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HISTOPATHOLOGICAL FEATURES OF EARLY ONSET INDONESIAN BREAST CANCER POINTING TO BRCA1/2 GERMLINE MUTATIONS

Purnomosari D[1], Van Der Groep P[2], Wahyono A[3], Aryandono T[3], Pals G[4], Van Diest PJ[2]

[1]Department of Histology and Cell Biology Faculty of Medicine, Gadjah Mada University, Yogyakarta
[2]Department of Pathology, University Medical Center Utrecht, the Netherlands
[3]Department of Surgery, Faculty of Medicine, Gadjah Mada University, Yogyakarta
[4]Departments of Clinical Genetics, VU University Medical Center, Amsterdam

ABSTRACT

Background: Breast cancer under 40 years concerns a relatively small subgroup of cases that tend to display a more aggressive phenotype. Compatible with this, early age of onset has been known as one of clinical characteristic of hereditary breast cancers associated with germline BRCA1 or BRCA1 mutations. As early onset breast cancer is frequent in Indonesia, we investigated the histopathological and immunohistochemical characteristics of early onset (< 40 years) Indonesian breast cancer patients, as such features can be used to distinguish between BRCA and non-BRCA germline mutation carriers among these young women.
Method: Thirty-five formalin-fixed and paraffin-embedded tissue sections of young women (mean 36 years, range 22-40 years) who underwent surgical resection at the Department of Surgery of the Sardjito Hospital Yogyakarta were examined for pathological features, estrogen and progesterone receptor status, proliferation as determined by Ki67 labeling, EGFR and CK5/6 and the presence of HER-2/neu and p53 protein. Additionally, mutation analysis for BRCA1 and BRCA2 was performed in 30 young women. The control group consisted of carcinomas from women above 50 years (mean 59.02, range 50-80 years).
Result: Carcinomas occurring in women aged below 40 years were more often advanced stage and higher proliferating (p=0.006). Among the early onset breast cancer patients, ductal type, grade 3, ER and HER-2/neu negativity, high Ki67 index and CK5/6 and EGFR positivity were typical for BRCA1 patients. Unfortunately, there were no typical phenotypical features for BRCA2 carriers. However, grade I and lobular cases were never BRCA1/2 germline mutated.
Conclusion: Early onset Indonesian breast cancer shows increased proliferation compared to late onset patients. Within the early onset group, the strongest features pointing to a sporadic cancer seem to be grade I and lobular differentiation. Features increasing the chance of a germline BRCA1/2 mutation are CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling. This is potentially useful to optimize selection of early onset breast cancer patients for BRCA1/2 mutation testing.

Keywords: breast cancer, early onset, histopathology, immunohistochemistry, BRCA1, BRCA2

INTRODUCTION

Breast cancer in patient under 40 years old is uncommon. The National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program reveals that 75% of breast tumors occur in women age >50 years, only 6.5% in women age <40 years, and a mere 0.6% in women age <30 years.[1,2] Carcinomas from younger patients tend to be more aggressive with a higher incidence of poorly differentiated (grade III) carcinomas and more hormone insensitive (ER/PR negative) tumors. Additionally, the tumors are often rapidly proliferating and express abnormal p53.[2,3,4]

Of the relatively small fraction of young patients, a disproportionally large fraction has hereditary breast cancer. It has been known that over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian cancer carry a germline BRCA1 or BRCA2 mutation.[5,6] Therefore, a diagnosis of breast carcinoma at young age greatly impacts these patients and their families.

Clinically, a hereditary basis of breast cancer is, apart from early onset and family history of (early onset) breast cancer, recognized by contra lateral breast cancer, male breast cancer, and ovarian/ Fallopian tube cancer.[7,8] Sometimes, however, family history is incomplete, even in developed countries. In Indonesia, incompleteness of family history is common as a significant number of patients do not seek medical treatment, families tend to be bigger, and patients are thereby often poorly informed on the exact medical problems in their families. So, young age at presentation is in developing countries even more important to indicate a potential hereditary condition.

