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## EDITORIAL

## Making Breast Cancer Molecular Subtypes Robust?

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Since first being described in 2000 (1), gene expression-based molecular subtypes have become an integral part of both basic and translational breast cancer research. Moreover, incremental research has found the subtypes to provide sufficient information on prognosis and systemic treatment selection to merit inclusion in international guidelines for breast cancer treatment (2). Although various subtype classifiers have been developed (3–6), the different classifiers generally agree on a taxonomy of breast cancer typically encompassing four subtypes (luminal A, luminal B, HER2-enriched, and basal-like).

However, there has been considerable controversy regarding the ability of different molecular subtyping methods to robustly assign the same molecular subtype to a specific sample (7). First, it is not clear that molecular subtypes can be robustly defined in general (8), and the definition of the subtypes has changed over time from the initial classifier by Sorlie et al. (3) to the PAM50 classifier (5) often used today. Although each version could be considered an improvement (9), development is still ongoing, exemplified by the recent revision of the PAM50 classifier in the commercial ProSigna assay (NanoString Technologies). Second, current classifiers cannot robustly assign subtype to individual samples in a truly independent manner. Briefly, to put gene expression measurements of a sample on a scale that is required by the classifier methods, gene expression data for each individual test tumor have to be centered against a large and heterogeneous reference tumor set (10). This gene-centering step makes the assignment of subtype to a tumor highly dependent on the composition of other tumors in a reference set (11).

The lack of robustness in assigning subtypes is not limited to gene expression assays like PAM50 (5) and SCMGENE (6), but is also observed in surrogate classifications based on clinico-pathological factors. For instance, there is considerable variation between laboratories in Ki-67 immunohistochemical measurement, which is used as a proliferation surrogate (2). Nevertheless, it has been argued, pending improved standardization, that local laboratory experience of Ki-67 levels could be used to stratify patients into Ki-67 “high” and “low”

groups, classifications that may influence therapy decision (2). This argument parallels the de facto situation for gene expression-based molecular subtyping, where slightly different implementations are used in different studies. Although these differences may have limited impact on broad characterizations of the subtypes in terms of, for example, patient outcome, it is not satisfactory that classifications of individual patients are not robust. Thus, there is an imminent need for robust, standardized methods for the assignment of breast cancer molecular subtype to individual tumors independent of data from other tumors.

The article published in this issue of the Journal by Paquet and Hallett describes an approach to making gene expression-based tumor subtyping of individual tumors robust and truly independent (12). At the heart of designing a true single sample predictor lies eliminating the gene-centering step. To this aim, the investigators developed a subtyping approach, AIMS, that relates raw expression measurements of subtype-specific genes to the levels of other genes within each tumor sample, instead of using a gene-centering step. To prove the concept, the investigators applied AIMS to mimic the PAM50 subtyping scheme and conducted a number of rigorous evaluations demonstrating that AIMS is a robust subtype classifier for new samples. AIMS is based on a collection of binary rules, for example, samples with higher raw expression levels for *FOXC1* than *ESR1* are more likely to be classified as basal-like. The stability of subtyping was evaluated on tumor sets with different proportions of specific subtypes. In these analyses, conventional subtyping tools were found to be highly unstable when single subtypes were excluded from the test set. For instance, 20% to 30% of samples changed subtype when PAM50 was applied to test sets with Luminal A tumors excluded, whereas AIMS was fully independent of the composition of the test set. The absolute nature of AIMS is of profound value when subtyping is applied to studies focusing on restricted cohorts of patients, for example, when characterizing responders to different regimes of therapy in a cohort of only ER-positive/HER2-negative cases or in neoadjuvant trials. In the first

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scenario, the investigators showed that conventional subtyping assigns the ER-positive/HER2-negative cases to all subtypes in proportions inherent to the method (for example, approximately 20% were incorrectly classified as basal-like by PAM50). In neoadjuvant trials, robust subtyping is even more critical, as such trials typically are small and often underpowered for clinically relevant endpoints, making them sensitive to inflated chance findings based on unstable conventional subtyping. The investigators' analyses also demonstrate that AIMS is consistently more stable than PAM50 when subtyping is applied to samples with missing measurements for some predictor genes, or to samples with a large proportion of non-neoplastic cells.

Subtyping approaches based on raw expression measurements also have limitations. One concern is that such methods may be highly sensitive to changes in the technology used to quantify expression levels. Since AIMS depends on the relative relations between expression levels of different genes in a sample, differences in measurement bias for a gene could affect its expression level relative to other genes in the sample, potentially causing instability in the subtyping. To explore this concern, the investigators analyzed samples profiled by both microarrays and RNA-sequencing and found that AIMS consistently assigns subtypes to samples across multiple platforms. Presumably, this stability is because of the construction of AIMS using relationships between genes having large differences in expression levels between subtypes, thereby making the relationships less sensitive to measurement bias.

The development of methods that can robustly assign subtype to individual tumors, based on absolute measurements, opens the door to address the issues with different subtype classification schemes in breast cancer. It may serve as a first step to standardize methodologies. The separation of luminal tumors into A and B is based on expression of proliferation-related genes, but it is likely that proliferation in luminal tumors is a continuum (11). An absolute classifier is critical to optimize cutpoints in proliferation that reproducibly separates luminal tumors with respect to response to systemic treatments, and to standardize risk of recurrence scores that are based on subtype assignments. Eventually, such studies may return more stringent subtyping tools even better suited for incorporation into clinical trials and routine clinical practice.

## Notes

The authors have no conflicts of interest to declare.

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