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A survey of immunohistochemical biomarkers for basal-like breast cancer against a gene expression profile gold standard

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Gene expression profiling of breast cancer delineates a particularly aggressive subtype referred to as 'basal-like', which comprises ~15% of all breast cancers, afflicts younger women and is refractory to endocrine and anti-HER2 therapies. Immunohistochemical surrogate definitions for basal-like breast cancer, such as the clinical ER/PR/HER2 triple-negative phenotype and models incorporating positive expression for CK5 (CK5/6) and/or EGFR are heavily cited. However, many additional biomarkers for basal-like breast cancer have been described in the literature. A parallel comparison of 46 proposed immunohistochemical biomarkers of basal-like breast cancer was performed against a gene expression profile gold standard on a tissue microarray containing 42 basal-like and 80 non-basal-like breast cancer cases. Ki67 and PPH3 were the most sensitive biomarkers (both 92%) positively expressed in the basal-like subtype, whereas CK14, IMP3 and NGFR were the most specific (100%). Among biomarkers surveyed, loss of INPP4B (a negative regulator of phosphatidylinositol signaling) was 61% sensitive and 99% specific with the highest odds ratio (OR) at 108, indicating the strongest association with basal-like breast cancer. Expression of nestin, a common marker of neural progenitor cells that is also associated with the triple-negative/basal-like phenotype and poor breast cancer prognosis, possessed the second highest OR at 29 among the 46 biomarkers surveyed, as well as 54% sensitivity and 96% specificity. As a positively expressed biomarker, nestin possesses technical advantages over INPP4B that make it a more ideal biomarker for identification of basal-like breast cancer. The comprehensive immunohistochemical biomarker survey presented in this study is a necessary step for determining an optimized surrogate immunopanel that best defines basal-like breast cancer in a practical and clinically accessible way.
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For many years pathologists have recognized biological heterogeneity among breast cancers. Since the pioneering gene expression profile studies by Perou and colleagues that identified intrinsic subtypes of invasive carcinoma,[1] the concept of breast cancer as a collection of different diseases has gained widespread acceptance.[2–6] Subsequent studies have confirmed that the five intrinsic molecular subtypes (luminal A, luminal B, HER2-enriched, basal-like and normal-like) possess distinct etiologies and clinicopathologic features with implications for treatment selection.[1,7–12] Of particular relevance to patient management, the 'basal-like' subtype comprises ~15% of all invasive breast cancers and is responsible for a disproportionately high number of metastatic breast cancer cases and breast cancer-related deaths.[13,14] This aggressive subtype is also associated with early age of onset,[15–18] BRCA-related hereditary cancers[19–22]

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and has a particularly high incidence in women of African ethnicity.[23–25] Despite its characteristically poor prognosis and resistance to established molecularly targeted therapies (eg, tamoxifen, aromatase inhibitors and trastuzumab), basal-like breast cancer is usually a diagnosis of exclusion in the clinical setting, based on lack of expression of hormone receptors and HER2.[14,26] With limited therapeutic options, cytotoxic chemotherapy is the principal systemic treatment for women with this type of breast cancer.

At present, microarray-based gene expression profiling technologies are not practical for hospital diagnostic laboratories and routine analysis of patient specimens. In fact, the relatively high cost and complexities associated with sample preparation, assay and data analysis by gene expression profiling have resulted in few published reports on basal-like breast cancer using this gold standard for its identification.[27] Most studies characterizing this subtype have performed investigations using formalin-fixed paraffin-embedded tissue specimens, typically obtained from clinical biopsy and excision samples found in pathology department archives. With immunohistochemistry as a universally available and inexpensive technique for analysis of formalin-fixed paraffin-embedded tissue, the literature has become dominated by the use of immunohistochemical surrogate definitions for basal-like breast cancer, most commonly the 'triple-negative phenotype' (TNP; characterized by the lack of expression of ER, PR and HER2),[26] a basal cytokeratin definition (characterized by positive expression of basal cytokeratins 5, 14 and/or 17)[20,28,29] or the immunopanel proposed in Nielsen *et al*[14] (negative ER and HER2 but positive expression of CK5/6 and/or EGFR, later modified to a 5-marker immunopanel with inclusion of negative expression of PR), the latter identifying wider prognostic differences than TNP.[16] However, against gene expression-based subtype assignment originally used to identify basal-like breast cancer, these immunohistochemical surrogates possess only moderate accuracy, with 76–79% sensitivity and 72–100% specificity.[14,30]

The past few years have seen a plethora of biomarkers described as having an association with the basal-like or triple-negative phenotypes.[31,32] Only some have been validated on independent series, very few have been compared with a gene expression gold standard, and no study has compared large numbers of these candidate biomarkers in parallel in a single validation series. In line with current pathology practices that rely on immunohistochemistry and concurrent morphological examination, we sought to evaluate 72 proposed basal-like biomarkers, drawn from recent gene expression profile data and published literature, on sections from the same breast cancer tissue microarray in which intrinsic subtype has been assigned by a PAM50 gene expression profile assay.[33,34] In doing so we sought to identify the best individual immunohistochemical biomarkers for this aggressive form of breast cancer.

