Clinicopathologic and molecular characterization of low-grade, early-stage, and HER2-positive invasive breast carcinoma

Natasha Hunter, MD[1]; Lisa Han, MD[2]; Haley Corbin, MD[3]; Eric Q. Konnick, MD, MS[4,]; William R. Gwin III, MD[1]; Shaveta Vinayak, MD[1]; Hannah Linden, MD[1]; William Audeh, MD[5]; Lavanya Samraj, MDS, MS[5]; Andrea R. Menicucci, PhD[5]; FLEX Investigators Group; T. Rinda Soong, MD, PhD[3,*]*

[1]Division of Hematology and Oncology and [4]Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, United States; [2]Lakeridge Health, Oshawa, ON, Canada; [3]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States; [5]Agendia, Irvine, CA, United States

Abstract

Objectives: Breast carcinomas overexpressing human epidermal growth factor receptor 2 (HER2) are typically associated with higher tumor grade and faster progression. HER2 positivity is rare in low-grade breast carcinomas with unclear biological implications. We aimed to characterize their clinicopathologic and molecular profiles in this study.

Methods: There were 2 cohorts of Nottingham grade 1, HER2-positive invasive breast carcinomas examined: (1) an institutional series (n = 14) and (2) tumors from patients (n = 59) enrolled in the FLEX multicenter clinical registry with MammaPrint and BluePrint profiling.

Results: Most (79%) in the case series were both estrogen receptor (ER) and progesterone receptor (PR)-positive. Over half were pathologic or clinical T1N0 tumors. In the 9 cases with adequate material for next-generation sequencing, the majority (66%) demonstrated *ERBB2* copy number variations. Most (66%) received HER2-targeted therapy. No recurrences were observed, with a median follow-up time of 43 months. In the FLEX cohort, most tumors were ER-positive (86%) and PR-positive (68%), and over half were clinical T1. Most (70%) were of the luminal phenotype, and over half (54%) were low-risk on MammaPrint.

Conclusions: Low-grade HER2-positive breast carcinomas constitute mostly low-stage, luminal-type, and apparently low-risk tumors, warranting investigation into whether therapy de-escalation could achieve favorable outcomes with less toxicity in this population.

KEY POINTS

- Low-grade human epidermal growth factor receptor 2 (HER2)–positive (HER2+) invasive carcinomas in breast are rare and present mostly as early-stage tumors with good prognosis as well as estrogen receptor (ER) and progesterone receptor (PR) positivity.
- More than half of low-grade HER2+ breast carcinomas are associated with the luminal molecular phenotype, with a significant proportion showing a low-risk MammaPrint profile in this study.
- Compared with conventional high-grade HER2+ breast tumors, low-grade HER2+ breast carcinomas may represent a distinct subset that could benefit from therapeutic de-escalation strategies.

Key words:

invasive breast carcinoma; low-grade and early-stage breast carcinoma; HER2-positive; HER2-directed therapy

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*Corresponding author: T. Rinda Soong, rsoong@pitt.edu

INTRODUCTION

About 15% to 20% of breast carcinomas overexpress the human epidermal growth factor receptor 2 (HER2).[1] Histologically, these tumors are generally characterized by higher tumor grade and lower frequency of estrogen receptor (ER) and progesterone receptor (PR)

expression.[2] HER2-positive (HER2+) node-negative tumors have been shown to have a higher risk of local recurrence compared to HER2-negative tumors.[3-5] The prognosis for HER2+ tumors dramatically improved with the incorporation of HER2-directed therapy (trastuzumab) in standard regimens but required combinations of chemotherapy and HER2-targeted therapies to achieve comparable gains to similar-stage ER/PR-positive tumors.[2,6,7]

Currently, most patients with HER2+ T1b or higher tumors receive at least a taxane-based regimen with trastuzumab per guidelines. Standard therapy for HER2+ tumors measuring less than 3 cm without positive lymph nodes includes 12 cycles of weekly paclitaxel plus trastuzumab, followed by trastuzumab every 21 days to complete 1 year of therapy.[8] Larger and node-positive tumors are treated more aggressively, usually with a regimen such as TCHP (docetaxel, carboplatin, trastuzumab, and pertuzumab) with HER2-directed therapy.[9,10] These treatments are often associated with significant side effects (eg, allergy, rash, fatigue, cytopenias, infection, neuropathy). Identifying a subgroup of HER2+ patients for whom therapy could potentially be de-escalated may provide significant benefit.

In a major update to the breast cancer staging system, the American Joint Committee on Cancer in 2017 incorporated ER/PR/HER2 status and Nottingham grade into its standard breast cancer staging algorithm, recognizing that these features play key prognostic roles.[11] Specifically, tumor grade is an independent prognostic indicator, with low-grade tumors correlating with lower overall risk and better survival.[12] Low-grade breast cancer has also been shown to be less responsive to chemotherapy.[13] Current treatment guidelines and decisions are driven largely by tumor size and nodal status but not tumor grade. The intersection of low tumor grade and HER2 overexpression, while unusual, does occur. In a large series by Hoff et al[14] of HER2+ tumors stratified by tumor grade, only 1% of low-grade invasive ductal carcinomas overexpressed HER2, compared with 17% and 23% for intermediate- and high-grade tumors in the cohort. In a series by Yu et al[15] examining classical invasive lobular carcinomas, 12 of 52 lobular carcinomas were HER2+, only one of which was low grade. Of the 406 early-stage cases in the Adjuvant Paclitaxel and Trastuzumab (APT) trial for node-negative HER2+ breast carcinoma, only 44 (10.8%) were classified as grade 1.[7]

Little data exist, however, to determine whether these breast tumors are pathologically and biologically similar to higher-grade exemplars and whether the aggressive regimens employed for higher-grade HER2+ tumors may result in unnecessary toxicity to achieve a cure for low-grade HER2+ breast carcinomas. With this in mind, we characterized low-grade HER2+ tumors in this study from the histopathologic, molecular, and gene expression perspectives. This analysis offers a step toward establishing whether low-grade HER2+ breast tumors may represent a distinct phenotype that has potential implications for prognosis and treatment.

