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Advances in Brief

The Prognostic Implication of the Basal-Like (Cyclin E^{high}/$p27^{low}$/$p53^{+}$/ Glomeruloid-Microvascular-Proliferation^{+}) Phenotype of *BRCA1*-Related Breast Cancer

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Abstract

Previous studies have shown that *BRCA1*-related breast cancers are often high-grade tumors that do not express estrogen receptors, HER2, $p27^{Kip1}$, or cyclin D1, but do express p53 and cyclin E. In addition, the expression of cytokeratin 5/6 (CK5/6), indicating a basal epithelial phenotype, is frequent in *BRCA1*-related breast cancer. Here, in a series of 247 breast cancers, we demonstrate that CK5/6 expression was associated with nearly all of the features of *BRCA1*-related breast cancer and was also associated with a poor prognosis. In a parsimonious multivariable proportional hazards model, protein levels of cyclin E, $p27^{Kip1}$, p53, and the presence of glomeruloid microvascular proliferation all independently predicted outcome after breast cancer. In this model, only cyclin E and $p27^{Kip1}$ levels were independent predictors in lymph node-negative cancers, whereas glomeruloid microvascular proliferation and tumor size independently predicted outcome in node-positive disease. The molecular determinants of the basal epithelial phenotype encapsulate many of the key features of breast cancers occurring in germ-line *BRCA1* mutation carriers and have independent prognostic value. Basal breast cancer deserves recognition as an important subtype of breast cancer.

Introduction

Clinicopathological features can be used to distinguish *BRCA1*-related breast cancer from both *BRCA2*- and non-*BRCA1/2*-related tumors (1–3). In particular, *BRCA1*-related breast cancer tend to be high-grade (1), lymph node-negative (4) tumors that do not express estrogen receptors (ERs), HER2 (3), or $p27^{Kip1}$ (5), but do express p53 (6), cyclin E,[13] and cytokeratin (CK) 5/6 (7, 8). A previous study from our group showed that glomeruloid microvascular proliferation (GMP), a feature of glioblastoma multiforme, is significantly more likely to be present in *BRCA1*-related cancers than in other types of breast cancer (9). Several, but not all, studies have suggested an inferior survival rate for women developing a *BRCA1*-related breast cancer (10). Essentially, breast cancers have either a luminal (expressing CK8/18, 19) or a basal phenotype (CK5/6, 14, 17), but some tumors express both markers (11). Previous work has suggested that *BRCA1*-related breast cancer is very likely to be CK5/6-positive (7, 8), and CK8/18 negative (2), but the relationship between *BRCA1* mutation, CK5/6 status, and outcome has not been previously studied. Therefore, in this study, we first sought to establish what associations existed between CK5/6 expression and all of the above listed variables, with the aim of comparing the clinicopathological profile of CK5/6-expressing cancers with those occurring in *BRCA1* mutation carriers (hereafter "*BRCA1* carriers"). Secondly, the 10-year outcome after breast cancer was compared in tumors with different levels of the above variables, and a multivariable model was created to determine which factor(s) of all of the above most strongly and independently determines survival after breast cancer.

Materials and Methods

Clinicopathological Review and IHC. The study design is an ethnically restricted single hospital-based retrospective cohort study, as described in previous publications (5, 9). The anonymized study design was approved by the hospital's Institutional Review Board. Of 309 consecutive cases of Ashkenazi Jewish women age 65 or less diagnosed with a first primary, nonmetastatic, invasive breast cancer between January 1, 1980 and November 1, 1995 at the Sir Mortimer B. Davis-Jewish General Hospital, Montreal, QC, 17 (5.5%) were excluded [because (*a*) we were unable to locate path blocks; (*b*) we found only carcinoma *in situ* was present on the available path blocks; or (*c*) DNA could not be adequately amplified after repeated attempts] leaving 292 cases. Breast cancer blocks were identified from each of these women by N. W., and clinicopathological and follow-up information were obtained by chart review by N. W., P. O. C and J. R. G. All of the specimens were reviewed by one pathologist (L. R. B.) for histological type, nuclear grade, and lymph node status, and were stained for ER and p53 using immunohistochemistry (IHC), as described previously (5, 9). The results of the cyclin E and $p27^{Kip1}$ IHC assays were read by P. P. and L. K., respectively, after performing the IHC as described in previous publications (5, 12). HER2 staining was performed and then scored by M. T. and L. R. B as described previously (13), and CK5/6 and Factor VIII IHC assessment was performed by I. M. S., O. S., and L. A. A (8, 13). Specimens were coded and DNA was extracted from the paraffin wax-embedded blocks using standard techniques.

