielsen and colleagues.[43] According to this definition, HER2 tumours are defined as those that express HER2 (3+) or harbour *HER2* gene amplification regardless of the expression of other markers; luminal tumours are defined as HER2-negative cancers that express ER regardless of the expression of Ck 5/6 and/or EGFR; and basal-like cancers are HER2-negative, ER-negative tumours that express Ck 5/6 and/or EGFR.[43] Samples lacking these four markers were considered to be of indeterminate phenotype. Given the reported limited robustness of the microarray methods to define luminal A and luminal B subtypes,[44,45] the lack of stability of luminal A and luminal B transcriptomic profiles,[46] and the paucity of validated immunohistochemical surrogates for these ER-positive subgroups,[47,48] we did not divide luminal tumours into these two subgroups.

TMA sections were subjected to chromogenic *in situ* hybridisation (CISH) with SpotLight probes for *CCND1*, *MYC*, *HER2*, *TOP2A* and chromosome 8 centromere. Results not in relation to Drosha and Dicer expression have already been reported elsewhere.[49,50] Only unequivocal signals in non-overlapping nuclei were counted. Signals were evaluated at ×400 and ×630 magnification and 60 morphologically unequivocal neoplastic cells were counted for the presence of the gene probe signals. Amplification was defined as >5 signals per nucleus in >50% of cancer cells or when large gene copy clusters were seen.[49] The immunohistochemical and CISH scoring was performed by at least two observers who were blinded to the results of the qRT-PCR analysis.

2.4. *Statistical analysis*

The SPSS statistical software package version 11.5 was used for all statistical analysis. Correlations between categorical variables were performed using the chi-square test and Fisher's exact test where appropriate. Metastasis-free survival (MFS) was expressed as the number of months from diagnosis to the occurrence of distant relapse. Disease-free survival (DFS) was expressed as the number of months from diagnosis to the occurrence of distant, local relapse or death (disease-related death). Breast cancer-specific survival was expressed as the number of months from diagnosis to the occurrence of breast-cancer-related death. Cumulative survival probabilities were calculated using the Kaplan–Meier method. Cases with missing qRT-PCR values for Drosha and/or Dicer were excluded from the survival analysis. Differences between survival rates were tested with the log-rank test. All tests were two-tailed. A *P*-value of 0.05 was considered as statistically significant.

3. Results

Drosha and Dicer mRNA expression using qRT-PCR was assessable in 200 samples each and the expression values for both genes were available in 197 samples. The mRNA levels were normalised to two house-keeping genes and compared to the mRNA expression of the normal breast tissue (Fig. 1). Samples with decreased expression (⩽1 ratio) of Drosha and Dicer were found, respectively, in 18% (36/200) and 46% (92/200) of the samples examined compared to normal breast tissue.

Drosha down-regulation was significantly associated with high histological grade, high proliferation as defined by Ki-67 labelling index, lack of Bcl2 expression, HER2 overexpression and *HER2* gene amplification and *TOPO2A* gene amplification (Table 1). Drosha down-regulation was significantly less frequently observed in luminal tumours (13%) and most frequently down-regulated in HER2-positive cancers (35%).

Dicer down-regulation was significantly associated with lack of markers of 'luminal' differentiation, including ER, PR and Bcl2, and with expression of 'basal-like' markers, including high molecular weight Cks (Ck 5/6 and Ck 17), EGFR, CAV1, CAV2 and nestin (Table 1). In fact, a statistically significant association between Dicer down-regulation and triple-negative and basal-like phenotypes was observed; however, not all triple-negative and basal-like cancers displayed Dicer down-regulation. Tumours with Dicer down-regulation significantly more frequently displayed high Ki-67 labelling indices.

Drosha and Dicer were concurrently down-regulated in 29 cases (15%) and significantly associated with high histological grade and high proliferation rates as defined by Ki-67 labelling index (Table 2). In fact, all of the grade 1 cancers expressed normal levels of either Drosha or Dicer. Furthermore, Drosha