Genetic testing for BRCA1 and BRCA2 is complex and time-consuming due to the large size of both genes, and the presence of mutations throughout their entire coding regions. Therefore, it is important to find additional clinical or pathological factors that could suggest the presence of BRCA1 or BRCA2 mutations in a patient.

Several studies have compared the characteristics of breast cancers in BRCA1 carriers and sporadic controls. Distinct features of BRCA1-associated tumor have been proposed, such as high tumor grade, estrogen (ER) and progesterone receptor (PgR) negativity[9,10], accumulation of p53[9,10], expression of the epidermal growth factor receptor (EGFR)[11,12,13], and absence of amplification and over expression of HER-2/neu.[14,15] Additionally, hereditary breast cancers are preferentially of the ductal and medullary carcinoma types.[16,17] cDNA expression analyses and expression of cytokeratins 5/6 have suggested a basal epithelial phenotype for BRCA1 related cancers.[18] However, the phenotype of BRCA2 related cancers is much less outspoken, and seems to be in between that of BRCA1 related and sporadic cancers.[19] Whereas in Western countries most hereditary breast cancers are BRCA1 related, the reverse trend is seen in Indonesia where most hereditary breast cancers seem to be BRCA2 related.[20] In the current study, we therefore investigated the histopathological and immunohistochemical characteristics of early onset (< 40 years) Indonesian breast cancer patients, as such features can be used as to identify the BRCA1/2 germline mutation carriers among these young women. This could help to limit expensive mutation screening to those patients at highest risk to harbor a germline BRCA mutation.

MATERIALS AND METHODS

Tumor specimens

Formalin-fixed and paraffin-embedded tissue sections were obtained from 35 young women (mean age 36.11 years, range 22–40 years) who underwent surgical resection of primary breast cancer between 2002-2004 at the Department of Surgery, Sardjito Hospital, Yogyakarta, Indonesia. Tumors were staged according to the 5th edition of the American Joint Committee on Cancer TNM classification system.[21] Fourteen tumors were early stage (I/II) and nineteen tumors presented at late stage (III/IV). Specimens included 33 invasive ductal carcinomas (IDCs), and 2 invasive lobular carcinomas (ILCs) according to the WHO.[22] Three tumors were classified as Grade I, 15 as Grade II and 17 as Grade III according to the modified Elston and Ellis grading.[23] Concerning family history, 3/35 patients had a history of breast or ovarian cancers in first-degree female relatives and 3/35 patients had a family history positive for other cancer types. Germline mutation analyses of the entire coding region of BRCA1/BRCA2 was done in 30 of these patients by pooled denaturing gradient gel electrophoresis with direct sequencing of aberrantly moving bands as described before[20], complemented by multiplex ligation dependent probe amplification to detect genomic deletions.[24] There were two mutations in BRCA1 (deletion exon 13-15 and exon 16 p.Met165Ile) and five mutations in BRCA2 (c.3040_3043del 4, p.Met1149Ile, p.Glu699Leu, p.Leu824X all in exon 11 and c.9485-16T>C in exon 25). One of these latter patients (AZ) had in addition to a pathogenic c.2699_2704 delTAAATG mutation in exon 11 of BRCA2 an unclassified variant mutation in exon 16 of BRCA1 (p.Met165Ile).

