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Supplemental Appendix for:
Intratumoral Heterogeneity in Breast Cancer – Comparison of Primary and Metastatic Breast Cancers
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Appendix S1. Supplemental Methods.

Immunohistochemistry (IHC) and IHC scoring criteria

FFPE sections for IHC were cut at 4 microns on a rotary microtome. IHC staining at consecutive sections for ER, PR, Ki67, and HER2 was performed on an automated immunostainers (Ventana Discovery or Leica BondMaX) following deparaffinization, rehydration and antigen retrieval. All sections were visualized with diaminobenzidine and counterstained in hematoxylin.

Table Antibodies used for IHC analysis

Markers	Company	Clone	Dilution	Antigen retrieval	Incubation condition (min,°C)	Assessme
ER	Neomarkers	SP1	Pre-diluted	EDTA pH8	32,37	N
PR	Ventana	IE2	Pre-diluted	EDTA pH8	32,37	N
Ki67	Ventana	41912	Pre-diluted	EDTA pH8	32,37	N
HER2	Ventana	4B5	Pre-diluted	EDTA pH8	16,37	M

'N': nuclear; 'C': cytoplasmic; 'M': membraneous

Digital images were captured using the Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) under 20x objective magnification (0.5 µm resolution). Two to ten (1.5mm) regions were selected in different areas in each slides from primary or metastatic tumors depending on tumor size. Each whole slides and selected regions were scored for the intensity of staining in the nucleus, cytoplasm or membrane according to different antibodies by two of the authors blinded to the clinical information and the staining results of other markers. For ER, PR and Ki67, the reactivity assessed was nuclear. For HER2, the reactivity assessed was membranous. The staining intensity was graded from 0 to 3. The percentage of positively stained cells with each staining intensity was recorded for heterogeneity index calculation. For ER, PR, and Ki67, they were classified into four categories based on percentage cells with moderate to strong staining (score 0 to 3: 0%, 1-20%, 21-50% and >50% respectively). HER2 immunostaining was graded into 4 classes according to the ASCO guideline. Any discrepancies were resolved by reviewing at a multi-head microscope and a consensus reached. For assessment of cellular heterogeneity, percentage of cells stained for each intensity grade was recorded for individual regions. The tumors were also classified into the 4 different molecular subtypes using IHC staining score as surrogate as follows:
Luminal A: ER (score 1-3), PR (score 2-3), HER2 (IHC 0+ to 2+) and Ki67 (score 0-1);
Luminal B: ER (score 1-3), PR (score 0-1) or HER2 (IHC 3+) or Ki67 (score 2-3);
HER2 over-expressed: ER (score 0), PR (score 0), or HER2 (IHC 3+);
Triple negative: ER (score 0), PR (score 0), or HER2 (IHC 0+ to 2+).

Statistical analysis for heterogeneity score

Heterogeneity was measured in two levels, cellular level and regional level. Cellular heterogeneity is the variability of cells within a nest of tumor. Regional heterogeneity is the variability of regional expression across an entire tumor. Rao's quadratic entropy (QE) which takes into the taxonomic distance between species was used to evaluate the heterogeneity

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in staining score while Shannon's index estimating diversity for "separate" species was used for evaluation regional heterogeneity in breast cancer subtype for different tumor sites. The average value for cellular heterogeneity (QE_{cell}) obtained from different regions within a tumor site was used for the comparison. A pairwise comparison using Wilcoxon test was used to evaluate the differences in staining and heterogeneity score between primary tumor and metastatic sites. The heterogeneity scores at each tumor site between different biomarkers were compared using non-parametric Kruskal Wallis test. To examine whether the extent of heterogeneity at regional level related to that at cellular level, spearman's correlation was used to examine the relationship between QE_{reg} and average QE_{cell} at each tumor sites for each biomarker. The findings were analyzed using the statistical software SPSS for Windows, Version 21. Statistical significance was established at $p<0.05$.