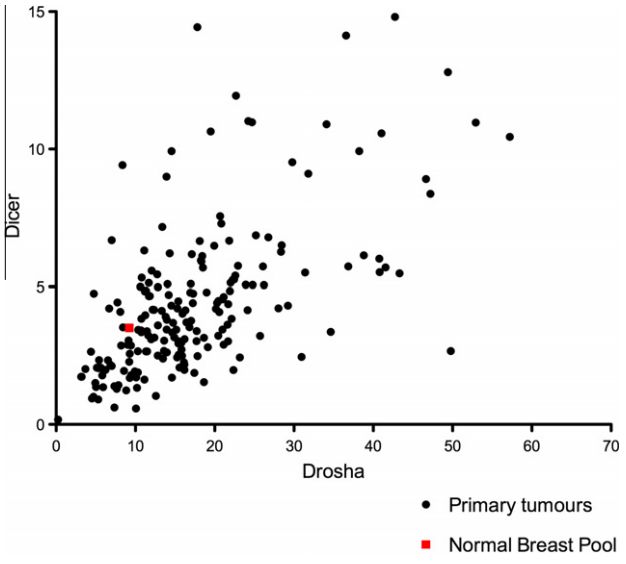

Fig. 1 – Drosha and Dicer quantitative real time PCR (qRT-PCR) expression values normalised according to two house-keeping genes (*TFRC* and *MRPL19*) in primary breast cancers and in a pool of normal breast samples.

Table 1 – Correlations between Dicer and Drosha down-regulation and clinicopathological features, immunohistochemical markers and amplification of key oncogenes.

Parameter	Drosha down-regulation				Dicer down-regulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
Size (TNM)	199			0.207[a]	199			0.872[a]
pT1		15 (14)	89 (86)			47 (45)	57 (55)	
pT2		17 (20)	66 (80)			40 (48)	43 (52)	
pT3		4 (33)	8 (67)			5 (42)	7 (58)	
Grade	**196**			**0.034**[a]	**196**			**0.013**[a]
1		1 (6)	15 (94)			3 (18)	14 (82)	
2		6 (10)	53 (90)			24 (41)	35 (59)	
3		29 (24)	92 (76)			64 (53)	56 (47)	
Histological type	198			0.5049[a]	**198**			**0.015**[a]
IDC		29 (19)	122 (81)			77 (51)	74 (49)	
ILC		2 (7)	25 (93)			7 (27)	19 (73)	
Mixed		3 (21)	11 (79)			3 (20)	12 (80)	
Other		1 (17)	5 (83)			4 (67)	2 (33)	
LVI	198			0.557[b]	**198**			**0.033**[b]
Absent		13 (21)	50 (79)			37 (58)	27 (42)	
Present		23 (17)	112 (83)			55 (41)	79 (59)	
LN metastasis	193			1.000[b]	193			0.361[b]
Absent		12 (18)	53 (82)			34 (52)	31 (48)	
Present		23 (18)	105 (82)			57 (45)	71 (55)	
ER	199			0.153[b]	**199**			**<0.001**[b]
Negative		10 (27)	27 (73)			28 (76)	9 (24)	
Positive		26 (16)	136 (84)			64 (40)	98 (60)	
PR	199			0.683[b]	**199**			**0.040**[b]
Negative		11 (20)	44 (80)			32 (58)	23 (42)	
Positive		25 (17)	119 (83)			60 (42)	84 (58)	
HER2	**199**			**0.011**[b]	199			0.139[b]
Negative		26 (15)	147 (85)			76 (44)	97 (56)	
Positive		10 (38)	16 (62)			16 (62)	10 (38)	
EGFR	199			0.215[b]	**199**			**0.002**[b]
Negative		30 (17)	149 (84)			76 (42)	103 (58)	
Positive		6 (30)	14 (70)			16 (80)	4 (20)	
Ck 14	198			0.755[b]	198			0.054[b]
Negative		32 (18)	147 (82)			79 (44)	100 (56)	
Positive		4 (21)	15 (79)			13 (68)	6 (32)	
Ck 5/6	191			0.544[b]	**191**			**0.020**[b]
Negative		29 (17)	141 (83)			74 (44)	96 (56)	
Positive		5 (24)	16 (76)			15 (71)	6 (29)	
Ck 17	**198**			**0.050**[b]	**198**			**0.004**[b]
Negative		28 (16)	146 (84)			74 (43)	100 (58)	
Positive		8 (33)	16 (67)			18 (75)	6 (25)	
Basal keratins	198			0.203[b]	**198**			**0.001**[b]
Negative		28 (17)	140 (83)			70 (42)	98 (58)	
Positive		8 (27)	22 (73)			22 (73)	8 (27)	
Any basal marker	198			0.080[b]	**198**			**<0.001**[b]
Negative		26 (16)	139 (84)			67 (41)	98 (59)	
Positive		10 (30)	23 (70)			25 (76)	8 (24)	
p53	184			0.426[b]				0.426[b]
Negative		22 (17)	104 (83)			57 (45)	70 (55)	
Positive		13 (22)	45 (78)			30 (52)	28 (48)	

(*continued on next page*)

Table 1 – (*continued*)