Materials and methods

Tissue Microarray Construction and PAM50 Molecular Subtype Assignment

Breast cancer tissue microarrays were constructed from archival tumor blocks of 137 high-grade patients, who received surgical intervention at Washington University and Barnes-Jewish Hospital in St Louis from 1997 to 2003, as previously described.[35] Samples (both direct consent and waived consent) were obtained from the Alvin J. Siteman Cancer Center Tissue Procurement Core facility according to an Institutional Review Board-approved protocol. Duplicate 0.6-mm cores were extracted from each tumor block and transferred to the recipient tissue microarray block. Sample preparation and processing for PAM50 gene expression profiling using qRT-PCR from paraffin cores is described in Cheang *et al*[36] and Nielsen *et al*.[34] Of 137 cases, 127 were successfully assigned an intrinsic molecular subtype (basal-like, HER2-enriched, luminal A, luminal B or normal-like). Excluding duplicate cases and the normal-like subtype from analysis, the remaining 122 samples consisted of 42 basal-like and 80 non-basal-like breast cancers (58 luminals and 22 HER2-enriched). Details of the PAM50 qRT-PCR subtype predictor are provided in Parker *et al*.[33] This study was approved by the Clinical Research Ethics Board of the University of British Columbia and the British Columbia Cancer Agency.

Immunohistochemical Staining and Scoring

Seventy-two biomarkers were drawn from gene expression profile data and a survey of published literature performed in June 2011 (Table 1). Antibodies suitable for application on formalin-fixed paraffin-embedded tissue samples were acquired for 61 of 72 proposed biomarkers. Two EGFR antibodies were included in the analysis. Manual EGFR immunostaining was performed according to the PharmDX kit manufacturer's instructions (Dako Cytomation, Carpinteria, CA, USA). A rabbit monoclonal antibody for EGFR (Epitomics, Burlingame, CA, USA) was applied using a Discovery XT autoimmunostainer (Ventana Medical Systems, Tucson, AZ, USA). The Epitomics anti-EGFR possessed more consistent staining and superior ease of interpretation—in addition to the advantage of automated application—than the Dako PharmDX anti-EGFR. Thus, the Epitomics anti-EGFR was used for all immunohistochemical subtype definitions that included EGFR. An aliquot of anti-EZH2 was graciously provided by Dr Gulisa Turashvili

Table 1 Antibody details for biomarkers that produced technically satisfactory immunostaining for scoring on the breast cancer tissue microarray ($n=47$; including two EGFR antibodies)