METHODS

Study cohorts

Case inclusion criteria included (1) Nottingham grade 1 invasive mammary carcinoma; (2) positive HER2 status, confirmed by HER2 immunohistochemical study (3+ score) or HER2 fluorescence in situ hybridization (FISH) study positive for *ERBB2* amplification, as determined by clinical practice guidelines; and (3) no unexplained discordance in HER2 immunohistochemical and FISH study findings.

Clinicopathologic and molecular data were collected from 2 separate cohorts. One cohort included a single institutional case series that had detailed treatment and clinical outcome data, and the other cohort constituted a larger sample of tumors collected from patients enrolled in the national multicenter prospective MammaPrint, BluePrint, and Full-genome Data Linked with Clinical Data to Evaluate New Gene EXpression Profiles (FLEX) clinical trial,[16] which is further described below.

Institutional case series: clinicopathologic data collection

Invasive breast carcinoma cases diagnosed at the authors' institution from 2003 to 2019 and confirmed to be HER2+ by immunohistochemical study and/or FISH per standard clinical guidelines were reviewed.[17,18] Tumors that were scored as Nottingham grade 1 on both core biopsy samples and subsequent resections were identified. Tumor grade was determined using the Nottingham histologic grading score, which assigns a score of 1 to 3 for each of these 3 parameters: degrees of tubular formation, nuclear pleomorphism, and mitotic activity. The final histologic grade is based on a sum of the individual scores of the 3 parameters: 3, 4, or 5 = grade 1; 6 or 7 = grade 2; and 8 or 9 = grade 3.[19,20] Pathology reports, including tumor grade information, on each case were reviewed and cosigned by at least 2 pathologists at the times of diagnoses. Central review of identified cases was also conducted by an independent pathologist (T.R.S.) for this study to confirm that the histology, tumor grade, and HER2 status of reported cases met the study inclusion criteria.

Pathology data were collected on the tumor tissue, including the histologic subtype, tumor stage, hormone receptor and HER2 status, the HER2/CEP17 ratio, and mean HER2 signals/cell for each case. The ER, PR, and HER2 status of the cases were determined based on standard clinical guidelines and immunohistochemical protocols[21] (Supplementary Table S1; all supplementary material is available at *American Journal of Clinical Pathology* online).

All identified cases were reviewed in the electronic medical record on patient demographic features as well as clinical characteristics, including treatments received and clinical outcome status.

Institutional case series: molecular data collection

Next-generation sequencing (NGS) was performed on primary tumor samples for which tumor content was sufficient (tumor cellularity >20%) on formalin-fixed, paraffin-embedded tissue blocks. Samples underwent molecular analysis using the UW-OncoPlex assay, a clinically validated, hybrid-capture NGS assay that interrogates the full coding sequences of 340 genes (Supplementary Table S2) and is capable of detecting all classes of genomic alterations, including single nucleotide variants (SNVs), copy number variations (CNVs), insertions, deletions, structural mutations, microsatellite instability, and tumor mutational burden.[22,23]

Sequencing of prepared hybrid-capture libraries was performed on a HiSeq2000 or NextSeq500 sequencing system (Illumina) with

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2 × 101-bp, paired-end reads as previously described; the minimum acceptable average coverage for the entire panel was set at 150.[22,23]

The SNV and indel data were annotated with gene-based annotation, conservation scores, predicted effects at the protein level, and population frequency using ANNOVAR (BIOBASE). ANNOVAR was also used to annotate the frequency of variants in an internal database.[22,23] Bioinformatically called SNVs and indels were manually reviewed for biologically significant mutations, including (1) loss of function mutations (splice site disruption, frameshift, nonsense) and hotspot missense mutations for tumor suppressor genes, (2) missense hotspot mutations for oncogenes, and (3) pathogenic gene mutations listed in COSMIC (v95, released November 24, 2021), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), and/or cBioPortal (v3.7.27; https://www.cbioportal.org/).

For CNV analysis, copy number states for individual probes were initially called using CONTRA version 2.0.3 (http://sourceforge.net/projects/contra-cnv/files, last accessed July 2020) with reference to a CNV control comprising reads from 2 independent rounds of library preparation and sequencing of the HapMap individual NA12878. The CNV calls were made at the resolution of individual exons using custom Perl scripts. We defined copy number gain as a predicted copy number of 3 or 4 and amplification as a predicted copy number of 5 or higher.[22,23]

We also evaluated samples that yielded adequate RNA quality for gene expression profiling by Agendia's MammaPrint and BluePrint assays. MammaPrint, a validated clinical assay analyzing 70 genes associated with breast cancer recurrence, is used by clinicians as a tool to predict benefit from chemotherapy in patients with early-stage, T1-2, N0-1 breast cancers.[24,25] The BluePrint assay interrogates 80 genes to identify the underlying biology of an individual breast cancer, classifying tumors as luminal A, luminal B, HER2, or basal.[26]

FLEX registry cohort: clinicopathologic data collection

We then sought to examine if the pattern of clinicopathologic and gene expression characteristics observed in the case series was representative by reviewing a larger case cohort of low-grade HER2+ invasive breast carcinomas in the FLEX clinical trial. The FLEX study is a prospective observational trial maintained by Agendia, the precision genomic testing laboratory that enrolls patients 18 years or older with histologically proven stage I to III invasive breast carcinoma with up to 3 positive lymph nodes and who consent to clinically annotated full transcriptome data collection.[27] The registry contains full genome expression data for more than 15 000 tumor cases enrolled from 2017 to 2023 as of September 2023, with the primary aim of investigating new gene associations with prognostic and/or predictive values in luminal-type breast cancers.[26-33]

The database was queried for grade 1 invasive breast carcinoma cases with HER2 results from 2001 to 2014. We collected demographic data, clinicopathologic data including clinical T and N staging information, pathologic T and N staging categories, histologic subtypes, HER2 immunohistochemical and FISH classifications, as well as MammaPrint and BluePrint results for this study.