***BRCA1* and *BRCA2* Mutation Status.** Mutation analysis was carried out by N. H. as described previously (5), looking specifically for the recurrent mutations in the Ashkenazi Jewish population (*BRCA1*: 185delAG, 5382insC;

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BRCA2: 6174delT). Haplotype analysis was also used to confirm 5382insC mutation. The *BRCA2* mutation 6174delT was sought using single-strand conformation analysis, by a mutation-specific PCR-RFLP endonuclease digestion analysis and by direct sequencing. We also used a size assay as a second assay for all three mutations. *BRCA2* carriers ($n = 10$) were excluded from all further analysis because it is not established that they follow the same pathway to carcinogenesis as do *BRCA1* carriers in survival analyses, and constituted too small a group to be analyzed alone.

Statistical Analysis. Clinical, pathological, and molecular data were collected in a mutually blinded fashion. Patient characteristics were compared using nonparametric Wilcoxon's test and Fisher's exact test. Borderline statistical significance was defined as *P*s between 0.05 and 0.10. Survival rates were calculated from the date of primary surgery until death from breast cancer (breast cancer-specific survival). The median follow-up of those who did not die of breast cancer was 9.35 years ($n = 186$; overall follow-up was 7.93 years, $n = 247$). Ten-year survival curves were estimated using the Kaplan-Meier method, and significance was assessed with the log-rank test. To estimate the relative risk (RR) of death from breast cancer, a Cox proportional hazards model was used with which all of the measured prognostic factors were examined in the first model (*i.e.*, tumor size, axillary lymph node status, nuclear grade, age at diagnosis, ER, p53, HER2, GMP, cyclin E, $p27^{Kip1}$, CK5/6, and *BRCA1* mutation status). The final most parsimonious model was built using the log-likelihood ratio test, employing a backward and forward approach in which variables with the highest contribution to the likelihood function were kept in the model. This model contained only six variables: tumor size, nodal status, p53, $p27^{Kip1}$, cyclin E, and GMP.

We reanalyzed the survival data separately for node-negative and node-positive subsets, using this parsimonious model. Whenever possible, the multivariate models were adjusted for missing values. To do this, we included a dichotomized variable to identify whether or not the variable of interest was missing. This allowed us to include 241 of the 247 subjects in the final model. All of the data were censored at 10 years, and significance was assessed at the 5% level using two-sided tests. A Poisson regression model was built to examine the relationship between the number of positive lymph nodes and tumor size: $\ln(\mu) = \ln(N_{exam}) + \alpha + \alpha_{CK5/6+} + \beta * \text{Tsize} + \beta_{CK5/6+} * \text{Tsize}$, where: μ = average number of positive nodes, α = overall intercept, $\alpha_{CK5/6+}$ = extra intercept for CK5/6+ patients, β = overall slope, $\beta_{CK5/6+}$ = extra slope for CK5/6+ patients, and the natural logarithm of the number of nodes examined was used as an offset.

Results and Discussion

The main focus in this study was the relationship among *BRCA1*, CK5/6, and other variables previously associated with *BRCA1* mutation status, and the influence of these markers on outcome. Accordingly, we excluded individuals with no information on recurrences or outcome, subjects carrying a *BRCA2* mutation and those with missing CK5/6 status. This left 247 of the original 292 patients, and this forms the historical cohort analyzed here. There were 27 *BRCA1* carriers and 220 non-*BRCA1/2* carriers in this cohort, and 95 tumors expressed CK5/6, and 152 did not. Tumors that expressed CK5/6 were more likely to occur in younger women (median, 6.1 years younger, $P = 0.002$) with larger tumors (median 2.0 *versus* 1.5, $P = 0.005$), of high grade [odds ratio (OR) for grade 3 *versus* grade 1 = 9.6, $P = 0.0001$; Table 1). These tumors were more likely to be ER-negative (OR, 3.0; $P < 0.0001$), p53-positive (OR, 2.3; $P = 0.006$), to have GMP (OR, 4.3; $P < 0.0001$); and cyclin E-positive (OR. 4.7; $P = 0.0001$). CK5/6 expressing-cancers were also much more likely to occur in *BRCA1* carriers than in other women (OR, 5.5;

