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ORIGINAL ARTICLE

Epithelial-mesenchymal transition profiles in triple negative breast carcinoma may explain its aggressive nature

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Abstract

Epithelial-mesenchymal transition (EMT) is increasingly explored in cancer progression. Considering that triple negative (TN) breast cancer has the poorest survival among molecular subtypes, we investigated 49 TN, 45 luminal and 25 HER2-enriched female breast carcinomas for EMT expression (using E-cadherin and vimentin immunohistochemistry) against lymphovascular and/or lymph node invasion. E-cadherin and vimentin expressions were semi-quantitated for positive- cancer cells (0=0-<1%, 1=1-10%, 2 =11-50%, 3=>50%) and staining intensity (0=negative, 1=weak, 2=moderate, 3=strong), with final score (low=0-4 and high=6-9) derived by multiplying percentage and intensity scores for each marker. Low E-cadherin and/or high vimentin scores defined EMT positivity. Low E-cadherin co-existing with high vimentin defined "complete" (EMT-CV), while low E-cadherin (EMT-C) or high vimentin (EMT-V) occurring independently defined "partial" subsets. 38 (31.9%) cancers expressed EMT, while 59.2 % TN, 13.3% luminal and 12% HER2-enriched cancers expressed EMT ($p<0.05$). Among the cancers with lymphovascular and/or lymph node invasion, EMT positivity by molecular types were 66.7% TN, 7.4% luminal and 11.8% HER2-enriched ($p<0.05$). Although EMT-V, associated with stem-cell properties was the dominant TN EMT profile, EMT-CV, a profile linked to vascular metastases, was encountered only in TN. EMT appears important in TN cancer and different EMT profiles may be associated with its aggressive nature.

Keywords: Breast carcinoma, E-cadherin, epithelial-mesenchymal transition, molecular subtype, triple negative, vimentin

INTRODUCTION

Although breast cancers continue to reign as the malignancy with the highest incidence amongst women worldwide[1-4], much has progressed in the understanding of its biology which has resulted in improved management and survival. Apart from the conventional categorisation by histological subtype, grade and stage, there have been moves towards a more refined classification which encompasses the individual tumour's intrinsic characteristics that can assist in determining treatment options. The use of DNA microarray and hierarchical clustering of gene expressions by Perou *et al*. delivered the landmark molecular characterization of breast cancers in 2000.[5] A year later, Sorlie *et al*. demonstrated the association of survival with different molecular subtypes[6], underscoring the importance of molecular subtyping of breast cancers. Nevertheless, while detailed genotyping seems most ideal for customising treatment, it remains impractical due to the lack of readily available facilities in routine diagnostic laboratories and cost constraints. Thus, most resort to use the of commonly available immunohistochemical markers i.e. estrogen receptors (ER) and progesterone receptors (PR) and amplification of human epidermal growth factor receptor 2 (HER2) to create a surrogate molecular subtyping system that is generally able to capture predictive and prognostic heterogeneity.[7] Using this system, breast carcinomas can be classified

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as (1) luminal, (2) HER2-enriched and (3) triple negative tumours (TN).[8,9] In terms of natural history, luminal tumours which constitute about 60-70% of breast carcinomas[10-12] fare better than HER2-enriched and TN cancers[6,7,13], the latter two occurring in approximately equal proportions. The introduction of target-specific treatment has however provided survival advantage to HER2-enriched tumours, presently leaving TN as the only "target-orphan".[7] That TN generally takes an aggressive path, affects younger patients, presents as larger and higher grade tumours with high risk of distant metastases[12,14-16], highlights an urgency for identifying therapeutic targets for these tumours.

The poor survival and aggressive nature of TN tumours is largely due to its highly metastatic behaviour with frequent metastases to brain and lung within 3-5 years.[17,18] It is believed that the migratory and invasive property of tumour cells is through acquisition of mesenchymal and loss of epithelial characteristics (epithelial-mesenchymal transition or EMT)[11,19-21], an important phenomenon that should be better understood in TN tumours. Based on this, we were interested to determine if EMT has a role in breast cancers, focusing in particular on TN using loss of E-cadherin (epithelial-cadherin), a cell surface protein which allows for cell-to-cell adhesion that is coded by the CDH1 (cadherin 1) gene, and a gain of vimentin, a type III intermediate filament expressed in mesenchymal cells[10,22] as markers of EMT acquisition.