Antigen	*Antibody type*	*Source*	*Clone*	*Dil.*	*Scoring system*	*Positive/*n *(%)*
αBC*[38–41]	Mouse mAb	Stressgen	1B6.1-3G4	1/20	Neg *vs* any staining	19/99 (19.2)
Anillin[33,42]	pAb	Bethyl Labs		1/100	Staining in $<10\%$ *vs* $\geq 10\%$	54/103 (52.4)
CAIX[43,44]	pAb	Santa Cruz		1/25	Neg *vs* any staining	19/108 (17.6)
CAV1[45–49]	pAb	BD Biosciences		1/250	Staining in $<10\%$ *vs* $\geq 10\%$	29/104 (27.8)
CAV2[48,50]	Mouse mAb	BD Biosciences	65	1/50	Neg *vs* any staining	8/105 (7.6)
CD44[51–54]	Rabbit mAb	Abcam	EPR1013Y	1/25	Allred score system	46/103 (44.6)
CD44v6[55–57]	Mouse mAb	BenderMed	VFF48	1/500	Staining in $<25\%$ *vs* $\geq 25\%$	67/108 (62.0)
c-Kit*[14,58,59]	pAb	Dako		1/200	Any staining *vs* strong in $\geq 20\%$	16/95 (16.8)
CLDN4*[60–62]	Mouse mAb	Zymed	3E2C1	1/50	Multiplicative quickscore	41/101 (40.6)
Cyclin E*[19,63]	Mouse mAb	Neomarkers	13A3	1/10	Staining in $<10\%$ *vs* $\geq 10\%$	49/107 (45.7)
CK5*[20,64–67]	Mouse mAb	Thermo	XM26	1/25	Neg *vs* any staining	30/96 (31.3)
CK5/6*[14,68]	Mouse mAb	Zymed	D5/16B4	1/100	Neg *vs* any staining	28/111 (25.2)
CK14*[20,28,69,70]	Mouse mAb	Santa Cruz	LL002	1/100	Neg *vs* any staining	10/106 (9.4)
CK17*[67,69]	Mouse mAb	Dako	E3	1/50	Neg *vs* any staining	21/94 (22.3)
EGFR*[14,24,58,71,72]	Mouse mAb	Dako PharmDX	2-18C9	Pre-dil	Neg *vs* any staining	19/104 (18.3)
EGFR*[14,24,58,71,72]	Rabbit mAb	Epitomics	EP22	1/50	Neg *vs* any staining	28/105 (26.6)
ER*[14,73]	Rabbit mAb	Thermo	SP1	1/25	Staining in $<1\%$ *vs* $\geq 1\%$	53/112 (47.3)
EZH2[74–78]	Mouse mAb	BD Biosciences	11	1/50	Staining in $<5\%$ *vs* $\geq 5\%$	76/102 (74.5)
FABP7[79–81]	Polyclonal	Abcam		1/100	Staining in $<10\%$ *vs* $\geq 10\%$	52/107 (48.5)
Fascin*[82–84]	Mouse mAb	Dako	55K-2	1/100	Neg *vs* any staining	27/108 (25.0)
FOXC1[33,85,86]	pAb	LifeSpan Biosciences		1/50	Neg *vs* any staining	27/108 (25.0)
HER2[87]	Rabbit mAb	Neomarkers	SP3	1/500	Binarized with FISH correction	12/104 (11.5)
IMP3*[88–91]	Mouse mAb	Dako	69.1	1/50	Neg *vs* any staining	9/105 (8.6)
INPP4B*[92–94]	Rabbit mAb	Epitomics	EPR3108Y	1/50	Staining in $\leq 5\%$ *vs* $>5\%$	23/106 (21.7)
Integrin $\beta 4$[95]	Rabbit mAb	eBiosciences	439-9B	1/25	Staining in $<5\%$ *vs* $\geq 5\%$	41/104 (39.4)
Ki67*	Rabbit mAb	Neomarkers	SP6	1/200	Staining in $<13.5\%$ *vs* $\geq 13.5\%$	72/111 (64.8)
Laminin5[96–98]	Mouse mAb	Dako	4G1	1/25	Staining in $<5\%$ *vs* $\geq 5\%$	62/97 (63.9)
Met[99–101]		House-made			Negative/weak/moderate staining *vs* strong staining $>10\%$	41/120 (34.1)
Moesin*[102,103]	Mouse mAb	Santa Cruz	38/87	1/100	Neg *vs* any staining	35/100 (35.0)
Nestin*[104–108]	Mouse mAb	Santa Cruz	10c2	1/50	Staining in $<1\%$ *vs* $\geq 1\%$	23/108 (21.3)
NGFR*[109,110]	Mouse mAb	Abcam	NGFR5	1/25	Neg *vs* any staining	8/103 (7.8)
p16*[111–113]	Mouse mAb	mtm Laboratories	E6H4	1/2	Staining in $\leq 80\%$ *vs* $>80\%$	36/95 (37.8)
p27[19,114]	Mouse mAb	BD Biosciences	57	1/50	Staining in $<50\%$ *vs* $\geq 50\%$	27/106 (25.4)
p53*[115–117]	Mouse mAb	Dako	DO-7	1/400	Staining in $<10\%$ *vs* $\geq 10\%$	33/105 (31.4)
p63[64,65,118]	Mouse mAb	CellMarque	4A4	1/200	Neg *vs* any staining	10/96 (10.4)
P-cad*[65,70,119–122]	Mouse mAb	BD Biosciences	56	1/20	Weak staining in $<10\%$ *vs* Any other staining	55/105 (52.3)
P-gp[123]	Mouse mAb	Abcam	C494	1/50	Any Staining *vs* Strong in $\geq 20\%$	34/95 (35.7)
PPH3*[124–127]	pAb	Upstate		1/100	Staining in $<1\%$ *vs* $\geq 1\%$	62/105 (59.0)
PR*[128]	Rabbit mAb	Neomarkers	SP2	1/200	Staining in $<1\%$ *vs* $\geq 1\%$	37/111 (33.3)
pS6rp	Rabbit mAb	Cell Signaling	91B2	1/250	Staining in $<5\%$ *vs* $\geq 5\%$	46/95 (48.4)
PTEN	Rabbit mAb	Cell Signaling	138G6	1/25	Neg *vs* any staining	77/94 (81.9)
S100A9*[129–131]	pAb	Santa Cruz		1/100	Staining in $<$median *vs* $\geq$median	35/105 (33.3)
Skp2*[63,114,132]	Mouse mAb	Zymed	2C8D9	1/25	Staining in $<10\%$ *vs* $\geq 10\%$	32/96 (33.3)
SMAD4	Mouse mAb	Santa Cruz	B-8	1/50	Allred score system	39/95 (41.1)
TRIM29*	Goat pAb	Santa Cruz		1/100	Bkgd or lower in $<100\%$ *vs* above bkgd	36/97 (37.1)
VEGF-A[133–135]	Mouse mAb	Lab Vision	JH121	1/25	Staining in <185 *vs* ≥ 185	62/105 (59.0)
Vimentin[70,96,136,137]	Mouse mAb	Zymed	V9	1/50	Staining in $<1\%$ *vs* $\geq 1\%$	18/100 (18.0)

