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Molecular sub-typing of invasive bladder cancer: time to divide and rule?

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Therapeutic decisions for muscle-invasive bladder cancer (MIBC) are largely based on histopathologic characteristics. In this issue of *Cancer Cell*, Choi and colleagues report three molecular subtypes of MIBC with potential to guide prognosis, patient stratification, and treatment.

Despite increased molecular understanding, there has not been a significant advance in the treatment of muscle-invasive bladder cancer (MIBC) in recent years. These tumors frequently become metastatic, which is associated with very poor outcome (median survival: approximately 1 year). The standard of care for patients with localized MIBC is radical cystectomy preceded by cisplatin-based chemotherapy (neoadjuvant chemotherapy; NAC), which aims to abolish undetected metastases (Sternberg et al., 2013). However, responses to NAC are recorded in only 40-60% of cases, and metastatic disease is frequently detected at the time of surgery. Cisplatin-containing combination chemotherapy is also the mainstay of treatment in the metastatic setting, where both *de novo* and acquired resistance present major problems.

What is needed to improve this dismal situation? Two issues currently under investigation may have impact. First, predicting response to chemotherapy may allow selection for NAC responders, avoiding unnecessary toxicity in patients unlikely to respond. Expression signatures associated with sensitivity have been derived from tumors (Takata et al., 2005) and tumor cell lines with known response (Lee et al., 2007), and validation in relevant clinical trials is eagerly awaited. Second, identification of therapeutic targets and development of personalized treatment strategies is urgently needed. While several druggable targets are present in MIBC, e.g. HER2, EGFR, FGFR1 and FGFR3, adequate biological understanding has been lacking and few trials with targeted agents have been initiated.

The significant heterogeneity in MIBC clinical behavior suggests more than one disease entity. This is already supported by genome-wide expression and DNA-based analyses, which report distinct molecular subtypes. Encouragingly, the signatures of some of these subtypes show prognostic value (Sjodahl et al., 2012). However, this information has yet to have impact on clinical management and predictive information has not been derived from such data.

In this issue of *Cancer Cell*, Choi et al. (2014) used whole-genome gene expression profiling to identify molecular subtypes of MIBC and provided evidence for their biological basis and clinical significance. Their findings have exciting implications for the clinical management of MIBC as they include not only prognostic information but also suggestions for subtype-directed targeted therapy and potential to predict response to cisplatin-based chemotherapy.

Choi et al. (2014) initially profiled 73 fresh-frozen MIBCs and, using unsupervised hierarchical cluster analysis, revealed 3 major clusters which they term basal, luminal and “p53-like”. Basal and luminal

designations reflect enrichment for markers previously reported in basal and luminal-type breast cancers, respectively (Sorlie, 2004). Basal MIBCs characteristically expressed CD44, KRT5, KRT6, KRT14, and CDH3, and lacked KRT20 expression. Such differential expression of cytokeratins 5 and 20 is related to urothelial differentiation states, the least differentiated of which characterises cells in the basal layer of the normal urothelium ($KRT14^{+}KRT5^{+}KRT20^{-}$)(Volkmer et al., 2012) and bladder cancer cells with stem cell-like features (Chan et al., 2009). The basal subtype also expressed "mesenchymal" markers (TWIST1/2, SNAI2, ZEB2, and VIM), low miR-200, which is implicated in mesenchymal marker regulation, and elevated levels of EGFR and its ligands. This subtype was enriched for tumors with sarcomatoid and squamous features, exhibited more aggressive disease at presentation, and had shorter disease-specific and overall survival. The luminal subtype expressed luminal breast cancer biomarkers (CD24, FOXA1, GATA3, ERBB2, ERBB3, XBP1, and KRT20), "epithelial" biomarkers, E-cadherin, miR-200 family, and showed both expression and mutation of FGFR3. The "p53-like" subtype also expressed luminal biomarkers but was distinguished by an activated wild-type p53 gene signature.

Choi et al. (2014) then developed a classifier based on genes differentially expressed between subtypes. Using this, they were able to identify the same 3 subtypes in a local validation cohort consisting of 57 formalin-fixed paraffin-embedded (FFPE) MIBC samples and in a publically available gene expression dataset. As in the initial analysis, these validation cohorts revealed an association between basal subtype and poor survival. A molecular taxonomy for bladder cancer described by Sjödahl et al. (2012) included a subset of squamous cell carcinoma-like (SCCL) MIBCs that expressed basal keratins and showed poor prognosis. Recently, these authors have suggested that the term "basal" is more appropriate for this group (Sjodahl et al., 2013). Choi et al. (2014) applied their classifier to this dataset and confirmed that the SCCL group corresponded to their basal subtype. This analysis also revealed that the luminal and "p53-like" subtypes shared features with the "urobasal A" and "infiltrated" subtypes of Sjödahl et al., respectively. To investigate the association of squamous features with the basal subtype, Choi et al. (2014) interrogated data from a previous study that reported a "squamous cluster" with KRT5 and KRT14 expression and found a subset of tumors enriched with squamous features that expressed the basal signature. They also examined expression of cytokeratins that were characteristic of basal (CK5/6) or luminal (CK20) tumors in a tissue microarray derived from 332 MIBCs and found inverse correlation of these markers and association of high CK5/6 with squamous features.

To investigate the biology of the subtypes, a bioinformatics approach was used to seek upstream regulators of basal and luminal gene expression. This implicated transcription factors reportedly active in the basal/stem cell compartment of the normal urothelium (Stat-3, NFkB, Hif1, and p63) (Ho et al., 2012) as potential regulators in basal MIBCs. Activation of PPARγ and estrogen receptor pathways was identified in luminal MIBC. p63 and PPARγ were then examined in tumor-derived cell lines. p63 knockdown yielded decreased basal marker expression and increased PPAR pathway activation, while treatment with a PPARγ-selective agonist activated PPAR and other luminal pathways and decreased basal transcription factor expression, clearly demonstrating the role of these opposing pathways in control

of these two major phenotypes. Delineation of these regulators holds promise for improved therapeutic options through the use of pathway-specific targeted agents.

Finally, Choi et al. (2014) noted that all of the NAC-treated "p53-like" MIBCs in their discovery set (n=7) showed resistance. Strikingly, this pattern was confirmed in an expanded local NAC cohort and 23 archival tumors from a Phase III chemotherapy trial. The "p53-like" signature was also identified in cell lines, where it associated with resistance to cisplatin-induced apoptosis. It should be noted however, that while the "p53-like" gene expression signature is characteristic of an activated wild-type p53 gene signature, it was not related to *TP53* mutation status, an observation that is reflected in the long-debated prognostic and predictive value of mutant p53 in bladder cancer. To confirm the link between "p53-like" and chemoresistance, the authors compared gene expression profiles from a cohort of matched pre- and post-treatment samples from a prospective Phase II clinical trial of NAC. The lowest response rate was observed in tumors initially classified as "p53-like". A particularly pleasing feature of this study is that FFPE samples were successfully used for validation studies, providing promise for routine clinical application. Intriguingly, enrichment of a p53-like signature in post-treatment tissues from patients whose pre-treatment tissue did not express this signature was observed. Whether this reflects global expression changes or selection of resistant subclones remains to be demonstrated. As not all non-responding tumors could be identified by this profile, including those exhibiting basal and luminal signatures, it is clear that other biomarkers of resistance remain to be elucidated, as does the possible contribution of intratumor heterogeneity. Nevertheless, these findings represent an important step towards the goal of more rational selection of patients for chemotherapy.

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