MATERIALS AND METHODS

Selection of cases

Histologically-confirmed invasive female breast carcinoma, at first presentation or recurrent between 2012 till 2017, which had undergone mastectomy or excision were recruited from the archives of the Department of Pathology, University of Malaya Medical Centre (UMMC). Lobular carcinomas were excluded due to their inherent lack of E-cadherin. Recruitment was conceptualised for retrieval of about 60 TN tumours based on the surgical pathology reports, and approximately equal numbers of consecutive non-TN tumours during the same period, allowing for a slight excess of this second group to try to increase the number of HER2-enriched tumours. All the histological sections of the cases, including the immunohistochemically (IHC) stained ER, PR and HER2 and the silver in-situ hybridisation (SISH) HER2 sections were retrieved and re-evaluated. Cases with insufficient archived material for immunohistochemical assessment for EMT were excluded from the study.

Histological typing, grading and molecular classification

Histological sub-typing was according to the 2012 WHO Classification of Tumours of the Breast[23,24] and histological grading was based on the Modified Bloom and Richardson Scoring System.[25] Interpretation of ER, PR and HER2 status was carried out according to the Guidelines of the American Society of Clinical Oncology/College of American Pathologists.[26-29] In this study, "HER2 positive" would mean 3+ staining (overexpression) on IHC and/or HER2/CEP17 ratio ≥2.0 with an average of ≥4.0 HER2 signals/cell or HER2/CEP17 ratio <2.0 but with an average of ≥6.0 HER2 signals/cell on SISH. Following re-evaluation, the cases were categorised into molecular subtypes using the consensus of the 12th St Gallen International Breast Cancer Conference in 2011[9], as luminal A (ER positive and/or PR positive, HER2 negative, of low histological grade i.e. grade 1 or 2 and/or with Ki67 labeling index <14%), luminal B1 (ER positive and/or PR positive, HER2 positive), luminal B2 (ER positive and/or PR positive, HER2 negative, of high histological grade i.e. grade 3 and/or with Ki67 labeling index>14%), HER2-enriched (ER negative, PR negative, HER2 positive) and triple negative (ER negative, PR negative, HER2 negative). Although Ki67 expression can be used to distinguish between HER2 negative luminal A and luminal B2 tumours[9], using Ki67 for distinguishing between the two was not routinely practiced at our centre. As Ki67 is presently not recommended by the American Society of Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) as a routine determinant marker due to inconsistencies in cut-off value as well as reproducibility[30,31], it was decided that for the purpose of this study, differentiation of luminal A from B2 would be based on histological grade, without additional determination of Ki67.

Epithelial-mesenchymal transition (EMT)

For the establishment of epithelial-mesenchymal transition, one paraffin block of the tumour showing tumour-stromal interface was selected during the histological review for immunohistochemical staining. Prior to use of the paraffin block, it was ascertained that sufficient tumour tissue should remain in the

paraffin block for further review after sectioning for the EMT stains. Two 4 μm sections were cut from the selected paraffin block on to platinum-coated slides (Matsunami Glass Industries, Japan) for staining with mouse-monoclonal antibodies to E-cadherin (1:50; Invitrogen: Clone NCH-38) and vimentin (1:500; Invitrogen:Clone V9) respectively on a Ventana BenchMark ULTRA automated system (Ventana Medical Systems Inc., Tucson, Arizona). E-cadherin expression was based on cytoplasmic membrane staining while vimentin was cytoplasmic. Expressions of both were semi-quantitated for percentage of malignant cells that expressed E-cadherin or vimentin as 0 (0-<1%), 1 (1-10%), 2 (11-50%), 3 (>50%) and intensity of staining as 0 (negative), 1(weak), 2 (moderate) and 3(strong). The final expression score for each antibody was obtained by multiplying the percentage of tumour cells stained and intensity scores for E-cadherin and vimentin respectively. Final scores of 0-4 were classified as low and scores of 6-9 as high expression. EMT was considered to be present if the tumour showed a low E-cadherin and/or a high vimentin final score. Figure 1 illustrates "high" and "low" E-cadherin while Figure 2