Abbreviations: bkgd, background; dil., dilution; mAb, monoclonal; pAb, polyclonal antibody.
Biomarkers that failed to progress to analysis due to lack of a commercial antibody demonstrated to work for IHC applications on breast tissue included: ALDH1, CD109, CD123, CD146, E2F-5, OATP2, Osteopontin, S100A2 and S100A7. Those that failed to progress due to nonspecific staining on controls tested in our laboratory included: Aurora A, Aurora B, CD68, CD280, CEP55, Chromogranin A, c-Myc, CXCR4, KNTC2, MELK, MIA, RAD51, Sox2, SPARC and YB-1. BRCA1 immunostaining results were excluded after mutational analysis performed by an external laboratory determined that nuclear staining was spurious, likely introduced by prolonged antibody storage. VEGFR2 showed discrepant staining relative to the positive control and was not subjected to further analysis.
*Biomarkers significantly associated with basal-like breast cancer after correction for multiple comparisons.

(BC Cancer Agency, Vancouver, BC, Canada). INPP4B was obtained from Epitomics. An antibody for Met was made in-house and previously stained by an external laboratory,[35,37] generating the data utilized in the current study. During immunohistochemical optimization using standard laboratory staining protocols programmed into the Discovery XT auto-immunostainer, 15 of the 61 candidate immunohistochemical biomarkers demonstrated nonspecific staining on control tissues or tissue microarrays after multiple attempts with varying antibody dilutions and

protocols. The remaining 46 were stained on 4 μm sections of the above-described breast cancer tissue microarray. Stained slides were scanned using a BLISS system (Bacus Laboratories/Olympus America, Lombard, IL, USA), and a pathologist scored each biomarker using, wherever possible, the scoring system described in the original literature associating that biomarker with basal-like breast cancer (Table 1).

Statistical Analysis

PASW Statistics 18 for Windows (SPSS, 2009, Chicago, IL, USA, www.spss.com) was used to perform contingency table analyses. Pearson's χ^2 analysis (or the Fisher's exact test, when appropriate) was used to compare biomarker expression in basal-like and non-basal-like cases defined by PAM50 gene expression profile. *P*-values were adjusted for multiple comparisons using a modified Bonferonni correction method previously described by Holm,[138] after which $P<0.05$ defined statistical significance. Ninety-five percent confidence intervals (95% CI) for sensitivity and specificity for each biomarker were generated in R version 2.11.1 (www.r-project.org) using a bootstrap methodology.

Results

Tissue Microarray Staining

Following antibody evaluation and optimization, immunostaining of 46 proposed biomarkers for basal-like breast cancer was technically satisfactory for scoring on the breast cancer tissue microarray (containing 42 basal-like and 80 non-basal like cases, as determined by PAM50 expression profile). Table 1 includes details of these antibodies and lists the number of cases available for analysis of each immunostain. Missing data reflects loss of cores from the tissue microarray section or exhaustion of tumor tissue in cores as sections went deeper into the tissue microarray block. Representative staining of each positively expressed basal-like breast cancer biomarker is illustrated in Figure 1. Described immunostains can be viewed in full through a