RESULTS

Institutional case series: clinicopathologic profile

A total of 14 cases meeting the study inclusion criteria were identified **Table 1** (additional details in Supplementary Table S3). In this series, patients were all White, with a median age of 57 years (range, 35-69 years). All but 2 tumors were less than 2 cm in size at the time of clinical detection, and over half (57%) were node negative at the time of resection. The tumors were predominantly of the ductal subtype, with 2 cases showing focal to prominent mucinous features **Table 1**; **Figure 1**. Micropapillary or other variant features were not identified. All cases were positive for ER, and most were positive for PR (79%) **Table 1**; Supplementary Table S3.

Patients were diagnosed between 2003 and 2019. Clinical HER2 status and subsequent management were determined per standard practice guidelines at the time of diagnosis.[17,18] The update in guidelines for determining HER2 status in breast pathology specimens in 2018 was noted.[17] HER2 immunohistochemical and FISH results of tumors diagnosed before the 2018 update were re-reviewed. All but 1 case (patient 2) in the series had unchanged positive HER2 status based on the 2018 update on interpretations **Table 1**; Supplementary Table S3. This patient had been managed as a HER2-positive case per contemporary clinical regimens at the time of her diagnosis, tumor resection, and adjuvant therapy administration.

Most patients (71%) were documented to have received chemotherapy combined with HER2-directed therapy, either neoadjuvantly or adjuvantly **Table 1**. All subjects who received cytotoxic chemotherapy were also given HER2-directed therapy. The 3 patients who did not undergo combination chemotherapy/HER2-targeted therapy had contradiction(s) due to medical comorbidities or patient preference. All patients with available treatment data completed at least a partial course of endocrine therapy **Table 1**.

Two patients received neoadjuvant chemotherapy with HER2-targeted therapy for larger (cT2 or cT3) and clinically node-positive tumors. Neither of these patients achieved a pathologic complete response.

No disease recurrence or cancer-related death was observed, with a median follow-up time of 43 months (range, 1-199 months).

Institutional case series: molecular profiles

Of the 14 tumors in this series, 9 had adequate tumor available for NGS using the UW-OncoPlex assay.[22,23] Average tumor mutation burden was 1.5, and mean coverage was 493 (range, 321-796). Of these samples, 4 demonstrated high-level amplification of the *ERBB2* locus, and 2 more showed probable gain of 1 to 2 copies of *ERBB2* when corrected for neoplastic content **Figure 2**. No significant correlation between *ERBB2* amplification/gain and clinicopathologic features, including receptor status, tumor size, or nodal status, was observed. Of the evaluable tumor samples, 5 demonstrated *PIK3CA* mutations, with only 1 patient demonstrating both *ERBB2* amplification and a missense mutation in *PIK3CA*. Mutations of *TP53* and *KMT2C* were noted in 2 cases, respectively **Figure 2**; Supplementary Table S4.

Of the 5 tumors adequate to perform subsequent MammaPrint and BluePrint analyses, 3 were classified as high risk on MammaPrint.

Table 1 Pathologic and Clinical Features of Grade 1 Invasive Breast Carcinoma Determined to Be HER2 Positive at the Time of Diagnosis in Institutional Case Series

		Tumor features								Treatment(s) received			Clinical outcome		
Patient No.	Age, y	Histologic subtype	ER	PR	HER2 IHC status	pT	pN	cT	cN	HER2-directed therapy	Chemotherapy	Endocrine therapy	Follow-up[a]	Recurred	Died
1	41	IDC+M	Pos	Pos	3+	1b	0	1b	0	Yes	Adjuvant ddAC/T, capecitabine	AI and tamoxifen	199	No	No
2	59	IDC	Pos	Pos	2+[b]	1c	0	1c	0	No[c]	No	AI	6	No	No
3	60	IDC	Pos	Pos	2+[b]	y1c	y1a	2	1	Yes	Neoadjuvant TCH	AI	112	No	No
4	40	IDC	Pos	Neg	3+	1b	0	1c	0	Yes	Adjuvant taxol ×12	Tamoxifen	93	No	No
5	69	IDC	Pos	Pos	3+	1b	0	1b	0	Yes	Adjuvant TDM-1[d]	AI	92	No	No
6	46	IDC	Pos	Pos	2+[b]	1c	1(mi)	1c	0	Yes	Adjuvant TCH	Tamoxifen	51	No	No
7	58	IDC	Pos	Neg	2+[b]	1b	0	1c	0	No[c]	No	AI	14	No	No
8	62	IDC	Pos	Pos	3+	1c	0	1c	0	Yes	Adjuvant TH	AI	47	No	No
9	62	IDC	Pos	Pos	2+[b]	1c	0	1b	0	Yes	Adjuvant TH	Tamoxifen	39	No	No
10	48	IDC	Pos	Pos	3+	1b	1(mi)	1b	0	Yes	Adjuvant TH	Tamoxifen	71	No	No
11	60	IDC+M	Pos	Pos	3+	y1a	y1a	3	1	Yes	Neoadjuvant ddAC/TH, adjuvant TDM-1	AI	29	No	No
12	56	IDC	Pos	Pos	3+	1a	0	1a	0	No[c]	No	AI	8	No	No
13	52	IDC	Pos	Pos	3+	1c	1(mi)	1c	0	Unk	Unk	Unk	1	No	No
14	35	IDC	Pos	Neg	2+[b]	1b	1a	Tis	0	Yes	Adjuvant TCHP	Unk	8	No	No

Abbreviations: AC+T, adriamycin + cyclophosphamide + taxol/taxotere; AI, aromatase inhibitor; ER, estrogen receptor; FISH, fluorescence in situ hybridization; IDC, invasive ductal carcinoma; IDC+M, invasive ductal carcinoma with mucinous features; IHC, immunohistochemical; MC, mucinous carcinoma; Pos, positive; Neg, negative; TCH, taxol/taxotere + carboplatin + herceptin; TCHP, taxol/taxotere + carboplatin + herceptin + pertuzumab; T-DM1, trastuzumab emtansine (Kadcyla); TH, taxol/taxotere + herceptin; Unk, unknown.
[a]Duration (months) of follow-up was measured as the date of diagnosis to the date of last known clinical encounter.
[b]For all cases with equivocal (2+) HER2 immunohistochemical study results, HER2 FISH studies were performed. All these cases yielded HER2 FISH ratio ≥2 and were determined to be positive for HER2 amplification per clinical guidelines at the time of diagnosis. Additional information on HER2 copy number/cell is listed in Supplementary Table S3.
[c]Patient declined HER2-targeted drug treatment option offered at the time of clinical consultation.
[d]Therapy provided on clinical trial.