Fifty-four late onset breast cancer (>50 years) from the same hospital were included as a control group (mean 59.02, range 50 – 80 years). On this series, data concerning family history, BRCA mutation status, lymph node status, and EGFR and CK5/6 status were lacking.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick sections. After deparaffination and rehydration, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3 % hydrogen peroxide. After antigen retrieval in citrate buffer (autoclave except for ER where the microwave was used, a cooling off period of 30 minutes preceded the incubation (overnight at 4°C) with the primary antibodies (p53: DO7, Dako, 1:500; EGFR: Novocastra, 1:10; ER: Dako, 1:50; PR: Novocastra, 1:50; HER-2/*neu*: Novocastra 1:100; Ki67: Dako, 1:40). The primary antibodies were detected using a biotinylated rabbit anti-mouse antibody (DAKO). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with haematoxylin, dehydrated in alcohol and xylene and mounted. For Ki67, p53, ER, and PR, only nuclear staining was considered and diffuse cytoplasmic staining was ignored, leading to an estimated percentage of positively stained nuclei. HER-2/*neu* was scored according to the HERCEPT system as negative, 1+, 2+ or 3+, and EGFR staining was scored positive when a clear membrane staining pattern was seen. Scoring was done blinded to BRCA1/2 mutation status by a single experienced pathologist (PvD).

HER-2/neu Amplification Testing

In addition to immunohistochemistry, HER-2/neu was tested for amplification by multiplex ligation dependent probe amplification (MLPA) as described before.[25] In short, 50- 500 ng of target DNA was denatured and the probe mix was added afterward. The mixture was heated and incubated overnight (16 h). Ligation was performed with the temperature stable Ligase-65 enzyme (MRC-Holland) then was inactivated in the thermocycler. Ligated mixture was amplified with one unlabeled and one carboxyfluorescein labeled PCR primers that are complementary to the universal primer sequences. PCR was carried out for 33 cycles. The fragments were analyzed with an ABI model 310 capillary sequencer (Applied Biosystems, Torrence, CA, USA) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis was performed using Genescan software. DNA from Centre d'Etude Polymorphisme duHumain (CEPH) was used as a control sample and analyzed simultaneously with breast cancer samples in each run. To objectify interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample was determined using an excel template. For this purpose, the relative peak areas for each probe were calculated as fractions of the sum of peak areas in a given sample. Subsequently, the fraction of each peak was divided by the average peak fraction of the corresponding probe in control samples. Finally, the values were normalized using the values obtained for the autosomal control probes that served as a reference for the copy number of 2.0. Cases that showed a copy number greater than 2 for at least two of the probes on the HER-2/neu locus could be considered amplified. HER-2/neu was considered to be "positive" when it was either 3+ overexpressed or amplified by MLPA.

Statistics

Statistical analysis was carried out by SPSS software version 11 for Windows. Continuous variables were tested for differences between the non-mutated and mutated cases using the Mann-Whitney test, and discrete variables with the chi-square test using logical classes. P-values <0.05 were considered significant.

RESULTS

Table 1 shows the main clinic pathological features of our early onset Indonesian breast cancer study group compared to the control group and data from other relevant published studies. There was high prevalence of advanced stage, tumors with poor differentiation, and tumors with lymph node metastases. Most of the cancers were of the ductal type, and special type cancers were lacking. With regard to the immunophenotype, about half the cases were ER and PgR positive, 30% were EGFR positive, 23% were HER-2/neu positive, and 23% were CK5/6 positive. The mean percentages of p53 and MIB-1 positive cells were both 30%.

Comparison with control group of late onset (>50 years) breast cancer for several clinic pathologic features (Table 1) showed that high Ki67 (>10%) was more frequent in early onset patients (74% to 44%, p= 0.006). Tumor stage, tumor type and ER staining only showed tendency for significance (p=0.105, p=0.165 and p=0.175 respectively) while the other features showed no significance at all.

Comparison of continuous features between non-BRCA and BRCA mutation carriers did not reveal statistically significant differences (Table 2). Further comparison between non-BRCA and BRCA carriers among early onset breast cancer with regard to the discrete histopathological features did also not show significant differences although BRCA germline mutation carriers had more advanced stage and had never lobular or grade I carcinomas (Table 3), except for family history (p=0.025) where twenty-two non-BRCA cases all had negative family history for breast/ovarian cancer or other tumors.