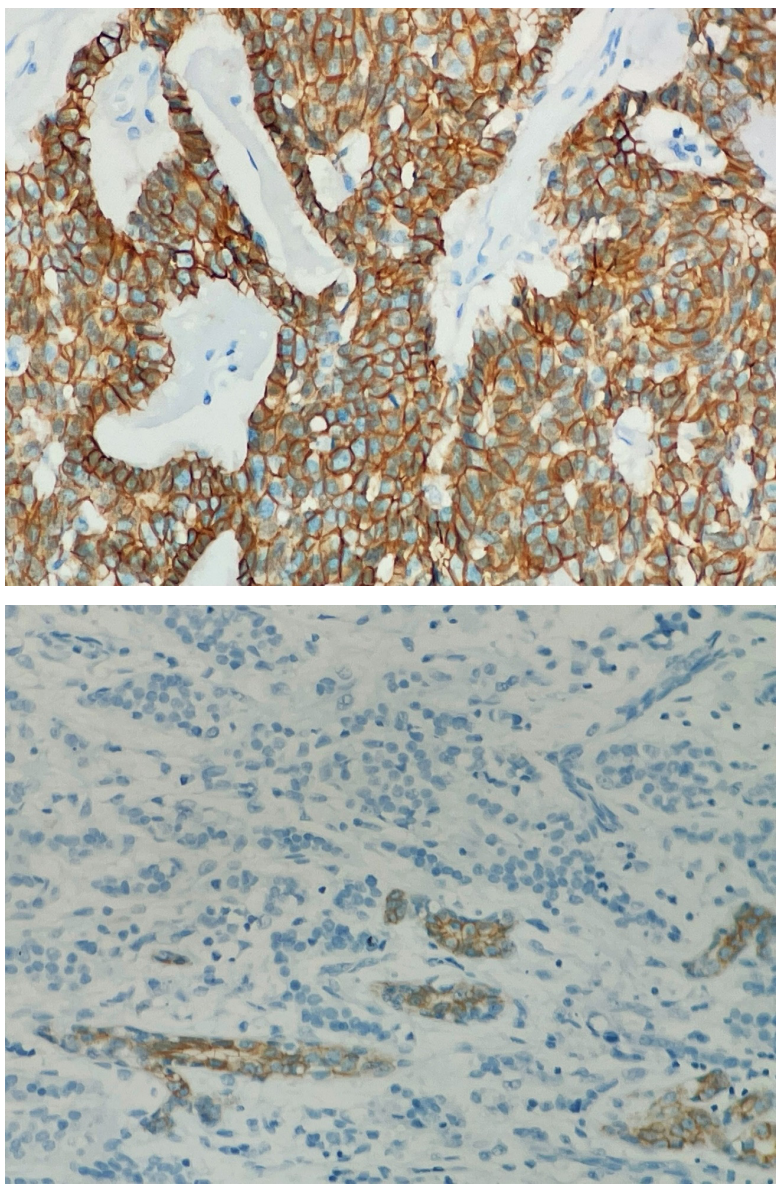

FIG. 1. (a) Breast carcinoma with "high" E-cadherin expression (x200) and (b) another case with loss of E-cadherin expression by the tumour (left) while a normal breast duct (right) demonstrates retained E-cadherin (x200).