Table 1 *Clinicopathological and prognostic characteristics of breast cancers (n = 247) dichotomized by cytokeratin 5/6 (CK5/6) status*

Variable	CK5/6−	CK5/6+	OR[a] [95% CI]	*P*	10-yr survival	No. of events	*P* value[b] (survival)
Age of diagnosis (yr)							
(25–50)	51	49	1.00		64.9%	31	...
[50–65]	101	46	0.47 [0.28–0.80]	0.005	75.3%	30	.048
Tumor size (cm)							
(0–2)	88	40	1.00		83.8%	17	...
[2–11]	56	53	2.08 [1.23–3.54]	0.008	58.6%	40	<.0001
Nuclear grade							
Grade 1	53	11	1.00		89.6%	5	...
Grade 2	70	26	1.79 [0.81–3.94]	0.18	72.4%	22	.007
Grade 3	29	58	9.64 [4.38–21.2]	0.0001	54.9%	34	<.0001
Lymph node status							
Negative	75	49	1.00		82.0%	19	...
Positive	60	42	1.07 [0.63–1.83]	0.89	57.5%	38	.0002
ER IHC							
Negative	41	50	1.00		55.4%	35	...
Positive	111	45	0.33 [0.19–0.57]	<0.0001	79.9%	26	<.0001
p53 IHC							
Negative	124	63	1.00		76.6%	36	...
Positive	27	32	2.33 [1.29–4.23]	0.006	54.2%	25	.0002
HER2 IHC							
Faint staining	139	80	1.00		74.3%	48	...
Weak/strong	13	15	2.01 [0.91–4.43]	0.099	45.8%	13	.004
BRCA1 germ-line mutation							
Negative	145	75	1.00		72.3%	52	...
Positive	7	20	5.52 [2.24–13.7]	0.0001	61.6%	9	.20
$p27^{Kip1}$ IHC							
Negative	79	62	1.00		65.0%	42	...
Positive	62	27	0.56 [0.32–0.97]	0.051	80.4%	14	.005
Cyclin E IHC							
Negative	128	51	1.00		77.0%	35	...
Positive	22	41	4.68 [2.54–8.62]	0.0001	51.4%	26	<.0001
GMP IHC							
None	128	64	1.00		74.5%	41	...
Few	12	22	3.67 [1.71–7.88]	0.0001	56.4%	14	.008
Moderate/many	1	6	12.0 [1.41–101.8]	0.008	21.4%	5	<.0001
Chemotherapy							
No	85	42	1.00		79.7%	22	...
Yes	64	51	1.61 [0.96–2.72]	0.09	61.2%	38	.0023
Hormonal therapy							
No	68	53	1.00		61.2%	42	...
Yes	76	34	0.57 [0.33–0.99]	0.057	83.5%	16	.0008

[a] OR, odds ratio; CI, confidence interval; ER, estrogen receptor; IHC, immunohistochemistry; GMP, glomeruloid microvascular proliferation.