Parameter	Drosha down-regulation				Dicer down-regulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
Ki-67	**183**			**0.010**[a]	**184**			**0.002**[a]
Low		6 (8)	65 (92)			24 (33)	49 (67)	
Intermediate		18 (21)	67 (79)			44 (52)	40 (48)	
High		9 (33)	18 (67)			19 (70)	8 (30)	
HER2–CISH	**188**			**0.024**[b]	189			0.273[b]
Not amplified		27 (16)	137 (84)			73 (44)	92 (56)	
Amplified		9 (38)	15 (62)			14 (58)	10 (42)	
TOPO2A – CISH	**188**			**0.003**[b]	189			0.196[b]
Not amplified		27 (16)	145 (84)			77 (45)	96 (55)	
Amplified		8 (50)	8 (50)			10 (63)	6 (37)	
TOPO2A – IHC	173			0.243[b]	175			0.095[b]
Low		12 (15)	70 (85)			32 (39)	50 (61)	
High		20 (22)	71 (78)			49 (53)	44 (47)	
Molecular subtypes[c]	**193**			**0.022**[a]	**193**			**0.001**[a]
Basal-like		6 (24)	19 (76)			19 (76)	6 (24)	
HER2		9 (35)	17 (65)			15 (58)	11 (42)	
Luminal		19 (13)	123 (87)			53 (37)	89 (63)	
Triple-negative	195			0.290[b]	**196**			**0.001**[b]
No		29 (17)	139 (83)			70 (41)	99 (59)	
Yes		7 (26)	20 (74)			21 (78)	6 (22)	
CCND1-CISH	196			1.00[b]	196			0.667[b]
Not amplified		32 (19)	139 (81)			81 (47)	91 (53)	
Amplified		4 (16)	21 (84)			10 (42)	14 (58)	
MYC – CISH	161			0.110[b]	162			0.312[b]
Not amplified		26 (18)	118 (82)			65 (45)	80 (55)	
Amplified		6 (35)	11 (65)			10 (59)	7 (41)	
Caveolin 1	199			0.104[b]	**199**			**0.006**[b]
Negative		30 (17)	151 (83)			78 (43)	103 (57)	
Positive		6 (33)	12 (67)			14 (78)	4 (22)	
Caveolin 2	**173**			**0.042**[b]	**174**			**0.025**[b]
Negative		29 (17)	133 (83)			72 (44)	91 (56)	
Positive		5 (45)	6 (55)			9 (82)	2 (18)	
FOX1A	154			0.089[b]	156			0.132[b]
Negative		10 (27)	27 (73)			21 (55)	17 (45)	
Positive		17 (15)	100 (85)			47 (40)	71 (60)	
E-Cadherin	178			0.280[a]	179			0.787[a]
Absent		11 (22)	38 (78)			24 (50)	24 (50)	
Reduced		4 (33)	8 (67)			6 (50)	6 (50)	
Normal		19 (16)	98 (84)			53 (45)	66 (55)	
Nestin	148			0.516[b]	**148**			**0.019**[b]
Negative		23 (18)	108 (82)			59 (45)	72 (55)	
Positive		4 (24)	13 (76)			13 (76)	4 (24)	
Bcl2	**150**			**0.010**[b]	**152**			**0.002**[b]
Negative		18 (33)	37 (67)			35 (64)	20 (36)	
Positive		10 (11)	85 (89)			36 (38)	60 (62)	
CD44	150			0.499[a]	149			0.920[a]
Low		8 (27)	22 (73)			15 (52)	14 (48)	
Intermediate		5 (16)	27 (84)			15 (47)	17 (53)	
High		16 (18)	72 (82)			43 (49)	45 (51)	

Table 1 – (*continued*)

Parameter	Drosha down-regulation				Dicer down-regulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
Cyclin D1	183			0.206[a]	184			0.111[a]
Low		8 (32)	17 (68)			15 (60)	10 (40)	
Intermediate		5 (16)	27 (84)			19 (58)	14 (42)	
High		22 (17)	104 (83)			53 (42)	73 (58)	
Drosha or Dicer down-regulation	**197**			**<0.001**[b]	**197**			**<0.001**[b]
Yes		29 (32)	62 (68)			29 (81)	7 (19.4)	
No		7 (7)	99 (93)			62 (39)	99 (61)	

Parameters in bold where $P < 0.05$.

CISH: chromogenic *in situ* hybridisation; Ck: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; PR: progesterone receptor; IHC: immunohistochemistry; LN: lymph node; LVI: lympho-vascular invasion.

[a] Chi-squared test.

[b] Fisher's Exact Test.