All but 1 case were subtyped by BluePrint as luminal tumors, with only 1 tumor classified as HER2 type Figure 2. No definitive distinct association of clinicopathologic features was seen with high-risk vs low-risk tumors subgrouped by MammaPrint.

FLEX cohort: clinicopathologic features

Given that the institutional case series was limited in sample size, we interrogated the FLEX clinical trial database supported by Agendia to assess if similar clinicopathologic and gene expression profiles were seen in this larger population-based cohort.

Of over 10 000 early-stage tumors profiled in the FLEX database, we identified 59 cases (less than 1% of the database) of low-grade HER2+ tumors that met the case inclusion criteria Table 2. Features observed were largely consistent with those seen in the institutional case series. Most (80%) of the carcinomas in this cohort displayed ductal histology. Other less common histologic subtypes, including lobular and mucinous carcinomas, were also reported Table 2; Figure 1. The tumors in this cohort were predominantly ER-positive (86%) and PR-positive (68%). More than half (>50%) were of clinical T1 and clinical N0 disease Table 2; Supplementary Table S4.

FLEX cohort: MammaPrint/BluePrint profiles

Slightly above half (54%) of these patients had low-risk tumors on MammaPrint analysis, compared with approximately 50% of those in the MINDACT study used to validate the MammaPrint data.[25] Most (70%) of these tumors were of the luminal subtype, with only 27% being of the HER2 subtype based on the BluePrint assay. Luminal B (37%) and luminal A (33%) were the most common molecular subtypes. One case was noted to be of the basal subtype based on the BluePrint assay Table 2; Table 3.

Clinical treatment and outcome data were not available for the FLEX cohort. Unequivocal distinct patterns of clinicopathologic features could not be ascertained between MammaPrint high-risk vs low-risk tumors based on data accessible in this cohort.

DISCUSSION

Multiple studies have demonstrated HER2 heterogeneity within traditional breast cancer subgroups, raising questions about the classification and clinical implications of the complex role that HER2 plays in breast cancer.[34,35] This increasingly nuanced understanding demands similarly refined models to outline the expected response for a given HER2 phenotype, with corresponding pathologic markers for prediction. The introduction of novel HER2-directed therapies, such as trastuzumab deruxtecan, has focused recent attention on establishing refined quantified metrics of HER2 expression level to better predict benefit from these new HER2-targeted therapies.[36] Much, however, remains to be learned about HER2-overexpressing breast tumors that do not otherwise fit the classic standard model of aggressive disease.

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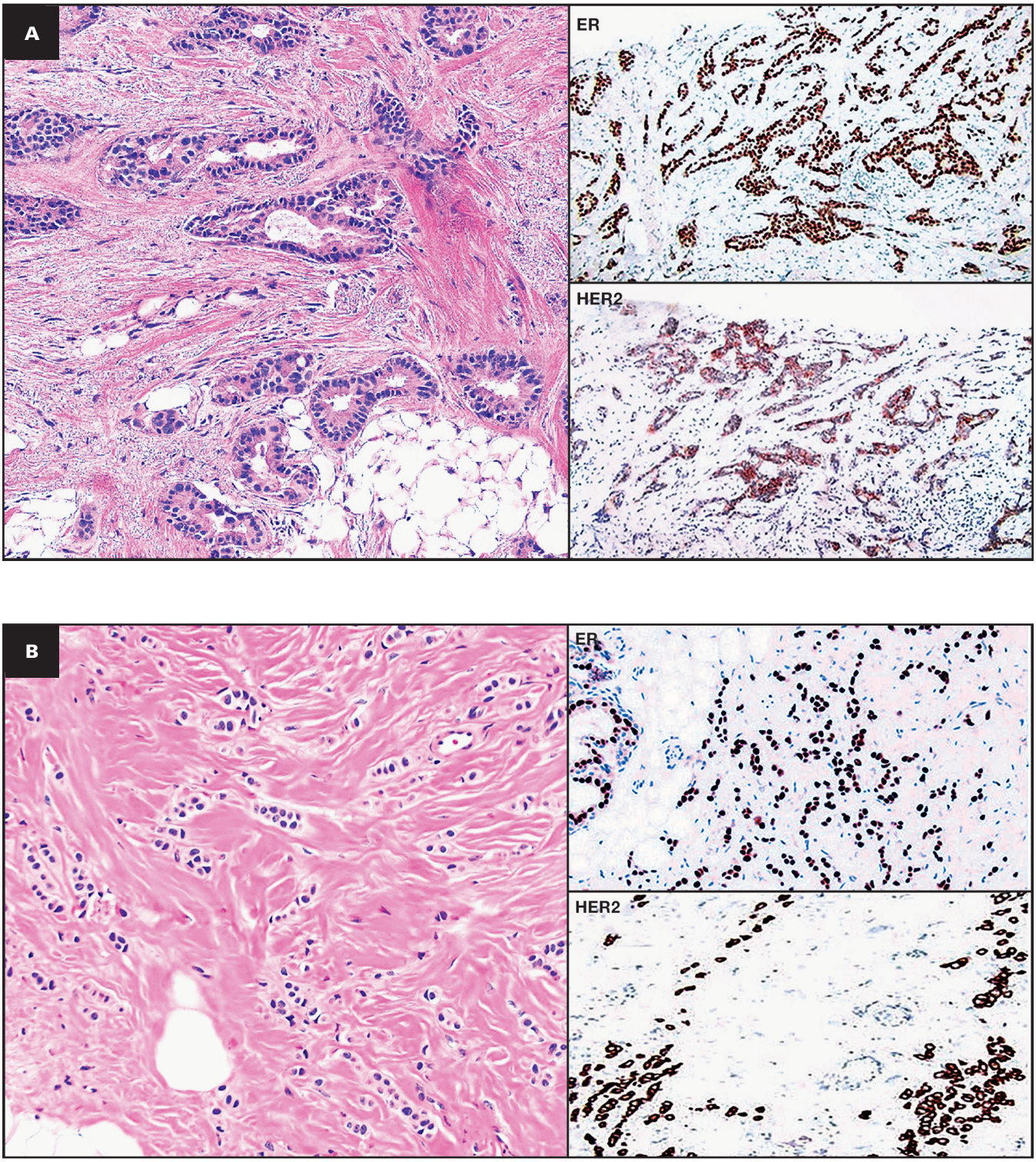