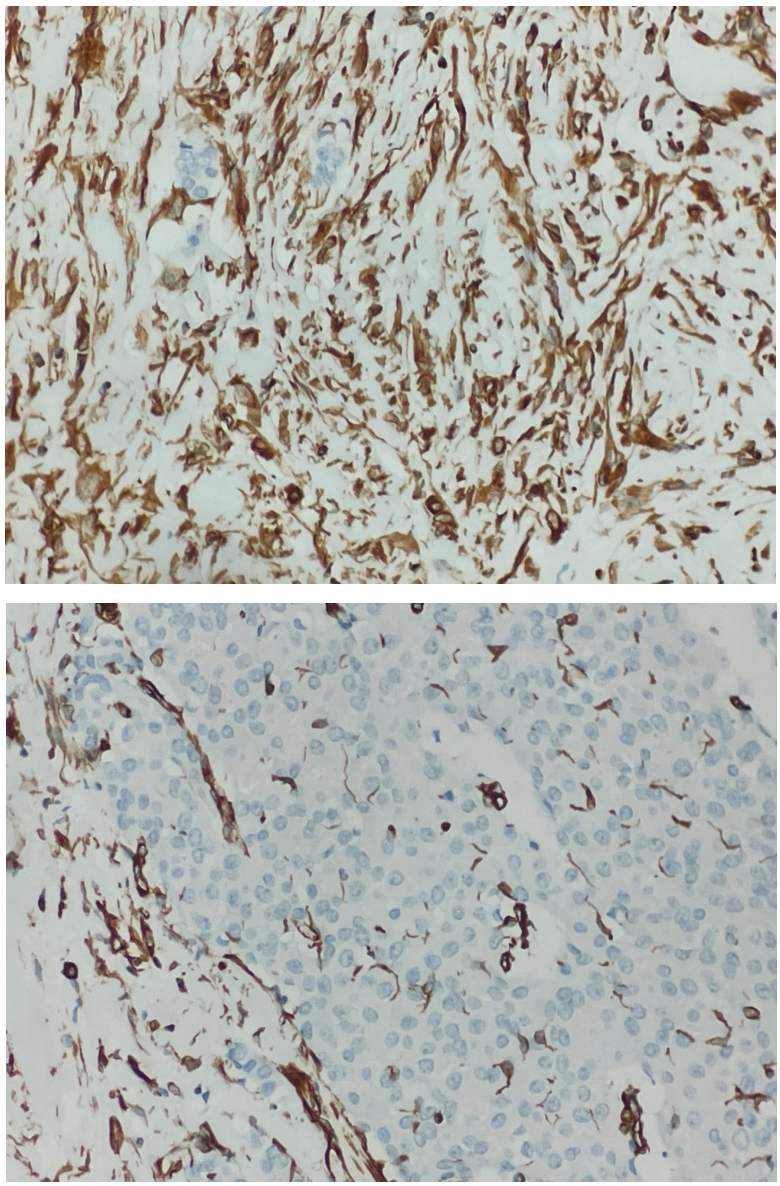

FIG. 2. (a) A case of breast carcinoma demonstrating a "high" vimentin score in which almost all the neoplastic cells demonstrate strong cytoplasmic expression of vimentin. In (b) vimentin score was considered "low" (x200).

illustrates "high" and "low" vimentin expression in the cases of breast carcinoma studied. In addition, EMT-positivity was sub-classified as "complete" (EMT-CV) when there was both low E-cadherin and high vimentin scores, and as "partial" with EMT-C equating low E-cadherin only and EMT-V equating high vimentin only.

Statistical analysis

Statistical analysis was performed using SPSS version 24.0 (IBM, Chicago, Illinois, USA). Categorical variables would be tested by Chi-square or Fisher's exact test, while continuous variables would be tested with One-way ANOVA for parametric data and Kruskal-Wallis for non-parametric data respectively. A p-value of <0.05 was considered statistically significant.

Ethical approval

The study was approved by the Institutional Review Board (UMMC-MREC-ID: 20161229-4720) and carried out in compliance with the Declaration of Helsinki. Informed, written consent to undergo resection of the breast

cancers was obtained from all patients. The Medical Research Ethics Committee/Institutional Review Board of UMMC accepts that additional consent from patients is not required for retrospective studies on archived material which are observational in nature, as in this study. Confidentiality of patients' personal information was maintained by restricting access of data to the authors of the present work only.

RESULTS

Demographic profile and tumour characteristics

A total of 155 non-lobular invasive breast carcinomas were retrieved from the archives but upon review, 36 tumours had insufficient material for further IHC study. Hence, only 119 tumours (49 TN, 45 luminal and 25 HER2-enriched) from 117 patients were finally entered into the study. Of the luminal tumours, 75.6% (34/45) were type A, 2.2% (1/45) type B1 and 13.3% (6/45) type B2. 4 of the 45 luminal tumours were considered "undetermined" for their luminal subtype as they had not been subjected to SISH confirmation although they were grade 2 with an equivocal (2+) HER2 expression on IHC. As the number of luminal B1, luminal B2 and "undetermined" were low in numbers, they were amalgamated with luminal A and categorised as "luminal" for this study. Two Malay patients had bilateral breast carcinomas. One patient had a grade II luminal A invasive carcinoma of no special type (ICONST) in the left and a grade II HER2-enriched ICONST tumour in the right. The second patient had a grade III luminal B2 ICONST in the right and a grade III TN ICONST in the left.

Table 1 captures the demographic profile and tumour characteristics of the cases entered into the study according to the molecular subtypes. Of the 117 patients, 48 (41.0%) were Chinese, 42 (35.9%) Malay, 24 (20.5%) Indian and 3 whose ethnicity could not be ascertained. The ages of the patients ranged between 25-80 years with a mean±SD of 55.5 y ±11.8 years. The molecular subtypes did not demonstrate ethnic predilection. Although age did not differ between the molecular subtypes, age was that recorded at the hospital visit within the period of this study, and not at first presentation of disease.