[c] Breast cancer molecular subtypes as defined by Nielsen et al.[43] criteria.

and Dicer were more frequently concurrently down-regulated in tumours lacking Bcl2, overexpressing HER2 and expressing 'basal-like' markers, namely EGFR, CAV1 and CAV2. In addition, down-regulation of Drosha and Dicer was less frequently found in tumours of luminal phenotype and more prevalent in tumours harbouring amplification of *TOPO2A* and *MYC*.

Ninety-nine (50%) of the tumours displayed Drosha and/or Dicer down-regulation. These tumours were significantly more frequently of high histological grade, lacked ER, Bcl2 and Cyclin D1 expression, expressed 'basal-like' markers (namely EGFR, Ck 17, CAV1, CAV2 and nestin) and more frequently displayed high Ki-67 labelling indices. Drosha and/or Dicer down-regulation was inversely correlated with luminal phenotype and directly associated with triple-negative phenotype.

Neither Drosha nor Dicer down-regulation reached statistical significance as predictors of metastasis-free, disease-free or disease-specific survival in this population of breast cancer patients treated with anthracycline-based chemotherapy ± endocrine therapy. However, a trend for shorter disease-free survival in cases with Drosha down-regulation ($P = 0.0506$) was observed (Fig. 2). No other associations with the outcome were observed in relation to Drosha, Dicer, Drosha and Dicer or either Drosha and/or Dicer down-regulation. Furthermore, neither subgroup analysis amongst patients with ER-positive or triple negative disease reached any significant association regarding the outcome (data not shown). In accordance with REMARK guidelines the clinical characteristics and standard prognostic factors of this series are displayed in Supplementary Table 1 and univariate analysis for DFS, MFA and OS is summarised in Supplementary Table 2.

4. Discussion

Down-regulation of two master regulators of the miRNA machinery, Drosha and Dicer, has been documented in a substantial proportion of invasive breast cancers (Table 3). Our findings support previous observations suggesting a higher prevalence of Dicer down-regulation in ER-negative breast cancers[22,51,52] and the association with non-luminal subgroups (HER2 and 'basal-like').[22] Here, we demonstrate that Dicer mRNA levels are down-regulated in 76% and 40% of ER-negative and ER-positive tumours, respectively. Grelier and colleagues reported the same association at the mRNA level, however, using the immunohistochemistry for Dicer protein detection, the correlation was inverse, showing lower staining in ER-positive cases.[22] In breast cancer cell lines, concordance between mRNA and protein levels was observed in only 72%.[22] Although Dicer mRNA levels appeared to be prognostic for metastasis-free survival, the protein expression was not informative for survival.[22] Furthermore, low levels of immunoreactivity were also observed in normal breast.[22] The fact that Dicer down-regulation is associated with ER-negative disease is further supported by its significant correlation with high histological grade, triple-negative phenotype, expression of 'basal-like' markers (EGFR, CKs, CAV1, CAV2, and nestin), lack of Bcl2, and high proliferation rates as defined by Ki-67 labelling index, suggesting that Dicer may be preferentially down-regulated in non-luminal subgroups of breast cancer. It is plausible that these associations stem from the association between Dicer down-regulation and higher histological grade and higher proliferation of non-luminal cancers. Consistent with this hypothesis, Dicer silencing by RNA interference induced increased growth rates in $Kras^{G12D}$-expressing mouse embryonic fibroblasts and an analysis of conditional mouse models lacking Dicer expression developed tumours that were of higher histological grade displayed greater nuclear pleomorphism and higher growth rates than tumours expressing wild-type Dicer.[53]

Drosha down-regulation significantly correlated with Dicer down-regulation, however, only 29 (15%) of the tumours displayed both Drosha and Dicer down-regulation. Unlike Dicer, which is preferentially down-regulated in triple-negative and basal-like cancers, Drosha down-regulation was significantly associated with HER2-positive disease (as defined by both immunohistochemistry and with an FDA approved CISH method).

We did not observe a significant association between down-regulation of Drosha and/or Dicer and poor outcome in a cohort of patients uniformly treated with adjuvant

Table 2 – Correlations between Drosha and Dicer down-regulation or Drosha and/or Dicer down-regulation and clinicopathological features, immunohistochemical markers and amplification status of key oncogenes.