Figure 1 Representative histologic images of HER2+ low-grade invasive mammary carcinomas. Panels demonstrate different histologic subtypes observed in this tumor group, including the most common subtype, invasive ductal carcinoma (**A**), followed by other less common histotypes: invasive lobular carcinoma (**B**) and invasive ductal carcinoma with mucinous features (**C**). **A**, A case of invasive ductal carcinoma included in the institutional case series, with positive ER expression and equivocal HER2 IHC expression (insets). This tumor had positive HER2 status confirmed by FISH. **B**, A case of grade 1 invasive lobular carcinoma that was enrolled in the FLEX trial, with positive ER expression and HER2 status (insets). **C**, The mucinous part of an invasive ductal carcinoma with mucinous features in the institutional case series, with positive ER expression, as well as positive HER2 IHC expression (insets). ER indicates estrogen receptor; FISH, fluorescence in situ hybridization; FLEX, MammaPrint, BluePrint, and Full-genome Data Linked with Clinical Data to Evaluate New Gene EXpression Profiles; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemical.

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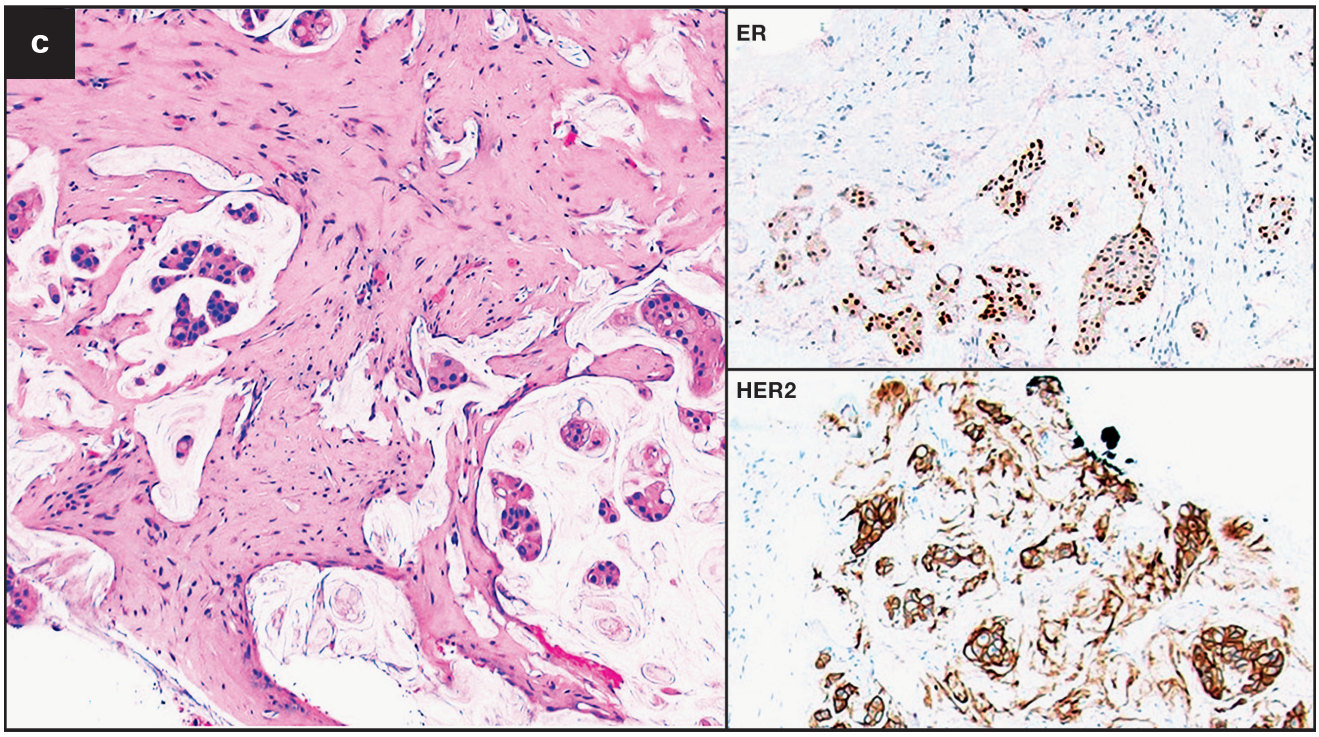

Figure 1 Continued

To our knowledge, this study presents the largest cohorts to date to describe the clinicopathologic, somatic mutational, and transcriptomic (MammaPrint/BluePrint) profiles of low-grade HER2+ invasive breast carcinomas. Most tumors identified in our cohorts displayed hormone receptor positivity. Some investigators suggest that "triple-positive" breast tumors should receive their own subtype designation, owing to the complex interactions between ER and HER2 pathways.[37-40] Higher ER expression has been associated with reduced benefit from trastuzumab in patients with early-stage breast cancer.[38] Moreover, in the metastatic setting, patients with HER2-overexpressing tumors typically undergo chemotherapy coupled with HER2-directed treatment, which has been shown to achieve worse outcomes for patients whose tumors express ER.[39] Given the significant toxicity that patients can experience from chemotherapy, recognizing a subgroup of HER2+ patients for whom therapy could potentially be de-escalated would provide significant benefit. The low-grade phenotype represented by our cohorts may provide one avenue of exploration given their distinct clinicopathologic and gene expression profiles compared with other classic higher-grade HER2+ tumor counterparts.