DISCUSSION

It is widely believed that breast cancer in young women is characterized by relatively unfavorable prognostic pathologic features. Published studies on this issue are however not easily compared because of differences in the age ranges, and the prognostic features considered. The majority of epidemiological studies have documented an adverse outcome of breast cancer in young patients[26,27] independent from other factors.[27]

With these considerations in mind the present study was undertaken to analyze some well-defined

Table 1. Clinicopathological Features of a Group of Early Onset (< 40 Years) Indonesian Breast Cancers Compare to Control Group (>50 Years) and Relevant Studies

		Present Study		Sidoni et al		Rodrigues et al	
		Early	Late	Early	Late	Early	Late
Feature		**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Stage	I/II	13 (41%)	32 (60%)	nd	Nd	Nd	Nd
	III/IV	19 (59%)	21 (40%)	nd	nd	Nd	Nd
		p=0.105					
Type	Lobular	2 (6%)	2 (4%)	2 (4%)	1 (2%)	Nd	Nd
	Ductal	33 (94%)	45 (83%)	44(88%)	45 (90%)	Nd	Nd
		p=0.165					
Grade	I	3 (9%)	2 (4%)	2 (4%)	4 (8%)	3 (15%)	7 (21%)
	II	15 (43%)	19 (36%)	27 (58%)	36 (75%)	9 (45%)	14 (41%)
	III	17 (48%)	32 (60%)	19 (38%)	8 (17%)	8 (40%)	13 (38%)
		p=0.438		**P=0.04**		**P=0.88**	
Nodal status	Negative	11 (37%)	nd	22(47%)	29 (58%)	Nd	Nd
	Positive	19 (63%)	nd	25(53%)	21 (42%)	Nd	Nd
Family history	Negative	25 (86%)	nd	26 (76%)	Nd	11 (55%)	22 (65%)
	Breast/ovarian		nd	8/34 (24%)	5/30 (17%)	8 (40%)	9 (26%)
	cancer	1 (3%)					
	Other cancers	3 (10%)	nd	nd	Nd	1 (5%)	Nd
ER	Negative	19 (58%)	23 (43%)	23 (46%)	11 (20%)	2 (10%)	7 (21%)
	Positive	14 (42%)	31 (57%)	27 (54%)	39 (80%)	18 (90%)	27 (79%)
		p=0.175		**P=0.01**		**P=0.31**	
PgR	Negative	19 (58%)	25 (46%)	25 (50%)	16 (30%)	4 (20%)	10 (30%)
	Positive	14 (42%)	29 (54%)	25 (50%)	34 (70%)	16 (80%)	23 (70%)
		p=0.307		**P=0.067**		**P=0.41**	
HER-2/neu	Not amplified	27 (77%)	43 (80%)	26 (52%)	37 (74%)	7 (35%)	21 (62%)
	Amplified	8 (23%)	11 (20%)	24 (48%)	13 (26%)	13 (65%)	13 (38%)
		p=0.78		**P=0.03**		**P=0.06**	
EGFR	Negative	23 (70%)	nd	nd	Nd	Nd	Nd
	Membranous	10 (30%)	nd	nd	Nd	Nd	Nd
CK5/6	Negative	27 (77%)	nd	nd	Nd	Nd	Nd
	Positive	8 (23%)	nd	nd	Nd	Nd	Nd
p53	Negative	21 (62%)	39 (72%)	35 (70%)	46 (92%)	16 (84%)	28 (82%)
	Positive	13 (38%)	15 (28%)	15 (30%)	4 (8%)	3 (16%)	6 (18%)
		p=0.305		**P=0.01**		**P=0.86**	
Ki67	Low	9 (26%)	30 (56%)	nd	Nd	12 (60%)	25 (74%)
	High	26 (74%)	24 (44%)	nd	nd	8 (40%)	9 (26%)
		p= 0.006				**P=0.30**	