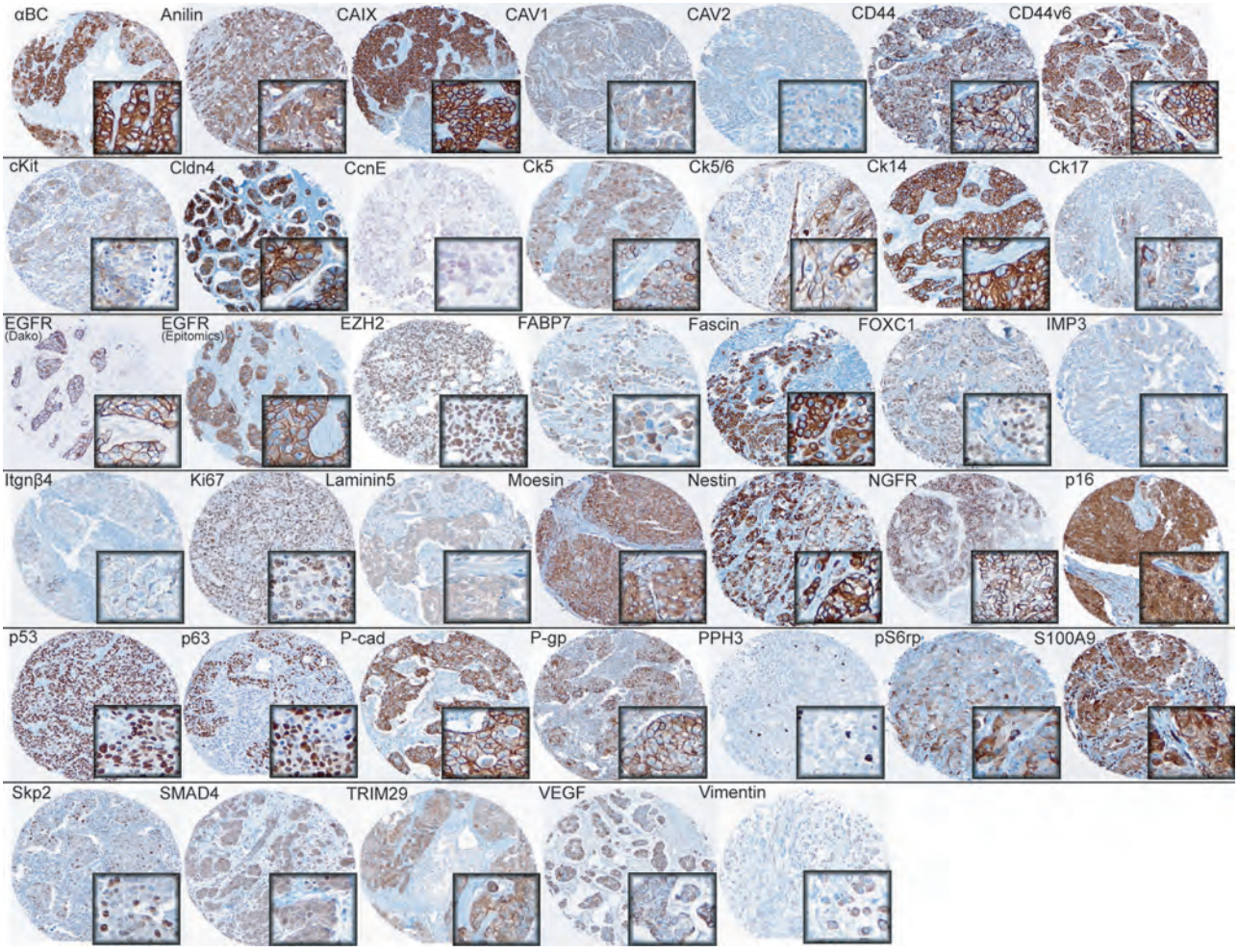

Figure 1 Representative staining of positively expressed basal-like biomarkers.

digital image archive accessible via the website of the Genetic Pathology Evaluation Centre (www.gpecimage.ubc.ca).

Immunohistochemical Interpretation and Univariate Analysis of Basal-Like Biomarkers

After correction for multiple comparisons, 25 of these 46 proposed basal-like biomarkers (labeled by an asterisk in Table 1) were significantly associated with basal-like breast cancer. Sensitivity, specificity, odds ratio (OR) as well as raw *P*-values for each of these biomarkers are presented in Table 2.

At an individual sensitivity of 92%, Ki67 (based on the previously established 13.5% cutpoint[36]) and phosphohistone-H3 (PPH3) were the most sensitive biomarkers for basal-like breast cancer. Ki67, a nuclear antigen expressed by proliferating cells, has been extensively characterized in the literature.[139,140] PPH3, a lesser known marker of mitotic figures,[126,141] lacks consistent established cutpoints that led to the adoption of a 1% cutpoint in the present study (corresponding to a commonly advocated cutpoint for ER). Of particular relevance to feasibility of clinical implementation, study investigators noted and confirmed that nuclear staining of PPH3 is strong, discrete and easy to interpret.[127] Lymphocyte staining and cytoplasmic staining in tumor cells were occasionally observed. Similarly, in support of common surrogate panels for basal-like breast cancer, lack of ER or PR expression was also sensitive (92%) for detection of basal-like breast cancer.

The biomarkers displaying the highest specificity (100%) for basal-like breast cancer in this study were cytokeratin 14 (CK14), insulin-like growth factor mRNA binding protein-3 (IMP3) and nerve growth factor receptor (NGFR). However, this apparently perfect specificity came at the price of poor individual sensitivity, ranging from 22% to 27%. Consistent with other basal cytokeratins, strong cytoplasmic and peri-membranous staining of CK14 was observed in tumor cells and the basal/myoepithelial layer of normal breast epithelial elements (disregarded during scoring). IMP3 staining was cytoplasmic and predominantly of weak intensity, but present in most tumor cells of a positive core. Essentially no background staining was observed for IMP3, making it possible for a trained pathologist to distinguish the characteristic weak positive staining in tumor cells. NGFR staining was membranous but, unlike CK14 and IMP3 staining, was not restricted to basal-like tumor cells and basal/myoepithelial cells of normal breast, as it was also seen in nerves and in occasional stromal and endothelial cells.

Negative INPP4B (inositol polyphosphate-4-phosphatase, type II) staining possessed the best combination of sensitivity (61.1%) and specificity (98.6%) with the overall highest individual OR (108.4) among the biomarkers tested in this study, suggesting its absence may be the best single diagnostic immunohistochemical biomarker for basal-like breast cancer. Although different staining intensities were observed (Figure 2a), cytoplasmic staining of INPP4B was only scored as percent positive tumor cells then later binarized using a 5% cutpoint.