Our smaller case series and the larger FLEX subset of patients with low-grade HER2+ tumors reveal similar profiles of early-stage luminal tumors, but both showed an apparently higher occurrence (46%-60%) of MammaPrint high-risk categorization than in the background population that is HER2 negative in the database. This suggests that low-grade HER2+ breast tumors may potentially be a group of breast cancer with intermediate risk, meriting more aggressive treatment than the more common luminal A and/or MammaPrint low-risk HER2-negative tumor but perhaps a different clinical approach from the standard aggressive regimens offered to patients with classic high-grade HER2+ breast cancer.

These observations raise questions on whether a more tailored approach may achieve favorable outcomes with less toxicity. Such de-escalation strategies have been tested previously with success, as in the APT trial, which demonstrated that patients with small, node-negative HER2+ tumors can receive less toxic treatment and still gain comparable benefit to those receiving a standard, more aggressive regimen. Recurrence rates in this population were extremely low, with 5-year disease-free survival across both treatment arms at above 97%.[8]

The low-grade HER2+ population described here may represent a category that could benefit from even further de-escalation and be spared related treatment toxicity. Focused investigation is needed to identify the pathologic and molecular features of candidate tumors for such strategies. Our small cohort may begin to capture the underlying biology of these less common tumors, opening the door to more exploration. For example, our research group recently demonstrated that ER expression on α-fluoroestradiol positrom emission tomography imaging could be robust in patients with ER+/HER2+ metastatic breast cancer, suggesting that additional stratification may already be achievable by combining pathologic data with novel imaging techniques.[41]

The proportion of cases with *PIK3CA* mutations observed in our case series appears higher than what has been seen in The Cancer Genome Atlas (TCGA), which showed *PIK3CA* mutations in ~39% of HER2-overexpressing breast cancers and also higher than what is demonstrated in cBioPortal.[42] Several studies have suggested that the presence of *PIK3CA* mutations in breast cancers with HER2

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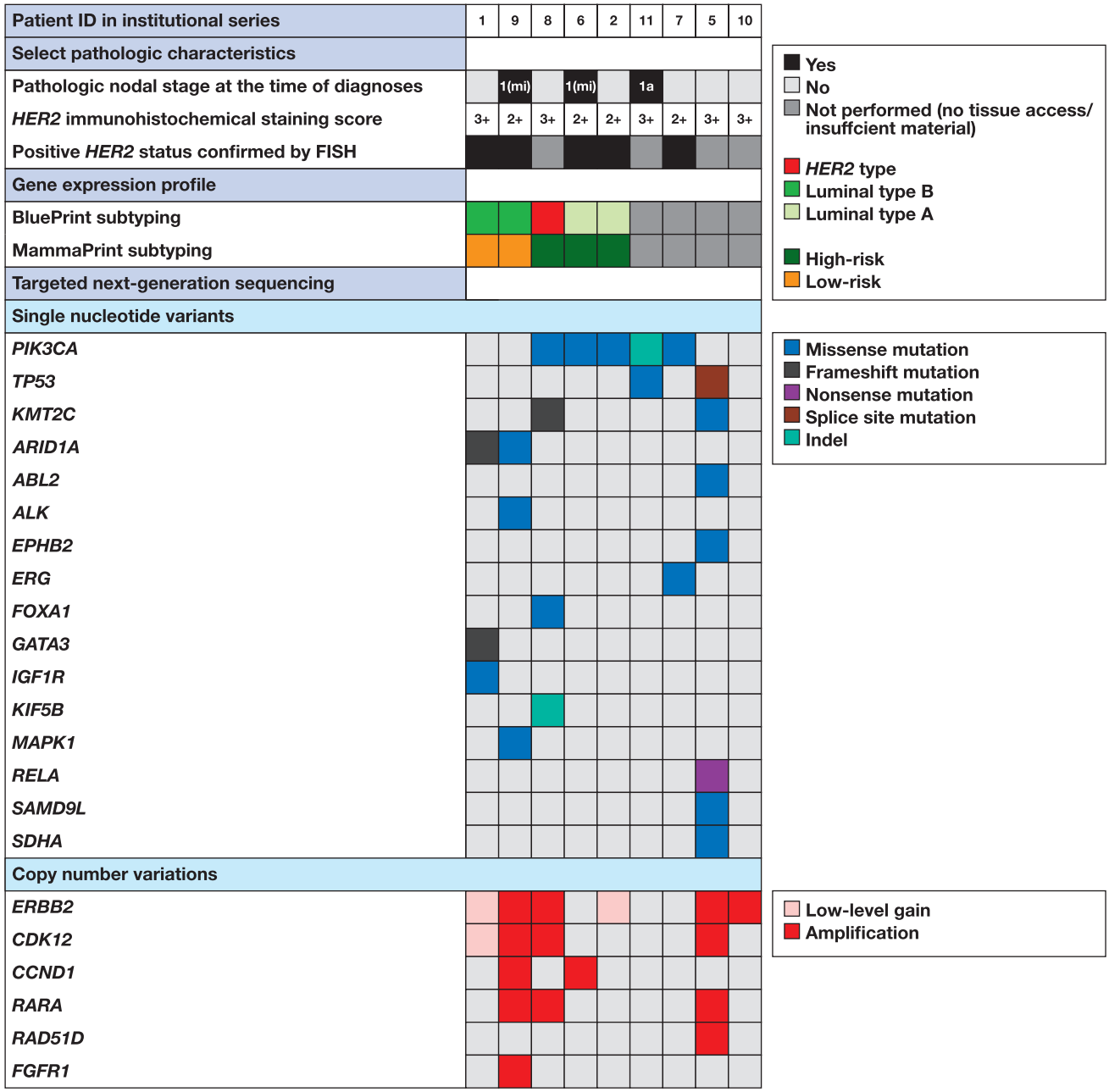