Histologically, ICONST constituted 95.0% (113/119) of the tumours, while 4 were medullary, 1 metaplastic and 1 mucinous carcinoma. It is noteworthy that ICONST was distributed across the 3 molecular subtypes. All 4 medullary and the single metaplastic carcinoma were triple negative while the only mucinous carcinoma was luminal A. The tumours ranged from 0.5-12.0 cm (mean±SD = 3.3±2.1 cm) in size. Even though the tumour size did not differ between the molecular subtypes, this should take into account that tumours were included irrespective of whether they were at first presentation, recurrent or had undergone neoadjuvant therapy. 52.1% (62/119) of the tumours were grade III, 43.7% (52/119) grade II and 4.2% (5/119) grade I. Due to low numbers of grade I tumours, grade I and II tumours which are generally considered "low" grade[8], were grouped together. HER2-enriched and TN tumours tended to be high grade compared with luminal tumours.

For lymphovascular (LV) and/or lymph node (LN) invasion, it was assumed that documented lymph node metastases equated concomitant presence of lymphovascular permeation, irrespective of whether the latter was observed in the tumour sample. Notwithstanding, LV and/or LN invasion could not be ascertained in 10 tumours which did not demonstrate LV permeation in the excised specimen but also did not have LN clearance performed. Hence, LV and/or LN invasion information could only be confirmed in 109 cases. 36.6% of TN tumours demonstrated LV and/or LN invasion, which was significantly less than HER2-enriched tumours (73.9%). Although TN tumours also exhibited less LV and/or LN invasion than luminal ones (60.0%), this did not reach statistical significance (p=0.051). While the proportion of confirmed nodal metastases amongst cases positive for LV and/or LN invasion was lowest in TN (66.7%) compared with luminal (85.2%) and HER2-enriched tumours (88.2%), this also did not reach statistical significance.

Expression of epithelial-mesenchymal transition

Epithelial-mesenchymal transition versus the breast cancer characteristics is shown in Table 2. 38 of the 119 (31.9%) cancers exhibited EMT. As the numbers of medullary, metaplastic and mucinous tumours were too small, and size of the tumours was not sufficiently reflective of the original presenting tumour, histological subtype and tumour size were not further analysed for association with EMT. The EMT-positivity rate was higher in TN (59.2%, 29/49) compared with luminal (13.3%, 6/45) and HER2-enriched tumours (12.0%, 3/25). 45.2% (28/62) of high-grade and 17.5% (10/57) of low-grade tumours expressed EMT. While TN showed increased EMT compared with luminal and HER2-enriched

TABLE 1: Demographic data of the cases and characteristics (histological subtype, size, histological grade and lymphovascular and/or lymph node invasion) of the breast cancers by molecular subtype.

		Luminal n (%)	HER2-enriched n (%)	Triple negative n (%)	p
Ethnicity (n=119)	Malay	14 (31.1)	14 (56.0)	16 (32.7)	0.164
	Chinese	22 (48.9)	8 (32.0)	18 (36.7)	
	Indian	8 (17.8)	2 (8.0)	14 (28.6)	
	Others	1 (2.2)	1 (4.0)	1 (2.0)	
Age (n=119)	Range (yrs)	37-80	30-78	25-79	0.681
	Mean±SD (yrs)	56.7±10.0	54.2±13.9	55.1±12.4	
Histological subtype (n=119)	No special type (ICONST)	44 (97.8)	25 (100.0)	44 (89.8)	0.130
	Medullary	0 (0)	0 (0)	4 (8.2)	
	Metaplastic	0 (0)	0 (0)	1 (2.0)	
	Mucinous	1 (2.2)	0 (0)	0 (0)	
Size (cm); (n=118)	Range	0.5-12.0	0.6-7.0	0.8-11.5*	0.183
	Mean±SD	3.0±2.2	3.5±1.5	3.4±2.4	
Histological grade (n=119)	Low grade (I+II) n (%)	38 (84.4)	9 (36.0)	10 (20.4)	<0.000
	High grade (III) n (%)	7 (15.6)	16 (64.0)	39 (79.6)	
	Luminal versus HER2-enriched				0.001
	Luminal versus triple negative				<0.000
	HER2-enriched versus triple negative				0.242
Lymphovascular and/or lymph node invasion n=109	Positive	27 (60.0)	17 (73.9)	15 (36.6)	0.009
	Negative	18 (40.0)	6 (26.1)	26 (63.4)	
	Luminal versus HER2-enriched				0.386
	Luminal versus triple negative				0.051
	HER2-enriched versus triple negative				0.009
Confirmed lymph node metastases (n=48)		23 (85.2)	15 (88.2)	10 (66.7)	0.272
Lymphovascular invasion only (n=11)		4 (14.8)	2 (11.8)	5 (33.3)	