$P = 0.0001$). There were borderline significant positive associations with HER2 overexpression (OR, 2.0; $P = 0.10$) and lack of $p27^{Kip1}$ expression (OR, 1.8; $P = 0.05$). When comparing these results with those obtained when the data were dichotomized by *BRCA1* mutation status, it was observed that the results were almost identical (data not shown), with nearly all of the effects being in the same direction, with the main differences being those of magnitude. However, HER2 expression showed no tendency to vary by *BRCA1* mutation status (OR, 0.98; $P = 0.99$). It has previously been suggested that tumor cells overexpressing HER2 may not be all equally likely to show positive immunohistochemical staining for p53 and, furthermore, that unlike breast cancers of luminal origin, basal-like breast cancers are likely to be HER2-negative but p53-positive (14). To test this, we compared the OR for the association between HER2 overexpression and p53 expression, firstly, in all cases, and then in CK5/6-positive and negative subgroups. Overall, the OR was 5.4 [95% confidence interval (CI), 2.4–12.3; $P < 0.0001$). In CK5/6-negative tumors, the OR was 15.0 (95% CI, 4.2–54), whereas for CK5/6-positive tumors, the OR was 1.9 (95% CI, 0.63–5.9; $P = 0.25$; P for test for heterogeneity = 0.01). This confirms the previous observations and illustrates the need for the molecular subclassification of tumors (2, 14–17).

Previous microarray studies have shown that the basal-like phenotype of breast cancer is associated with a particular gene expression profile (7, 15). Moreover, in a study of 89 breast cancers, 7 tumors showed very high levels of the ubiquitin ligase subunit, Skp2 (18). This protein regulates the levels of $p27^{Kip1}$ by promoting its degradation (19). Because $p27^{Kip1}$ is an inhibitor of cyclin E, Skp2 overexpression may play a key role in regulating progression through the cell cycle (18). Often, tumors overexpressing Skp2 also demonstrated reduced expression of HER2, ER, androgen receptor, cyclin D1, CK18, and MYB (18). All of these, except for MYB (20), have been reported to be also found at low expression/protein levels in *BRCA1*-related cancer (2, 21). Elevated expression of CK5/6, CK17, cyclins A2, B1, D3, and E1, CDK2 and CDK4, and MYC was also observed in the Skp2-positive group, thus defining them as basal-like breast cancers (18). Of these markers, we focused on CK5/6 and cyclin E in this study.

On univariate analysis, tumors that expressed CK5/6 had a significantly worse breast cancer-specific survival than those that did not (Fig. 1*A*). To establish the relationship between basal-related tumor markers and outcome after breast cancer, we compared the Kaplan-Meier survival curves for breast cancer-specific survival after dichotomizing by markers of prognosis that in our study are linked to the basal-like phenotype, namely, cyclin E (Fig. 1*B*), $p27^{Kip1}$ (Fig. 1*C*), p53 (Fig. 1*D*), GMP (Fig. 1*E*) and germ-line *BRCA1* mutation (Fig. 1*F*).

To determine which of these markers, if any, were independently predictive of outcome after breast cancer, we constructed a multivariable model. Neither CK5/6 nor *BRCA1* were present in the final models, but are strongly associated with most of the variables that were present in the final, most parsimonious model, shown in Table 2A. Of those variables most closely associated with the basal epithelial phenotype (Table 1), the adjusted RR for death from breast cancer was greatest for GMP (RR, 2.34; $P = 0.0002$), for any GMP compared with the absence of GMP. In this model, all of the included