Figure 2 Somatic mutations and copy number variations detected in institutional case series. The PIK3CA SNVs were the most common mutations (5/9 tumors) detected in this series. ERBB2 CNVs were seen in about two-thirds of the cases. No significant correlation between ERBB2 amplification/gain and clinicopathologic features, including receptor status, tumor size, or nodal status, was observed. CNV indicates copy number variation; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; SNV, single nucleotide variant.

overexpression may result in decreased rates of pathologic complete response[43-45], and that the emergence of *PIK3CA* mutations may potentially be a resistance mechanism for anti-HER2 therapies.[46,47] A number of clinical trials have investigated the utilization of PI3 kinase inhibitors in HER2+ breast cancer, pointing to a potential targeted approach for these tumors.[48-50]

Delving further into the somatic mutational and CNV profiles of the tumors examined in the institutional case series, we noted that *ERBB2* CNVs were not detected in a subset of tumors evaluated by NGS. Error in calling HER2 positivity on these samples was unlikely given consensus review by 2 pathologists per standard clinical guidelines at the time of diagnoses and additional central re-review by a third independent pathologist at the time of the study. Tumor cellularity and tissue quality may be a contributing factor, but all cases and materials were re-reviewed for adequacy for sequencing by at least 2 pathologists for this study. The finding may reflect the issue of HER2+ intratumor heterogeneity known in HER2+ tumors,[51-53] given that NGS assessments were performed in this study in a bulk fashion on tumor blocks obtained from resection specimens, which may harbor subclones not sampled in

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Table 2 Pathologic and Clinical Features of Grade 1 Invasive Breast Carcinoma Enrolled in the FLEX Trial

Clinicopathologic features	N (%)[a]; Total:59
Age (years) at diagnosis, median (range)	59 (36-72)
Race	
White	51 (86.4)
African American	6 (10.2)
Unknown	2 (3.4)
Histologic subtype	
Invasive ductal carcinoma	47 (80)
Invasive lobular carcinoma	8 (14)
Invasive mucinous carcinoma	2 (3)
Others[b]	2 (3)
Hormone receptor status	
ER+	51 (86)
PR+	40 (68)
No known ER and PR status	1 (2)
Clinical T stage[c]	
cT1(a-c)	31 (52)
cT2	9 (15)
cT3	1 (2)
Unknown	18 (31)
Clinical N stage[c]	
cN0	30 (51)
cN1	6 (10)
cN2	1 (2)
Not assessed/unknown	22 (37)
MammaPrint index	
Low risk	32 (54)
High risk	27 (46)
BluePrint subtype	
Luminal A	20 (34)
Luminal B	22 (37)
HER2	16 (27)
Basal	1 (2)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.
[a]Values are presented as number (%) unless otherwise indicated.
[b]Others: Invasive tubular carcinoma (n = 1); case with missing histologic subtype information (n = 1).
[c]Pathologic T stage and N stage information was available in only a subset of cases (<50% of this cohort), and information is provided in Supplementary Table S4.

prior biopsy specimens in which HER2 status was ascertained. The observation hints at the possibility that HER2+ low-grade breast carcinomas may be subject to a higher-than-expected level of intratumor heterogeneity than what had been reported in conventional HER2+ tumors, raising again the consideration of this tumor subgroup having different biologic profiles than conventional HER2+ breast carcinomas. Another possibility is that HER2 overexpression in this tumor subgroup is enriched for other unidentified underlying mechanisms. These cases would benefit from more intensive genomic studies regarding possible intratumor heterogeneity and alternative causes of HER2 overexpression to determine if there are different underlying biologic mechanism(s) at play in these cases.

Our study has several limitations. Given the rarity of this tumor phenotype, our institutional case series had a small sample size, and only a subset had enough materials for full NGS, as well as MammaPrint and BluePrint profiling. These limited the study power to assess treatment response and long-term clinical outcomes in association with molecular and clinicopathologic profiles, and restricted further investigation of the very minor subset of larger and/or node-positive tumors within this disease group that otherwise constituted early-stage carcinomas. The FLEX study cohort, although being an independent and multicenter case collection with transcriptomic annotations, lacked detailed clinical treatment and follow-up information for correlation. While the cases derived from the FLEX trial present the largest cohort to date in literature that focused on low-grade HER2+ invasive carcinomas, the lack of access to tissue, histologic, and clinical treatment data pertaining to the FLEX trial cases precluded our ability to assess parameters other than tumor stage and nodal status that could have affected BluePrint and MammaPrint risk profiles in this tumor subgroup.

Despite these limitations, our 2 separate cohorts highlighted the consistent observations that more than half of low-grade HER2+ tumors were early-stage and triple-positive carcinomas, with also over half having a luminal molecular phenotype and a significant proportion showing a low-risk MammaPrint profile. The findings suggest distinct differences between this tumor subset and the conventional clinical profile of HER2+ tumors, and they hint at potential clinical and molecular heterogeneity related to a minor subset within this low-grade tumor group. Our findings offer the first steps toward characterizing low-grade HER2+ breast tumors, and the observations merit further investigation. Validation of findings and subset analyses in independent larger-scale cohorts with clinicopathologic and molecular annotations would benefit follow-up studies to achieve more definitive subclassification of HER2-positive tumors that are intrinsically heterogeneous.