*Size available for only 48 tumours

TABLE 2: Epithelial-mesenchymal transition (EMT) versus molecular subtype, histological grade and lymphovascular and/or lymph node invasion of the breast carcinomas

			Epithelial-mesenchymal transition		
			Positive	**Negative**	**p**
Molecular subtype	Luminal n (%)		6 (13.3)	39 (86.7)	<0.000
	HER2 enriched n (%)		3 (12.0)	22 (88.0)	
	Triple negative n (%)		29 (59.2)	20 (40.8)	
	Luminal versus HER2-enriched				1.000
	Luminal versus Triple negative				<0.000
	HER2-enriched versus Triple negative				0.001
Histological grade	Low grade (I and II) n (%)		10 (17.5)	47 (82.5)	0.001
	High grade (III) n (%)		28 (45.2)	34 (54.8)	
Grade by molecular subtype	Low grade (I+II)	Luminal n (%)	5 (13.2)	33 (86.8)	0.397
		HER2-enriched n (%)	2 (22.2)	7 (77.8)	
		Triple negative n (%)	3 30.0)	7 (70.0)	
	High grade (III)	Luminal n (%)	1 (14.3)	6 (85.7)	<0.000
		HER2-enriched n (%)	1 (6.3)	15 (93.8)	
		Triple negative n (%)	26 (66.7)	13 (33.3)	
	High grade:	luminal versus HER2-enriched			0.526
		luminal versus triple negative			0.015
		HER2-enriched versus triple negative			<0.000
LV and/or LN invasion (n=109)	Positive n (%)		14 (23.7)	45 (76.3)	0.331
	Negative n (%)		17 (34.0)	33 (66.0)	
LV and/or LN invasion by molecular subtype	Positive	Luminal n (%)	2 (7.4)	25 (92.6)	<0.000
		HER2-enriched n (%)	2 (11.8)	15 (88.2)	
		Triple negative n (%)	10 (66.7)	5 (33.3)	
		luminal vs HER2-enriched			0.634
		luminal vs triple negative			<0.000
		HER2-enriched vs triple negative			0.003
	Negative	Luminal n (%)	4 (22.2)	14 (77.8)	0.216
		Her2-enriched n (%)	1 ((16.7)	5 (83.3)	
		Triple negative n (%)	12 (46.2)	14 (53.8)	

LV lymphovascular; *LN* lymph node

tumours, this increase was confined to high-grade and not low-grade TN tumours. Tumours with or without LV and/or LN invasion did not demonstrate significant difference in EMT-positivity rate. However, among tumours with LV and/or LN involvement, EMT was significantly increased in TN (66.7%, 10/15), compared with luminal (7.4%, 2/27) and HER2-enriched (11.8%, 2/17) tumours. Table 3 shows further analysis of the EMT profile, viz complete or partial, of the 38 EMT positive cases against the different molecular subtypes, tumour grade and LV and/or LN invasion. 10 (26.3%) showed complete EMT-CV with both low E-cadherin and high vimentin scores, 12 (31.6%) were EMT-C with only low E-cadherin and 16 (42.1%) were EMT-V with only high vimentin. The low E-cadherin and high vimentin profile (EMT-CV) was only observed in TN tumours. All EMT-positive luminal tumours expressed partial EMT expression of which 50.0% (3/6) were EMT-C and 50.0% (3/6) were EMT-V. All (3/3) EMT-positive HER2-enriched tumours were EMT-C. Of the 29 EMT-positive TN tumours, 34.5% (10/29) were EMT-CV, 20.7% (6/29) EMT-C and 44.9% (13/29) EMT-V.

DISCUSSION

For a general landscape of the cases enrolled in this study, it should first be recalled that the cases were recruited based on an attempt to retrieve about 60 non-lobular, triple negative breast (TN) carcinomas between 2012 till 2017, followed by retrieval of approximately equal numbers of consecutive non-lobular, non-TN tumours during the same period, and that the findings be interpreted in this context. Ethnically, the molecular subtypes did not demonstrate preponderance of any of the major ethnic groups in Malaysia. In view of the findings in a large study of over 1000 breast cancers in Sarawak (East Malaysia) which showed predilection of TN and HER2-enriched tumours for Malays[32], the possibility of ethnic partiality cannot be summarily dismissed from the limited number of cases in this study. Similarly, although there was no difference in age of patients with TN tumours compared with HER2-enriched and luminal ones, it should be remembered that patients' ages recorded in this study were not those at first presentation.