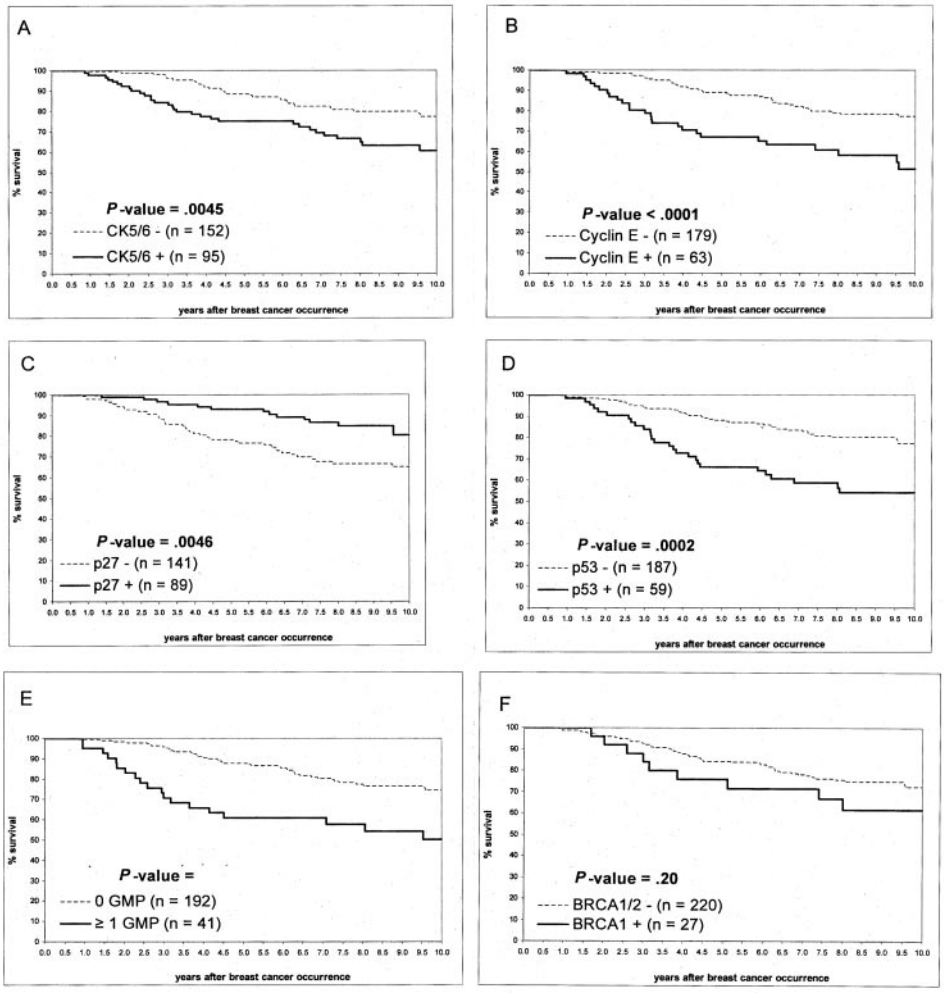

Fig. 1. Kaplan-Meier survival plots are shown for cytokeratin 5/6 (*CK5/6*) (*A*); cyclin E (*B*); $p27^{Kip1}$ (*C*); p53 (*D*); glomeruloid microvascular proliferation (GMP; *E*), and *BRCA1* (*F*). Log-rank *P*s for the comparison in breast cancer-specific survival (BCSS) at 10-year follow-up are shown in boxes inside each subfigure.

Table 2 *Final proportional hazards model*[a] *for breast cancer specific survival at 10 years follow-up*

Variable	Univariate		Multivariate	
	RR[b] (95% CI)	*P*	RR (95% CI)	*P*
A. All patients (n = 241)				
Tumor size				
<2 cm	1.00		1.00	
≥2 cm	3.09 (1.75–5.46)	0.0001	2.08 (1.14–3.80)	0.02
Lymph nodes				
Negative	1.00		1.00	
Positive	2.78 (1.60–4.83)	0.0003	2.58 (1.45–4.56)	0.001
p53				
Negative	1.00		1.00	
Positive	2.57 (1.54–4.28)	0.0003	1.70 (0.99–2.90)	0.05
$p27^{Kip1}$				
Negative	1.00		1.00	
Positive	0.43 (0.23–0.78)	0.006	0.51 (0.28–0.95)	0.03
Cyclin E				
Negative	1.00		1.00	
Positive	2.63 (1.58–4.38)	0.0002	1.99 (1.16–3.41)	0.01
GMP				
None	1.00		1.00	
>0	2.68 (1.55–4.62)	0.0004	2.34 (1.31–4.21)	0.004
B. Node-negative disease (n = 120)				
Tumor size				
<2 cm	1.00		1.00	
≥2 cm	1.68 (0.68–4.13)	0.26	0.99 (0.39–2.55)	0.99
p53				
Negative	1.00		1.00	
Positive	2.48 (0.98–6.30)	0.06	1.20 (0.44–3.24)	0.73
$p27^{Kip1}$				
Negative	1.00		1.00	
Positive	0.21 (0.06–0.72)	0.01	0.24 (0.07–0.85)	0.03
Cyclin E				
Negative	1.00		1.00	
Positive	5.13 (2.01–13.0)	0.0006	3.53 (1.34–9.28)	0.01
GMP				
None	1.00		1.00	
>0	1.85 (0.67–5.14)	0.24	1.78 (0.60–5.25)	0.30
C. Node-positive disease (n = 100)				
Tumor size				
<2 cm	1.00		1.00	
≥2 cm	3.45 (1.43–8.32)	0.006	3.95 (1.47–10.6)	0.007
p53				
Negative	1.00		1.00	
Positive	2.20 (1.15–4.22)	0.02	1.99 (0.99–4.00)	0.05
$p27^{Kip1}$				
Negative	1.00		1.00	
Positive	0.74 (0.36–1.52)	0.41	0.87 (0.41–1.83)	0.70
Cyclin E				
Negative	1.00		1.00	
Positive	2.03 (1.02–4.03)	0.04	1.24 (0.59–2.61)	0.58
GMP				
None	1.00		1.00	
>0	3.16 (1.56–6.44)	0.002	3.16 (1.44–6.95)	0.004