Subjectivity in pathologic tumor grading could potentially raise reproducibility issues in defining low-grade (Nottingham grade 1) invasive breast carcinomas. The possibility of interrater variability is acknowledged. The institutional case series was developed with at least 2 pathologists agreeing on the tumor diagnosis, grade, and HER2 status at the time of pathology reports, in addition to a separate blinded central review for the institutional cases series at the time of this study. Central review of the cases identified from the FLEX trial could not be performed due to lack of access to most of the tumor slides, but the largely consistent findings observed between the single-institutional case series and the cohort identified from the multicenter FLEX trial suggest that the impact of interrater variability on tumor grading was likely not substantial in our study to bias case identification.

In summary, our findings support early-stage low-grade HER2+ breast carcinoma as a tumor subgroup with a largely "triple-positive" phenotype. More than half of evaluable tumors were of the luminal subtype by BluePrint, and a significant proportion

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Table 3 BluePrint and MammaPrint Profiles of Cohort Identified in FLEX Clinical Trial

	Select pathologic characteristics		Gene expression profiles		
Case No.	pN stage[a]	HER2 IHC score	HER2 amplification confirmed by FISH	BluePrint subtyping[b]	MammaPrint subtyping
1	pN1	3+	na	Luminal A	High risk
2	pN2a	3+	na	Luminal A	Low risk
3	pN1	3+	na	HER2 type	Low risk
4	pN1	3+	na	HER2 type	Low risk
5	pN1(mi)	3+	na	Luminal B	Low risk
6	pN1(mi)	3+	na	Luminal A	High risk
7	pN0(mol+)	2+	Yes	Luminal A	High risk
8	pN0(i+)	2+	Yes	Luminal A	High risk
9	pN0	2+	Yes	Luminal B	High risk
10	pN0	3+	Yes	Luminal B	High risk
11	pN0	2+	Yes	Luminal A	High risk
12	pN0	2+	Yes	Luminal A	High risk
13	pN0	3+	na	Luminal A	High risk
14	pN0	2+	Yes	Luminal A	High risk
15	unknown	2+	Yes	Luminal A	High risk
16	unknown	2+	Yes	Luminal A	High risk
17	unknown	3+	na	Luminal A	High risk
18	unknown	3+	na	Luminal A	High risk
19	unknown	2+	Yes	Luminal A	High risk
20	unknown	2+	Yes	Luminal A	High risk
21	unknown	2+	Yes	Luminal A	High risk
22	unknown	2+	Yes	HER2 type	High risk
23	unknown	3+	na	HER2 type	High risk
24	pN0	2+	Yes	HER2 type	High risk
25	pN0	3+	na	HER2 type	High risk
26	pN0	3+	Yes	HER2 type	High risk
27	pN0	3+	na	HER2 type	High risk
28	pN0	3+	na	HER2 type	High risk
29	pN0	3+	na	HER2 type	High risk
30	pN0	3+	na	HER2 type	High risk
31	pN0	3+	na	Basal type	High risk
32	pN0	3+	na	Luminal A	Low risk
33	pN0	3+	na	Luminal A	Low risk
34	pN0	3+	na	Luminal A	Low risk
35	pN0	2+	Yes	Luminal A	Low risk
36	pN0	3+	na	Luminal B	Low risk
37	pN0	3+	na	Luminal B	Low risk
38	pN0	2+	Yes	Luminal B	Low risk
39	pN0	3+	na	Luminal B	Low risk
40	pN0	2+	Yes	Luminal B	Low risk
41	pN0	2+	Yes	Luminal B	Low risk
42	pN0	3+	na	Luminal B	Low risk
43	Unknown	2+	Yes	Luminal B	Low risk
44	Unknown	2+	Yes	Luminal B	Low risk
45	Unknown	2+	Yes	Luminal B	Low risk
46	Unknown	3+	na	Luminal B	Low risk
47	Unknown	2+	Yes	Luminal B	Low risk
48	Unknown	2+	Yes	Luminal B	Low risk

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Table 3. Continued

Case No.	Select pathologic characteristics		Gene expression profiles		
	pN stage[a]	HER2 IHC score	HER2 amplification confirmed by FISH	BluePrint subtyping[b]	MammaPrint subtyping
49	Unknown	3+	na	Luminal B	Low risk
50	Unknown	3+	na	Luminal B	Low risk
51	Unknown	3+	na	Luminal B	Low risk
52	Unknown	2+	Yes	Luminal B	Low risk
53	Unknown	2+	Yes	Luminal B	Low risk
54	Unknown	3+	na	Luminal B	Low risk
55	Unknown	3+	na	HER2 type	Low risk
56	Unknown	3+	na	HER2 type	Low risk
57	Unknown	2+	Yes	HER2 type	Low risk
58	Unknown	3+	na	HER2 type	Low risk
59	Unknown	3+	na	HER2 type	Low risk

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemical; na, not applicable/not performed.
[a]Pathologic nodal stage at the time of resection: pN0: no regional lymph node metastasis identified histologically; pN0(i+): isolated tumor cells (ITCs)—malignant cells <0.2 mm and ≤200 cells; pN0(mol+), ITCs in a lymph node only detectable using molecular tests; pN1mi: micrometastases (malignant cells measuring 0.2-2.0 mm, or >200 cells); pN1, metastases in 1 to 3 axillary lymph nodes (at least one >2.0 mm); pN2a, metastases in 4 to 9 axillary lymph nodes.
[b]Luminal B and luminal A were the most common molecular subtypes, with close to half being classified as low risk on MammaPrint assay.

were classified as low-risk by the MammaPrint assay, suggesting a less aggressive phenotype than conventional HER2+ tumors. Most of these early-stage low-grade HER2+ tumors were treated with HER2-targeted therapy in our case series, and none have recurred during follow-up. Further studies are warranted to examine the disease pathway of these tumors and to define whether a more tailored therapeutic approach and de-escalation can be considered for some of these patients to reduce treatment-related toxicity while maintaining similar favorable clinical outcomes.

Supplementary material

Supplementary material is available at *American Journal of Clinical Pathology* online.

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Conflicts of interest

None declared.

Data availability

The datasets analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was performed with the approval of the Institutional Review Board of the University of Washington in accordance with the Declaration of Helsinki.

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