Invasive carcinoma of no special type (ICONST) constituted 95% of the cases, and as expected were distributed across the 3 molecular subtypes, with 39% being luminal, 39% TN and 22% HER2-enriched. The ratio of cases with luminal, TN and HER2-enriched profiles has to be taken in the context of case selection in this study, and do not represent a ratio prevalence of breast cancer molecular subtypes in the Malaysian population, for which there is already well-documented information.[32-35] However, the molecular subtype heterogeneity within histological subtypes, and in this case ICONST, is not surprising and is well-acknowledged.[31] All the medullary and metaplastic carcinoma were triple negative while the mucinous cancer was luminal similar to findings of others.[31,36,37] That the size of the tumours did not differ between the molecular subtypes, has to be interpreted with the understanding that use of neoadjuvant treatment or tumour size at original presentation in the cases of recurrent tumours had not been taken into account in this study. Akin to the increasing frequency of high grade tumours from luminal

TABLE 3: EMT types as compared with grade, lymphovascular and/or lymph node invasion in breast cancers and molecular subtype

		EMT-CV	EMT-C	EMT-V	p
Molecular subtype	Luminal	0	3	3	0.030
	HER2-enriched	0	3	0	
	Triple negative	10	6	13	
Grade (n=38)	Low grade (I and II)	1	5	4	0.294
	High grade (III)	9	7	12	
LV and/or LN invasion (n=31)	Positive	4	4	6	0.805
	Negative	3	6	8	

LV lymphovascular; *LN* lymph node; *EMT-CV* low E-cadherin and high vimentin scores; *EMT-C* low E-cadherin only; *EMT-V* high vimentin only

to HER2-enriched to TN observed by Zhen *et al*.[38], a similar trend, with high grade (grade III) tumours constituting 16% of luminal, 64% HER2-enriched and 80% TN, was also noted. Although Zhen *et al*. did not find any association between lymph node metastases, and molecular subtype[38], we noted that prevalence of LV and/or LN invasion was significantly less in TN (37%) compared with HER2-enriched (74%) tumours and possibly with luminal (60%). It should be mentioned that Zhen *et al*.'s study did not include lymphovascular invasion and whether this contributed to the different observations, can only be speculated on. However, in a large study of over 11,000 breast cancers with over 1000 TN tumours, Ugras *et al*., like us, reported that lymph node metastases and lymphovascular invasion was least frequent in TN tumours when contrasted with the other molecular subtypes[39], raising the provocative question then as to the reason for the aggressive nature of TN breast cancers.

Interestingly, epithelial-mesenchymal transition was highest in TN tumours at 59% compared with luminal and HER2-enriched tumours at 13% and 12% respectively. The preponderance of EMT in TN compared with luminal and HER2-enriched tumours has also been shown by Orlandini *et al*.[22] That high grade tumours exhibited increased EMT as in this study, has also been previously reported.[40,41] While EMT was seen in the majority of TN tumours when compared with the other molecular subtypes, this difference appeared to reach statistical significance only in high grade TNs. Notably, HER2-enriched tumours, the other molecular subtype with predominance of high-grade tumours, did not exhibit significantly increased EMT. Although not reaching statistical significance, EMT in low grade TN tumours (30%), was interestingly also marginally higher compared with similar grade HER2-enriched (22%) and luminal (13%) tumours. Taken altogether, we are inclined to believe that TN as a molecular subtype and not the high grade per se influenced EMT acquisition but this needs to be confirmed with much larger samples. Indisputably, other possibilities which could have resulted in the lack of EMT in high-grade HER2-enriched tumours should also be considered. Even targeted treatment may have intercepted the epithelial-mesenchymal transition of HER2-enriched tumours, considering the plethora of factors that have been described to be associated with EMT.[11,17,41] Supported also by Orlandini *et al*.'s observation[22], in this study EMT appeared not to influence LV and/or LN invasion in breast cancers as a group. Nevertheless, on further analysis, this finding may not apply across the molecular subtypes of breast cancers. In the current study, EMT was present in 66.7% of TN tumours with LV and/or LN invasion which was significantly higher compared with luminal (7.4%) and HER2-enriched (11.8%) tumours with similar LV and/or LN invasion. Thus, under-representation of TN tumours in a study sample would easily bias the findings of a general breast cancer population, again reiterating the heterogeneity of breast cancers. Analysis of the different EMT profiles, complete and partial transition, in the EMT-positive cases showed further noteworthy observations. Although the number of cases was small, it is interesting that only TN tumours exhibited complete EMT with loss of epithelial and gain of mesenchymal characteristics. This is very noteworthy in the light of recent work. In 2009, Giampieri *et al*. using intravital imaging recorded two distinct modes of breast cancer cellular motility.[42] When cells invaded and moved in clusters, they were restricted to lymphatic spread and only single cell invasion resulted in blood-borne metastases. Following on this observation, using animal breast cancers, Mohammed *et al*. recently demonstrated that blood-circulating breast cancer cells showed complete EMT while those circulating via lymphatics expressed high vimentin without loss of E-cadherin.[43] In other words, the blood-circulating tumour cells existed as single cells, after losing their E-cadherin and cell-cell adhesion while gaining mesenchymal vimentin. Contrarily, lymphatic-circulating cancer cells were motile after acquiring mesenchymal properties but moved in small clusters as they still retained their cell-cell adhesion through E-cadherin. Incorporating these observations and that from this study, it seems logical to suggest that the generally accepted higher frequency of haematogenous spread in TN tumours[44] may be linked to the tumour cells undergoing complete EMT, therefore permitting them to invade as single cells, a circumstance which occurred only in TN but not the other molecular subtypes. It is pertinent at this juncture to refer back to our observations that LV and/or LN invasion was lowest in TN compared with luminal and HER2-enriched tumours, and yet among tumours with LV and/or LN invasion, TN showed the highest (66.7%) EMT in comparison with luminal (7.4%) and HER2-enriched (11.8%)