Parameter	Drosha and Dicer downregulation				Drosha and/or Dicer downregulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
Size (TNM)	200			0.428[a]	198			0.805[a]
pT1		12 (11)	93 (89)			50 (48.5)	53 (51.5)	
pT2		15 (18)	68 (82)			42 (50.6)	41 (49.4)	
pT3		2 (17)	10 (83)			7 (58.3)	5 (41.7)	
Grade	**197**			**0.026**[a]	**195**			**0.018**[a]
1		0 (0)	17 (100)			4 (25)	12 (75)	
2		5 (9)	54 (91)			25 (42)	34 (58)	
3		24 (20)	97 (80)			69 (57)	51 (43)	
Histological type	**199**			**0.041**[a]	**197**			**0.028**[a]
IDC		25 (17)	126 (83)			81 (54)	70 (46)	
ILC		1 (4)	26 (96)			8 (31)	18 (69)	
Mixed		2 (13)	13 (87)			4 (29)	10 (71)	
Other		0 (0)	6 (100)			5 (83)	1 (17)	
LVI	199			0.284[b]	197			0.067[b]
Absent		12 (19)	52 (81)			38 (60)	25 (40)	
Present		17 (13)	118 (87)			61 (46)	73 (54)	
LN metastasis	194			1.0[b]	192			0.286[b]
Absent		10 (15)	56 (85)			36 (56)	28 (44)	
Present		19 (15)	109 (85)			61 (48)	67 (52)	
ER	200			0.197[b]	**198**			**<0.001**[b]
Negative		8 (22)	29 (78)			30 (81)	7 (19)	
Positive		21 (13)	142 (87)			69 (43)	92 (57)	
PR	**200**			0.656[b]	198			0.056[b]
Negative		9 (16)	46 (84)			34 (62)	21 (38)	
Positive		20 (14)	125 (86)			65 (45.5)	78 (54.5)	
HER2	**200**			**0.031**[b]	198			0.057[b]
Negative		21 (12)	153 (88)			81 (47)	91 (53)	
Positive		8 (31)	18 (69)			18 (69)	8 (31)	
EGFR	200			**0.049**[b]	**198**			**0.008**[b]
Negative		23 (13)	157 (87)			83 (47)	95 (53)	
Positive		6 (30)	14 (70)			16 (80)	4 (20)	
Ck 14	199			0.490[b]	197			0.146[b]
Negative		25 (14)	155 (86)			86 (48)	92 (52)	
Positive		4 (21)	15 (79)			13 (68)	6 (32)	
Ck 5/6	192			0.185[b]	190			0.062[b]
Negative		22 (13)	149 (87)			81 (48)	88 (52)	
Positive		5 (24)	16 (76)			15 (71)	6 (29)	
Ck 17	199			0.057[b]	**197**			**0.004**[b]
Negative		22 (13)	153 (87)			80 (46)	93 (54)	
Positive		7 (29)	17 (71)			19 (79)	5 (21)	
Basal Cks	199			0.160[b]	**197**			**0.002**[b]
Negative		22 (13)	147 (87)			76 (46)	91 (54)	
Positive		7 (23)	23 (77)			23 (77)	7 (23)	
Any basal marker	**199**			**0.032**[b]	**197**			**<0.001**[b]
Negative		20 (12)	146 (88)			73 (45)	91 (55)	
Positive		9 (27)	24 (73)			26 (79)	7 (21)	
p53	185			1.00[b]	184			0.204[b]
Negative		19 (15)	108 (85)			60 (48)	66 (52)	
Positive		9 (16)	49 (84)			34 (59)	24 (41)	
Ki-67	**184**			**0.013**[a]	**183**			**0.001**[a]
Low		4 (6)	68 (94)			26 (36)	46 (64)	
Intermediate		16 (19)	69 (81)			46 (55)	38 (45)	
High		7 (26)	20 (74)			21 (78)	6 (22)	

Table 2 – (*continued*)