Table 2. Means of Continuous Clinicopathological Features of Early Onset Indonesian Breast Cancers (=40 Years) According to BRCA*1/2* Mutation Status. None of the Values were Statistically Significant between BRCA Mutation Carriers and Patients Without Mutations

	N	No Mutation		BRCA Mutation		BRCA1 mutation		BRCA2 Mutation	
		Mean	N	Mean	N	Mean	n	Mean	N
Age	30	35.96	23	36.14	7	40	2	34.6	5
Tumor size	29	3.09	23	3.33	6	4	2	3	4
ER	28	29.81	21	**41.43**	7	**0**	2	58	5
PgR	28	24.95	21	24.43	7	25	2	24.2	5
Ki67	30	25.96	23	32.14	7	20	2	37	5
p53	29	24.23	22	**53.14**	7	**90**	2	38.4	5

Table 3. Frequencies of Discrete Clinicopathological Features of Early Onset Indonesian Breast Cancers (< 40 Years) According to BRCA1/2 Mutation Status

Features	n	No Mutation		BRCA Mutation		BRCA1 Mutation		BRCA2 Mutation	
		freq	(%)	freq	(%)	freq	(%)	freq	(%)
stage	30								
I/II	12	9	39	3	25	0	0	3	60
III/IV	18	14	61	4	22	**2**	**100**	2	40
type	30								
lob	2	2	9	**0**	**0**	0	0	0	0
duc	28	21	91	7	100	2	100	5	100
grade	29								
well	3	3	14	**0**	**0**	0	0	0	0
mod	11	7	32	4	57	0	0	4	80
poor	15	12	55	3	43	**2**	**100**	1	20
nodal status	29								
negative	10	8	36	2	29	1	50	1	20
positive	19	14	64	5	71	1	50	4	80
Fam history	29								
no	25	22	100	3	43	1	50	2	40
yes	1	**0**	**0**	1	14	1	50	0	0
other	3	**0**	**0**	3	43	0	0	3	60
				p= 0.025					
ER	28								
negative	15	11	52	4	57	2	100	2	40
positive	13	10	48	3	43	**0**	**0**	3	60
PgR	28								
negative	14	11	52	3	43	1	50	2	40
positive	14	10	48	4	57	1	50	3	60
her2	30								
not ampl	23	17	74	6	86	**2**	**100**	4	80
amplified	7	6	26	1	14	0	0	1	20
EGFR	28								
negative	19	16	76	3	43	0	0	3	60
positive	9	5	24	4	57	**2**	**100**	2	40
CK5/6	30								
negative	24	20	87	4	57	0	0	4	80
positive	6	3	13	3	43	**2**	**100**	1	20
p53	29								
negative	18	15	68	3	43	0	0	3	60
positive	11	7	32	4	57	**2**	**100**	2	40
Ki67	30								
negative	8	7	30	1	14	1	50	0	0
positive	22	16	70	6	86	1	50	**5**	**100**

pathological prognostic factors in a series of 35 invasive breast cancer patients below 40 years of age and compared them with a control group of postmenopausal aged over 50 years.

The results of our study show that breast carcinoma in Indonesian women below 40 years of age differ especially in proliferation as measured by Ki67 positivity. As rate of proliferation is of overriding prognostic importance and the most important component of grade[28], this is consistent with those studies showing higher prevalence of grade 3 cancers[29,30] and worse prognosis in young women[3,26,31], although Rodrigues et al.,[30] found no significance for Ki67 in their study. Grade itself was not significant in our study, and the results on grade in the literature and conflicting.[29,30] ER, PR, p53 and HER-2/neu were not significant in our study as well, and also for these features other studies are conflicting.[29,30]

Early onset breast cancer is often hereditary.[32] In recent years, the histopathological features of BRCA1 germline mutation related tumors have been well characterized. They are most frequently of ductal and medullary histological type[6], more frequently poorly differentiated[6,17,33,34,35,36], usually ER[14,17,34,35,36,37,38], PR[14,34,35,36,39] and HER-2/neu[40,41] negative, and show a high degree of positivity for CK5/6[40], EGFR[11,12,13] and p53 accumulation.[39,42,43] Fully consistent with this, our few BRCA1 related cases were advanced stage, ductal type, grade 3, ER and HER-2/neu negative, and EGFR, CK5/6 and p53 positive.