Table 2 Test characteristics of statistically significant basal-like breast cancer biomarkers after correction for multiple comparisons—arranged by odds ratio (OR)

Biomarker	*Sensitivity (95% CI)*	*Specificity (95% CI)*	*OR (95% CI)*	*Raw* P-*value*
INPP4B negative	61.1 (43.8–75.9)	98.6 (91.5–100)	108.4 (13.5–872.0)	1.7E – 12
Nestin	54.1 (37.2–70.0)	95.8 (88.2–98.7)	26.7 (7.1–100.3)	1.9E – 09
ER negative	92.1 (78.6–97.6)	67.6 (56.1–77.6)	24.3 (6. 8–87.0)	2.1E – 09
CK5	70.6 (52.8–84.2)	90.3 (80.3–96.2)	22.4 (7.3–68.6)	7.4E – 10
cKit	42.4 (25.6–59.4)	96.8 (88.2–100)	22.1 (4.6–106.1)	2.9E – 06
p16	78.8 (61.3–90.5)	83.9 (72.7–91.7)	19.3 (6.6–56.6)	2.0E – 09
Fascin	57.9 (41.5–73.0)	92.9 (84.4–97.2)	17.9 (5.9–54.5)	6.0E – 09
PPH3	91.7 (77.8–97.5)	58.0 (45.9–69.2)	15.2 (4.2–54.3)	3.5E – 07
Moesin	71.4 (53.3–84.4)	84.6 (73.7–91.9)	13.8 (5.1–37.2)	2.1E – 08
CK17	50.0 (31.0–65.7)	91.9 (82.5–96.9)	11.4 (3.6–35.9)	3.7E – 06
ki67	92.1 (78.4–97.7)	49.3 (37.8–60.8)	11.4 (3.2–40.2)	7.3E – 06
PR negative	92.1 (78.6–97.6)	46.6 (35.0–58.2)	10.2 (2.9–36.1)	3.5E – 05
TRIM29	71.0 (51.9–85.2)	78.8 (67.2–87.3)	9.1 (3.4–24.1)	2.2E – 06
α-B-crystallin	41.2 (25.0–58.3)	92.3 (83.3–97.0)	8.4 (2.7–26.3)	5.9E – 05
S100A9	62.2 (45.5–76.9)	82.4 (71.4–90.2)	7.7 (3.1–19.1)	3.8E – 06
CK5/6	50.0 (32.6–64.9)	87.7 (78.1–93.9)	7.1 (2.8–18.3)	1.4E – 05
Skp2	60.6 (42.4–76.0)	81.0 (69.4–89.1)	6.5 (2.6–16.7)	4.1E – 05
EGFR (Epitomics)	51.4 (34.3–67.6)	85.7 (75.4–92.4)	6.4 (2.5–16.3)	5.0E – 05
P-cadherin	77.8 (60.7–89.2)	60.9 (48.5–71.6)	5.4 (2.2–13. 7)	1.7E – 04
Claudin 4	63.9 (46.4–78.6)	72.3 (60.0–82.3)	4.6 (1.9–11.0)	3.9E – 04
Cyclin E	69.4 (51.9–82.9)	66.2 (54.4–76.4)	4.5 (1.9–10.6)	4.7E – 04
p53	52.8 (36.1–69.0)	79.7 (68.7–88.1)	4.4 (1.8–10.6)	6.6E – 04
CK14	27.0 (13.9–43.2)	100	—	1.1E – 05
IMP3	25.0 (11.8–40.6)	100	—	3.1E – 05
NGFR	22.2 (10.3–38.1)	100	—	1.3E – 04