Parameter	Drosha and Dicer downregulation				Drosha and/or Dicer downregulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
HER2 – CISH	189			0.064[b]	188			0.125[b]
Not amplified		22 (13)	143 (87)			78 (48)	86 (52)	
Amplified		7 (29)	17 (71)			16 (67)	8 (33)	
TOPO2A – CISH	**189**			**0.021**[b]	**188**			**0.038**[b]
Not amplified		23 (13)	150 (87)			81 (47)	91 (53)	
Amplified		6 (38)	10 (62)			12 (75)	4 (25)	
TOPO2A – IHC	174			0.130[b]	174			0.129[b]
Low		8 (10)	74 (90)			36 (44)	46 (56)	
High		17 (18)	75 (82)			52 (57)	40 (43)	
Molecular subtypes[c]	**194**			**0.031**[a]	**192**			**0.001**[a]
Basal-like		6 (24)	19 (76)			19 (76)	6 (24)	
HER2		7 (27)	19 (73)			17 (65)	9 (35)	
Luminal		15 (10)	128 (90)			57 (40)	84 (60)	
Triple-negative	196			0.248[b]	**195**			**0.001**[b]
No		23 (14)	146 (86)			76 (45)	92 (55)	
Yes		6 (22)	21 (78)			22 (81)	5 (19)	
CCND1-CISH	197			1.00[b]	195			0.669[b]
Not amplified		26 (15)	146 (85)			87 (51)	84 (49)	
Amplified		3 (12)	22 (88)			11 (46)	13 (54)	
MYC – CISH	**162**			**0.034**[b]	161			0.609[b]
Not amplified		20 (14)	125 (86)			71 (49)	73 (51)	
Amplified		6 (35)	11 (65)			10 (59)	7 (41)	
Caveolin 1	**200**			**0.029**[b]	**198**			**0.024**[b]
Negative		23 (13)	159 (87)			85 (47)	95 (53)	
Positive		6 (33)	12 (67)			14 (78)	4 (22)	
Caveolin 2	**174**			**0.015**[b]	173			0.058[b]
Negative		22 (13.5)	141 (86.5)			79 (48.8)	83 (51.2)	
Positive		5 (45.5)	6 (54.5)			9 (81.8)	2 (18.2)	
FOX1A	155			0.176[b]	155			0.064[b]
Negative		8 (22)	29 (78)			23 (61)	15 (39)	
Positive		14 (12)	104 (88)			50 (43)	67 (57)	
E-Cadherin	179			0.396[a]	178			0.677[a]
Absent		9 (18)	40 (82)			26 (54)	22 (46)	
Reduced		3 (25)	9 (75)			7 (58)	5 (42)	
Normal		15 (13)	103 (87)			57 (48)	61 (52)	
Nestin	149			0.719[b]	**147**			**0.010**[b]
Negative		19 (14)	113 (86)			63 (48)	67 (52)	
Positive		3 (18)	14 (82)			14 (82.4)	3 (17.6)	
Bcl2	**151**			**0.002**[b]	**150**			**0.001**[b]
Negative		15 (27)	40 (73)			38 (69)	17 (31)	
Positive		8 (8)	88 (92)			38 (40)	57 (60)	
CD44	151			0.961[a]	148			0.488[a]
Low		5 (17)	25 (83)			18 (62)	11 (38)	
Intermediate		5 (16)	27 (84)			15 (47)	17 (53)	
High		13 (15)	76 (85)			46 (53)	41 (47)	
Cyclin D1	184			0.438[a]	**183**			**0.034**[a]
Low		6 (24)	19 (76)			17 (68)	8 (32)	
Intermediate		4 (12.1)	29 (87.9)			20 (62.5)	12 (37.5)	
High		19 (15.1)	107 (84.9)			56 (44.4)	70 (55.6)	
Dicer down-regulation	**198**			**<0.001**[a]	**198**			**<0.001**[a]
Yes		29 (32)	62 (68)			92 (100)	0 (0)	
No		0 (0)	107 (100)			7 (7)	99 (93)	

(*continued on next page*)

Table 2 – (*continued*)

Parameter	Drosha and Dicer downregulation				Drosha and/or Dicer downregulation			
	n	Yes	No	*P*-value	*n*	Yes	No	*P*-value
Drosha down-regulation	**199**			**<0.001**[a]	**197**			**<0.001**[a]
Yes		29 (81)	7 (19)			36 (100)	0 (0)	
No		0 (0)	163 (100)			62 (39)	99 (61)	

Parameters in bold where $P < 0.05$.

CISH: chromogenic *in situ* hybridisation; Ck: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; PR: progesterone receptor; IHC: immunohistochemistry; LN: lymph node; LVI: lympho-vascular invasion.

[a] Chi-squared test.

[b] Fisher's Exact test.

[c] Breast cancer molecular subtypes as defined by Nielsen et al.[43] criteria.