The phenotype and genotype of breast cancers in BRCA2-mutation carriers seems to be less outspoken and lies between that of BRCA1 mutation carriers and sporadic controls.[19] Better defining the phenotype of BRCA2 related breast cancers is especially important in Asian populations like ours, as the prevalence of BRCA2 related cancers exceeds that of BRCA1 related cancers.[24] BRCA2 tumors are more frequently of ductal type[44] as in the present study where all BRCA2 related tumors were ductal. They are also more frequently grade 2 and 3 than sporadic controls.[6,34,35] In line with these previous studies, BRCA2 carriers had in the present study moderately or poorly differentiated tumors and none of them were well differentiated. The frequency of ER and PR expression in BRCA2 tumors has been reported as similar to that in sporadic breast tumors most studies[14,35,36,39], in line with the present study. As to p53, some studies have found p53 accumulation in around 20-50% of BRCA2 related carcinomas.[14] In the present study, 60% of the BRCA2 associated tumors showed p53 accumulation. Data on HER-2/neu expression in BRCA2-associated tumors vary from series to series, probably as a consequence of differences in the techniques employed. For example, Armes et al.[39]and Eerola et al.[36] found no differences in the expression of HER-2/neu in BRCA2 and sporadic breast tumors. However, other studies revealed low frequencies between 0 and 3% in HER-2/neu over expression in BRCA2 tumors.[14,17,35] Combining immunohistochemistry with a new amplification test, we found a 20% frequency of HER-2/neu positivity in BRCA2 associated tumors.

Concerning cytokeratin 5/6, 20% of our BRCA2 cases were positive, compared to 15.4% (N=48) in the literature.[13] There are few data on EGFR and Ki67 index in breast cancers of BRCA2 mutated cases. One study reported a 100% EGFR positivity[19] in BRCA2 related breast cancer (N=5), another one 8% (N=48)[13] compared to 40% in the present study. As to Ki67, one study reported a median Ki67 of 20% in BRCA2 cancers, a little lower than the 37% mean in our study. These studies, like ours, concern however just a few cases, so these results will not be significantly different.

The results of the present study add to the concept that breast cancer arising in BRCA1 and BRCA2 mutation carriers of mutation in the genes differ from sporadic breast cancer of age matched controls. This is especially clear for BRCA1 related cancers. Unfortunately, also our study has not firmly established a clear phenotype for BRCA2 related breast cancers. One of the most important applications of this information would be its use as a guide for genetic testing. Although we have developed a fast and relatively cheap method for BRCA1/2 mutation detection[20], such testing is still difficult to afford for most Indonesian patients. Currently, young age and positive family history are the best predictors of a high likelihood of carrying a BRCA1/*2* mutation. However, to optimize screening, it would for early onset Indonesian patients that are often oblivious to their family history be quite useful if histopathological features, in conjunction with clinical data, could be used to raise the a priori chance of a germline mutation (Table 3). To this end, the strongest features pointing to a sporadic cancer seem to be grade I and lobular differentiation. Features increasing the chance of a germline BRCA1/2 mutation are CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling.

In conclusion, early onset Indonesian breast cancer is characterized by increased proliferation. Within the early onset group, the strongest features pointing to a sporadic cancer seem to be the absence of family history of breast and or ovarian cancer, grade I and lobular differentiation. Features increasing the chance of a germline BRCA1/2 mutation are family history of breast and or ovarian cancer, CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling. This may be potentially useful to optimize selection of early onset breast cancer patients for BRCA1/*2* mutation testing.

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