[a] Adjusted for all missing variables. Final model excludes cases for which adjustment was not possible (n = 6); cases with unknown nodal status are not included.
[b] RR, relative risk; CI, confidence interval; GMP, glomeruloid microvascular proliferation.

variables (tumor size, nodal status, p53, $p27^{Kip1}$, cyclin E, and GMP) independently predicted survival after breast cancer, and all (except nodal status) were strongly associated with the basal phenotype, which was itself strongly associated with the presence of a *BRCA1* mutation. In agreement with the findings reported here, most (16, 18, 22–25), but not all (26), previous studies of basal CK profiles and survival after breast cancer have found that the basal epithelial phenotype is associated with an inferior outcome.

To a certain extent, the strength and independence of the basal-related markers depended on the nodal status of the breast cancers. For example, in univariate Kaplan-Meier survival plots, for $p27^{Kip1}$, *BRCA1* and CK5/6, a difference in survival between tumors possessing, or not possessing, the marker of interest was seen for only node-negative disease (*P*s 0.006, 0.002, and 0.03 respectively). By contrast, GMP status predicted outcome in node-positive disease (P = 0.0008) but not in node-negative disease. Only cyclin E status was significantly associated with a poor outcome in both node-negative and node-positive disease (the results for p53 were of borderline significance). These results are reflected in the final multivariable proportional hazards model, in which node-negative disease (Table 2B; n = 120; 19 breast cancer deaths) is considered separately from lymph node-positive disease (Table 2C; n = 100; 38 breast cancer deaths). Notably, in node-negative disease, $p27^{Kip1}$ [RR, 0.24; 95% confidence intervals (CIs), 0.07–0.85] and cyclin E (RR, 3.53; 95% CI, 1.34–9.28) were the only independent predictors of breast cancer-specific survival (Table 2B). In node-positive disease, tumor size (RR, 3.95; 95% CI, 1.47–10.6) and GMP status (RR, 3.16; 95% CI, 1.44–6.95) were the only independent variables (Table 2C).

We observed a positive association between tumor size and CK5/6 staining. There was no similar association between nodal status and