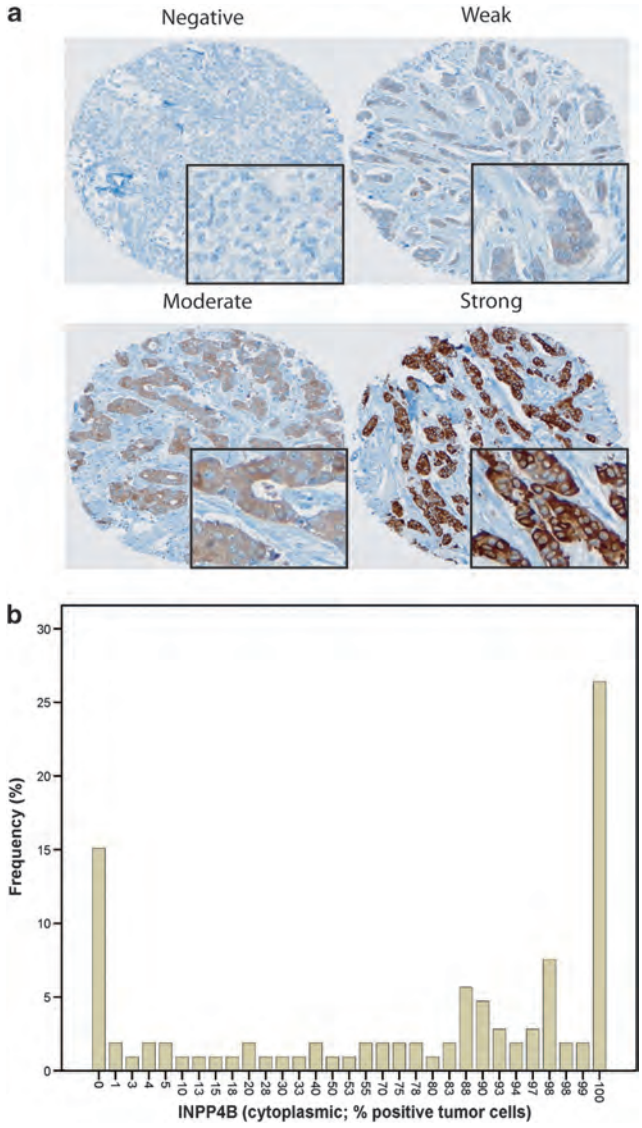

Figure 2 Immunohistochemical analysis of INPP4B in breast cancer. (**a**) The majority of cases expressing INPP4B demonstrated staining in most tumor cells regardless of staining intensity. (**b**) The frequency distribution of percent positive tumor cells regardless of INPP4B staining intensity confirmed this observation.

Very minimal background staining was noted. The pattern of INPP4B expression was observed to be predominantly dichotomous, with more than half of cases having all-or-none staining in tumor cells (Figure 2b).

Other single biomarkers with a relatively favorable OR include nestin (using a 1% cutpoint), negative ER staining (using a 1% cutpoint), CK5 (notably more sensitive than older CK5/6 antibodies; Table 2) and c-kit. Thus, the best positively expressed immunohistochemical biomarkers for basal-like breast cancer are nestin and CK5, both representing intermediate filaments belonging to a category of proteins that are relatively abundant and stably expressed—two features that are technically advantageous for immunohistochemical analyses.

Discussion

Gene expression profiling-based technologies originally used to identify basal-like breast cancer are not widely accessible in daily practice. DNA microarray-based testing platforms currently lack the robustness and cost-efficiency required for routine clinical use. Surrogate immunohistochemical definitions for basal-like breast cancer, despite moderate sensitivity and specificity, have been more frequently employed by both the research and medical communities. Building on the clinical ER/PR/HER2 TNP, basal cytokeratin definitions and the combined immunopanel described in our previous work,[14,16] a large and evolving body of research has since described additional biomarkers for basal-like breast cancer (reviewed in Choo *et al*[31]).

Validation of proposed biomarkers against a gold standard is a necessary step to identify the most useful biomarkers that can best define the intrinsic molecular subtypes of breast cancer by immunohistochemistry. The development of the PAM50 assay, a gene expression assay applicable to formalin-fixed paraffin-embedded blocks,[33,34] now greatly facilitates such endeavors. Specifically, this 50-gene bioclassifier stratifies breast cancers into prognostic groups that can be used to aid clinicians in making treatment decisions.[33,34,142] Furthermore, the PAM50 assay identifies breast cancer intrinsic subtypes (luminal A, luminal B, HER2-enriched or basal-like) with prognostic and predictive implications.[142,143] This study presents an immunohistochemical assessment of several dozen published biomarkers of the aggressive basal-like subtype of breast cancer in a cohort of molecularly defined breast cancer specimens.

On the basis of OR, loss of INPP4B expression (OR = 108.4, 61.1% sensitivity and 98.6% specificity) is the immunohistochemical assay that is most strongly associated with basal-like breast cancer among the 46 biomarkers tested. This class II phosphatase is one of the many players involved in the negative regulation of phosphatidylinositol signaling, a pathway of particular interest for targeted therapies in basal-like and triple-negative breast cancers.[144–148] Located on chromosome 4q31.21, the *INPP4B* locus is commonly deleted in basal-like breast cancers and cell lines.[92–94,149–151] Previous studies characterizing INPP4B as a tumor suppressor were primarily focused at the genetic level; however, Gewinner *et al*[93] recently demonstrated successful immunohistochemical analysis of INPP4B and correlation between loss of INPP4B expression and decreased overall breast cancer survival.

Nonetheless, relying on lack of expression of a biomarker for identification of basal-like breast

cancer cases can be misleading as negative staining can be caused by technical problems at any of the several steps. For instance, antigen fading was an issue that we encountered with the INPP4B antibody when applied to tissue microarray cohorts consisting of previously frozen, archival formalin-fixed paraffin-embedded pathology specimens collected more than 25 years ago (data not shown). Conversely, nestin—a positive biomarker for basal-like breast cancer—possessed the second highest odd ratio (OR = 28.7, 54.1% sensitivity and 95.8% specificity) among the 46 biomarkers surveyed. This type VI intermediate filament is an established marker of neural progenitor cells,[152,153] yet several studies have described its expression in the basal/myoepithelial layer of the mammary gland and in tumor cells of suspected basal-like and triple-negative breast cancer cases.[104–108,154] Parry *et al*[106] reported nestin positivity in 15 of 22 (68%) basal-like cases (as defined by the Nielsen *et al*[14] immunohistochemical definition) as compared with 3 of 117 (2%) non-basal-like cases. Similarly, Liu *et al*[108] detected nestin expression in 9 of 21 (57%) TNPs but only 12 of 129 (9%) non-TNPs. In this study, nestin displayed positive immunohistochemical expression in 20 of 36 (55%) cases defined as basal-like by expression profile, but only 3 of 72 (4%) non-basal-like cases. The observed consistency of nestin immunostaining and interpretation across different studies may reflect good antigen stability in clinical samples, typical of structural proteins.