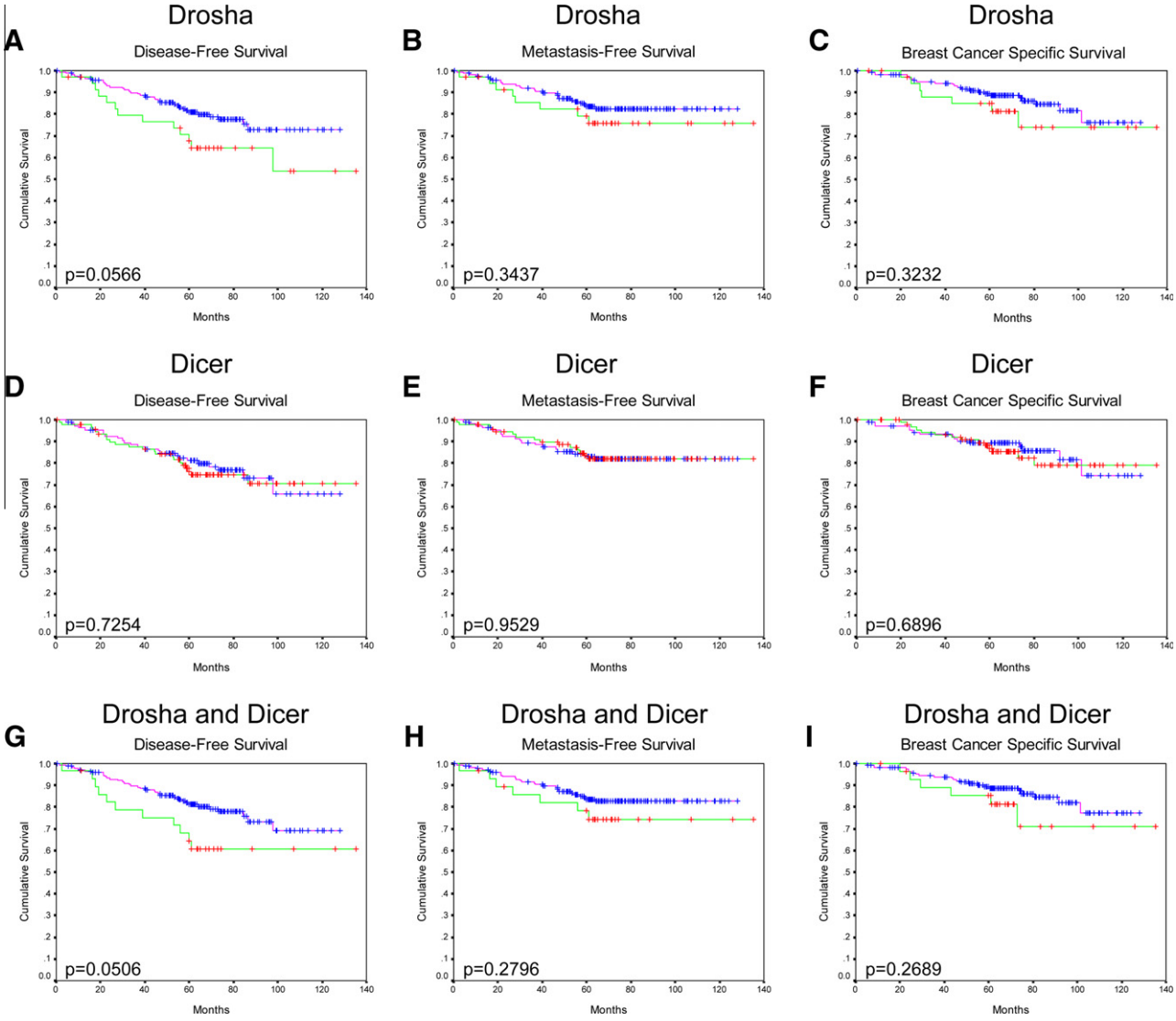

Fig. 2 – Kaplan–Meier survival curves for disease-free survival, metastasis-free survival and breast cancer specific survival (months) in cases categorised according to the mRNA expression levels of Drosha (A–C), Dicer (D–F), and concurrent Drosha and Dicer (G–I). Cases with down-regulation are shown with green lines, whereas cases with up-regulation are shown with pink lines. Ticks represent cases censored at last follow-up. Drosha (*n* = 199); Dicer (*n* = 199); Drosha and Dicer (*n* = 200). Please note that cases where either Drosha or Dicer was not down-regulated were considered as not-down-regulated for survival analysis.

Table 3 – Results and characteristics from previous studies on Drosha and / or Dicer expression levels and outcome of breast cancer patients.

Cohort	Number of patients	Years of inclusion	Samples	ER-positive (%)	% of cohort with adjuvant chemotherapy	Follow-up	Method	Gene investigated	Cut offs for low and high expression	Cases with low / high Dicer expression	Prognostic value for disease free survival	Reference
Grelier et al.	104	1992–1999	Fresh-frozen	79.00	NM	NM	qRT-PCR	Dicer	Cases grouped according to median levels of Dicer mRNA levels into low and high expressors	45 with low Dicer and 43 with high Dicer	Yes, only Dicer mRNA levels	[22]
Grelier et al.	86	1998	Formalin-fixed paraffin-embedded	82.60	NM	NM	IHC	Dicer	0: lack of immunoreactivity, 1: weak immunoreactivity, 2: strong immunoreactivity	intensity 0: 7, intensity 1: 58 intensity 2: 21	No	[22]
Chin et al. (re-analysed in Merritt et al.)	129	1989–1997	Fresh-frozen	67	50% with anthracycline-containing	Median 6.6 years	Based on published gene expression array data (E-TABM-158)	Drosha, Dicer	Cases grouped according to median levels of Dicer mRNA levels into low and high expressors	NM	Yes, only Dicer mRNA levels	[24,26]
Pawitan et al. (re-analysed in Merritt et al.)	159	1994–1996	Fresh-frozen	82.04	18.72% with mostly CMF	Mean 6.1 years	Based on published gene expression array data (GSE1456)	Drosha, Dicer		80 with high Dicer, 79 with low Dicer	Yes, only Dicer mRNA levels	[24,28]
Ivshina et al. (re-analysed in Merrit et al.)	249	1987–1989	Fresh-frozen	82.36	8.99%, NM type	Median 8.8 years	Based on published gene expression array data (GSE4922)	Drosha, Dicer		125 with high Dicer, 124 with low Dicer	Yes, only Dicer mRNA levels	[24,27]