tumours. Although not achieving statistical significance, it is interesting that confirmed LN metastases in the LV and/or LN invasion positive cases was lowest in TN (67%) when compared with luminal (85%) and HER2-enriched (88%); implying that in contrast, TN had the highest rate of LV permeation without concomitant nodal metastases. Granted that no attempt was made to differentiate between lymphatic and blood vessel tumour emboli when LV invasion was recorded in this study, the possibility of many being blood-borne emboli seems an attractive suggestion in view of the lower confirmed nodal involvement. Apart from the exclusivity of EMT-CV to TN tumours and the implication of its influence on blood-borne spread, we also noted that EMT-V was the most common EMT profile seen in TN tumours. Linking this to Mohammed *et al.'s*[43] elegantly mapped out characteristics of blood and lymphatic-circulating breast cancer cells, it would appear that TN tumours also had a high proportion of tumour cells which retained epithelial properties while acquiring mesenchymal properties (equivalent to EMT-V in our study), and hence capable of being lymphatic-circulating. However, what may perhaps be even more important could be their finding that EMT-V cells were enriched with stem cell properties which are linked to a more efficient metastatic potential. In gist they noted that these EMT-V cells exhibited greater ability than those with complete EMT (EMT-CV) to form mammospheres *in vitro*, and tumours *in vivo*. Whether, these two phenomena of EMT-CV which appears to support blood-borne spread and EMT-V which potentiates metastatic niduses, can explain the propensity for distant spread and early recurrence of TN tumours is important and needs to be properly proven.

Although this study reveals several interesting findings, it is appropriate to state that, apart from having limited number of cases, there are limitations which should be addressed in future. Being a snapshot study with recruitment of cases, irrespective whether at first presentation or recurrent and whether neoadjuvant therapy had been administered may not have stringently controlled bias in a dynamic process such as EMT. It can also be too simplistic to view TN breast cancers as a single entity, acknowledging that cluster analysis of gene expression profiles has been able to subtype TN into at least 6 categories viz basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR)[45], and these may have their individual EMT signatures. Information about distant metastases could have better defined the spread of the various molecular subtypes, however after consideration, the authors did not include this aspect into the study as several of the distant metastases were radiologically detected but not biopsy-proven, and at best only considered to be diagnostically presumptive.

In conclusion, and within its limitations, this study shows that epithelial-mesenchymal transition occurred most frequently among TN tumours. Interestingly, complete EMT i.e. loss of epithelial and gain of mesenchymal characteristics, a profile allegedly associated with blood-borne metastases, was observed only in TN and not luminal and HER2-enriched tumours. In addition, the most common EMT profile encountered in TN tumours was that with increased mesenchymal transition but with retention of epithelial features, a profile recently linked with a cancer stem cell disposition and accorded with increased metastatic potential.

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