Ki67 and PPH3, both markers of proliferation associated with poor prognosis and basal-like/triple-negative breast cancer,[124,126,127,141,155] possessed the highest sensitivity (~92%) for the basal-like subtype in the present study. Nevertheless, neither Ki67 nor PPH3 is particularly specific for the basal-like subtype, hardly surprising given that luminal B and HER2-enriched breast cancers are also characterized by strong proliferation signatures.[1,3,7] CK14, IMP3 and NGFR had the highest specificity (100%) for basal-like breast cancer but this came at the significant expense of sensitivity, which, in line with published observations,[84,109] ranged from 22% to 27%.

Given that highly specific biomarkers had low sensitivity while highly sensitive biomarkers suffered low specificity, a multi-marker immunopanel rather than a single biomarker might be more useful to account for phenotypic heterogeneity and increase overall sensitivity for detection.[156,157] Preferably, such a panel would also exhibit high sensitivity and specificity with a limited, practical number of biomarkers.[158] Interestingly, as individual biomarkers for basal-like breast cancer, INPP4B (61% sensitivity, 99% specificity) and nestin (54% sensitivity, 96% specificity) showed comparable sensitivities and specificities to existing multi-marker definitions, such as the TNP (83% sensitive, 87% specific) and the Nielsen *et al*[14] definition (67% sensitive, 93% specific). A 2-marker panel for identification of basal-like breast carcinomas comprised of INPP4B negativity and/or nestin positivity was observed to have 83% sensitivity and 96% specificity. Similarly, a 2-marker panel of INPP4B and CK5, another top basal-like biomarker from the current survey that is already an established immunohistochemical marker in diagnostic laboratories, possessed 83% sensitivity and 91% specificity. However, to avoid overoptimistic results due to over-fitting, fair comparisons against existing immunopanels and any attempts to determine a superior surrogate panel that best defines basal-like breast cancer need to be performed on a series independent from the one used herein to identify the best biomarkers, and ideally by independent research groups. Our available large tissue microarray series (most recently described in Mehta *et al*[159]), designed for biomarker correlations with long-term outcomes, appears unsuitable for this task due to the antigen-fading issue around INPP4B, suggesting a more contemporary series might be better suited for such work.

As with all reported statically significant basal-like biomarkers described above or listed in Table 2, large confidence intervals for OR values were observed, indicating that results should be interpreted with caution due to the limited sample size. As pointed out by Pepe *et al*,[160] the independent contribution of a biomarker to classification accuracy can be negligible despite a strong association with disease status (in this case, basal-like as opposed to non-basal-like breast cancer). However, in conjunction with reported sensitivity and specificity values, all lowest confidence interval values remain above the null value (OR = 1), supporting a true association between tested biomarkers and basal-like breast cancer.[161]

Although great strides have been made in automated immunostaining and antigen retrieval techniques, as well as commercialization of antibodies for an ever-growing list of antigens, it still remains to be determined whether or not immunohistochemistry is entirely up to task for recapitulating gene expression profile analyses. Subject to data reduction and statistical model building techniques on a series independent from the one used herein, the results of this comprehensive immunohistochemical survey may be able to contribute to the development of a clinically practical multi-marker immunopanel that best defines basal-like breast cancer in an inexpensive and widely accessible way.[162,163] Followed by rigorous evaluation of classification accuracy and validation on large independent data sets, application of such an assay in retrospective analyses and prospective clinical trials will help to accurately identify basal-like breast cancer cases, ultimately facilitating development of much needed therapies for breast cancer patients with this particularly aggressive form of the disease.

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Disclosure/conflict of interest

MJE, CMP, and PSB hold shares in, and TON has received consultant fees from, Bioclassifier LLC. Bioclassifier LLC holds the patent rights to the PAM50 test, which has been licensed to Nanostring Technologies. Nanostring Technologies is currently working to commercialize the PAM50 test in a format different from the research test used in the paper. Neither Bioclassifier LLC nor Nanostring Technologies financed any aspects of the current manuscript. PAM50 is an open source bioinformatics approach that can be applied to data generated with a variety of research-use-only platforms (in this case, a PCR-based platform different from the clinical assay being commercialized by Nanostring Technologies). The remaining authors declare no conflict of interest.

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