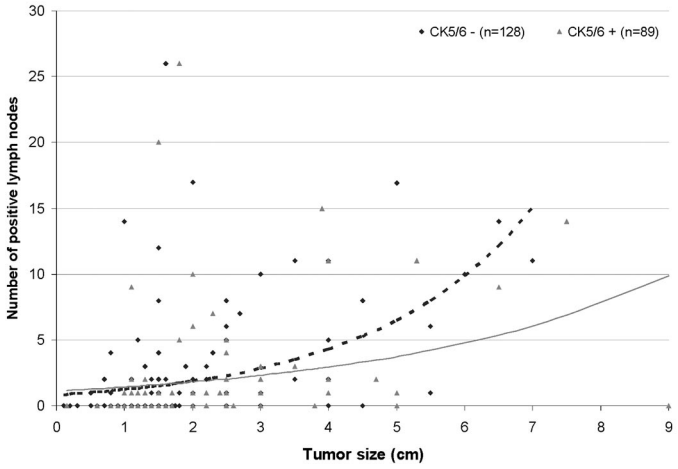

Fig. 2. The graph shows the Poisson regression curves to predict the number of positive lymph nodes for women with cytokeratin 5/6 (CK5/6) negative status ($n = 128$) and positive ($n = 89$). (See ''Materials and Methods'' for the model used.) Each individual is represented by either a dark diamond (CK5/6-negative immunohistochemical staining) or a pale triangle (CK5/6-positive immunohistochemical staining). The estimated curve for CK5/6-negative cases is shown by a dashed line and for CK5/6-positive cases by a solid line. The difference between the two log-transformed slopes is statistically significant. $\alpha = -2.69$, $P < 0.0001$; $\alpha_{CK5/6+} = 0.28$, $P = 0.095$; $\beta = 0.37$, $P < 0.0001$; $\beta_{CK5/6+} = -0.1668$, $P = 0.0002$.

CK5/6 staining. We wondered, therefore, whether the known positive correlation between tumor size and the number of lymph nodes that were involved by tumor would hold equally for CK5/6-negative and CK5/6-positive cancers. Using a Poisson regression model, we found that tumors that did and did not stain with CK5/6 demonstrated the expected positive correlation between tumor size and the number of lymph nodes involved by tumor, but that the log-transformed tumor size slopes differed significantly (Fig. 2; $P = 0.0002$). For tumors expressing CK5/6, the relationship between tumor size and the number of positive lymph nodes was weak, especially when compared with those tumors that did not express CK5/6. CK5/6-positivity is a feature of *BRCA1*-related breast cancer, and we have recently shown that the positive correlation between tumor size and lymph node status is also disrupted in *BRCA1*-related cancers (4). It is tempting, therefore, to speculate that this may be a general feature of basal-related breast cancers. If true, this could have significance for the reclassification of breast cancers based on pathways, rather than on strict morphological criteria. This finding also suggests that the favored route of metastatic spread may differ for basal and nonbasal breast cancers, because CK5/6-positive cancers are less likely than CK5/6-negative cancers to be node-positive when large in size (Fig. 2), but are nevertheless associated with a worse outcome (Fig. 1*A*).

The basal phenotype, originally recognized as being associated with CK5/6 positivity, can be extended to include cyclin $E^{high}/p27^{low}/p53^{+}/GMP^{+}$ staining. One of these four markers was found to be abnormal in almost 90% (50 of 56) of those who died of breast cancer, although 68% of those who survived 10 years also had at least one abnormal "basal" marker. Only six tumors expressed the full cyclin $E^{high}/p27^{low}/p53^{+}/GMP^{+}$ phenotype: four of these women died of breast cancer at 10 years follow-up; this compares with 52 breast cancer deaths among 209 women with all other phenotypes. Notably, CK5/6-positivity alone indicates a more than five times greater likelihood of identifying a *BRCA1* mutation than does CK5/6-negativity (Table 1), and this could be of value in itself, for example in deciding whether to first analyze *BRCA1* or *BRCA2* in a woman with breast cancer. In addition, our data show that markers associated with the CK5/6-positive phenotype have independent predictive value.

In conclusion, we report the following: (*a*) as previously shown, germ-line *BRCA1* mutations result in breast cancers that are highly likely to be basal in character, as defined by CK5/6 IHC; (*b*) the basal phenotype is also characterized by large tumors that express low levels of ER, HER2, and $p27^{Kip1}$ and high levels of cyclin E, and that feature both nuclear p53 and intratumoral vascular nests (GMP); (*c*) in univariate analysis, all of these factors are associated with a poor outcome; (*d*) those tumor markers most closely linked to the basal phenotype (p53, $p27^{Kip1}$, cyclin E, and GMP) are independent predictors of outcome; and (*e*) the relationship between tumor size and nodal status is significantly different when comparing tumors that do, and do not, express CK5/6. Therefore, we consider that the basal phenotype of breast cancer deserves recognition as a separate biological entity. The combined association and survival data presented here suggest that much of the inferior survival after breast cancer that is experienced by *BRCA1* carriers (particularly among women with lymph node-negative disease) is attributable to the basal epithelial phenotype of these cancers.

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