CMF: cyclophosphamide, methotrexate, 5-fluorouracil; ER: oestrogen receptor; IHC: immunohistochemistry; NM: not mentioned; qRT-PCR: quantitative reverse-transcription-PCR.

anthracycline-based chemotherapy. These results are in contrast to the report from Merrit and colleagues in ovarian, lung and breast cancer patients.[24] There might be several reasons for this discrepancy. First, in that analysis,[24] Drosha and Dicer expressions in breast cancer were obtained from microarray experiments and the cut-offs employed were based on the median expression of these genes in each cohort. Herein, we have employed qRT-PCR and normalised the results to the expression of these genes in normal breast tissue, as described for the analysis of Drosha and Dicer expression in ovarian cancers described by the same group.[24] Another important difference is that herein, all patients were uniformly treated with adjuvant anthracycline-based chemotherapy ± endocrine therapy, whereas in the breast cancer cohorts analysed by Merritt and colleagues,[24] patients received different systemic therapy regimens. Grelier and colleagues also reported an association between reduced levels of Dicer and shorter metastasis-free survival; regrettably, the systemic therapies of patients included in that study were not reported[22] and survival curves were not adjusted for therapies. Furthermore, in the study by Grelier and colleagues[22] the mRNA levels were not normalised to the expression of normal breast tissues. Instead, the median mRNA expression value was used to calculate a threshold separating low and high Dicer mRNA expressing tumours. Taken together, our results suggest that although Drosha and Dicer down-regulation may be associated with the outcome of breast cancer patients, they may not be significant prognostic factors in a population of patients treated uniformly with adjuvant anthracycline-based chemotherapy (which account for up to 70% of all patients with early breast cancer).

One of the limitations of this study is that expression of both enzymes was based on mRNA extracted from formalin-fixed, paraffin-embedded samples. It should be noted, however, that the qRT-PCR assays were designed for fragmented mRNAs and all amplicons were <80 bp. Although we have endeavoured to develop an immunohistochemical assay for Drosha and Dicer, as previously reported,[22,24] none of the antibodies tested specifically displayed the expected subcellular distribution or were optimal when applied to our *in vitro* and formalin-fixed paraffin-embedded controls (i.e. cell lines where the mRNA and protein expression levels of Drosha and Dicer were determined by qRT-PCR and Western blotting, respectively, and subsequently immunohistochemically tested after formalin fixation for 24 h and paraffin embedding; data not shown). Furthermore, in agreement with the results reported by Greiler and colleagues,[22] our pilot study also revealed a poor correlation between Dicer expression levels defined by qRT-PCR and immunohistochemistry, possibly due to the specificity of the antibody on formalin-fixed, paraffin embedded samples after antigen retrieval (data not shown). Owing to the unresolved issues about the specificity, sensitivity and dependence on pre-analytical variables for the currently immunohistochemical assays for Drosha and Dicer and our inability to optimise these antibodies using ideal controls, we have decided not to analyse the immunohistochemical expression of Drosha and Dicer in this cohort of patients. Further studies to develop and validate adequate immunohistochemical methods for the analysis of Drosha and Dicer in formalin-fixed paraffin embedded tissue sections are warranted.

In conclusion, contrary to previous reports,[22,24] our results suggest that mRNA levels of either Drosha or Dicer or both do not constitute robust prognostic markers in patients treated with anthracycline-containing adjuvant treatment ± endocrine treatment, using an approach previously employed to demonstrate the prognostic significance of down-regulation of these enzymes in ovarian cancer.[24] Down-regulation of Drosha and Dicer, however, seems to be preferentially found in HER2-positive disease and in triple-negative/'basal-like' cancers, respectively, suggesting that distinct components of the miRNA machinery may be dysfunctional in different molecular subgroups of breast cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.08.007.

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