Metastasis Unleashed: Hyposialylation Empowers Chemo-Evasive Circulating Tumor Cell Clusters in Breast Cancer

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Therapy resistance is frequently observed in cancer patients with distant metastases and effective management of metastatic disease remains challenging. Unraveling the cellular mechanisms and molecular targets fueling metastatic spread is crucial for advancing cancer therapies. In a recent issue of *Cancer Discovery*, Dashzeveg and colleagues revealed that loss of terminal sialylation in glycoproteins within circulating tumor cell clusters is a dynamic process that contributes to cellular dormancy, facilitates evasion of chemotherapy, and enhances metastatic seeding. Furthermore, the study identifies the glycoprotein podocalyxin (PODXL) as a potential target for counteracting the metastasis of quiescent tumor cells associated with paclitaxel treatment in triple-negative breast cancer.

Among breast cancer subtypes, triple-negative breast cancer (TNBC) is regarded as one of the most aggressive forms of breast cancer, comprising 10% to 20% of all cases. Approximately 40% of patients with TNBC develop lung metastases and have a poor outcome, with a median survival of 19 months (1). TNBCs are distinguished by the absence of expression of the estrogen and progesterone receptors as well as the lack of HER2 (*ERBB2*) gene amplification, typically present in other types of breast cancer, making them poorly responsive to targeted agents. Standard chemotherapeutic treatments remain the standard of care, yet are rather inefficient. For instance, only about half of patients with TNBC respond to chemotherapy such as the microtubule inhibitor paclitaxel or Nab-paclitaxel (PAX; ref. 2). The prognosis of TNBC relies not only on the early response to chemotherapy but also on the development of drug-tolerant, incurable metastases. It is now well established that circulating tumor cells (CTC), the pioneers of metastasis, circulate in the bloodstream as either single cells or multicellular clusters, with clusters exhibiting a greater metastatic potential compared with their single-cell counterparts (3). Of note, the impact of chemotherapy on CTC dynamics remains poorly understood.

In a recent study published in *Cancer Discovery*, Dashzeveg and colleagues (4) provide mechanistic insight into CTC dynamics in response to PAX. Using patient blood samples, various established human/mouse TNBC cancer models, patient-derived xenografts, as well as sophisticated glycan and glycoproteomic analysis, the authors investigated the role of glycosylation in CTC aggregation and cluster formation. Glycosylation, facilitated by glycosyltransferases, is a prevalent posttranslational protein modification that involves the addition of sugar residues to over 50% of proteins. The glycosylation of cell surface proteins undergoes dynamic alterations and is closely linked to the progression of cancer (5). Through the modulation of glycosylation patterns, cell surface polarity and protein binding affinity are finely tuned to regulate cell–cell and protein–protein interactions.

The authors first performed a longitudinal analysis of CTCs in patients with stage III–IV breast cancer, including TNBC and other subtypes. Blood samples were collected at baseline and after a 3-month period to evaluate CTC-relevant events in response to chemotherapeutic interventions. In line with the literature (6), the presence of CTC clusters at the baseline is correlated with an unfavourable prognosis. Post-chemotherapy, a significant rise in CTC cluster, but not single CTCs, was observed, suggesting a role of CTC clusters in chemo-evasion.

The authors then investigated the potential impact of cell surface glycosylation on the formation of CTC clusters. Glycan profiling was conducted by employing a range of fluorescently labeled lectins that selectively bind to specific sugar residues, allowing their quantitative detection using flow cytometry alongside the FDA-approved CellSearch platform for CTC enumeration. Of note, *Sambucus nigra* (SNA) lectin recognizes α2,6-sialic acid (SA). This approach determined a specific loss of α2,6-sialylation in clustered CTCs, in comparison with single CTCs, that was associated with PAX treatment both in patients and spontaneous metastatic patient-derived xenograft (PDX) models.

The authors next studied the effects of sialylation on human and mouse tumor cell clustering by gene modulation of β-galactoside α2,6-sialyltransferase 1 (*ST6GAL1*). This gene encodes for a type II transmembrane protein that catalyzes the addition α2,6-SA onto terminal glycans on glycoproteins (7). CRISPR-Cas9–mediated *ST6GAL1* depletion (ST6KO) and rescue overexpression (ST6OE) experiments revealed that CTC cluster formation, based on surrogate *in vitro* aggregation assays, is promoted by loss of α2,6-SA or *ST6GAL1*. Moreover, *ex vivo* PAX treatment led to decreased α2,6-SA in a dose-dependent manner, and ST6KO resulted in resistance to PAX treatments, suggesting that α2,6-SA and *ST6GAL1* deficiency are associated with evasion to PAX treatment. Assessment of the proliferative status and cell-cycle distribution in CTCs isolated from patients and PAX-treated animals revealed an association of CTCs clusters with low α2,6-SA levels and relative quiescence under PAX treatment pressure.

Differential gene expression analysis upon bulk RNA sequencing of wild-type (WT) and ST6KO tumor cells identified regulatory pathways in ST6KO cells, including suppression of cell cycle, and upregulation of adhesion and inflammatory responses, among others. Competitive

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proliferation assays with mixed WT and ST6KO cells over time and under PAX treatment further implicated that loss of *ST6GAL1* induces cellular quiescence and facilitates chemo-evasion.

The authors next compared α2,6-sialylation in primary tumor cells, CTCs, and disseminated tumor cells (DTC) in the lungs from PDXs and established xenograft mouse models using multiple approaches, including IHC, flow cytometry, and single-cell RNA sequencing. CTC clusters displayed lower expression of *ST6GAL1* as well as lower levels of α2,6-SA compared with single CTCs, primary tumor, and DTCs. Intriguingly, sorted SNA-low cells transitioned into SNA-high DTCs in the lungs, as observed at 24 hours and 9 days upon intravenous injection into mice. These findings suggest a transient loss of α2,6-SA in CTCs and dynamic gaining of sialylation in DTCs after seeding, which prompted the authors to further explore the regulatory roles of ST6GAL1 in TNBC metastasis.

Genetic modulation of *ST6GAL1* in various orthotopic and intravenous PDX models demonstrated that *ST6GAL1* depletion promotes CTC clustering and metastatic seeding, even though ST6KO PDX tumors displayed delayed initiation and slower growth in comparison with WT PDXs. *ST6GAL1* overexpression and upregulated levels of α2,6-SA, on the other hand, enhanced tumor growth, but impaired spontaneous lung metastasis. Loss of *ST6GAL1* also facilitated transendothelial migration *in vitro*.

Finally, glycoproteomic analysis of SNA-precipitated proteins of ST6WT and ST6KO membrane fractions of whole cell lysates was conducted to determine glycoprotein targets of ST6GAL1 that might impact tumor cell clustering and metastatic seeding. Among proteins already known to facilitate CTC cluster formation, such as CD44, EGFR, ICAM1, and DSG2, the authors identified new potential targets, including adhesion molecules PODXL, ECE1, ALCAM1, immune regulator HLA-A2, and several exosomal proteins. Dashzeveg and colleagues directed their attention to the cell adhesion machinery, speculating that newly identified adhesion molecules might contribute to CTC clustering and metastatic seeding. They hypothesized that the levels or activity of these adhesion molecules might be regulated by ST6GAL1-mediated sialyation. siRNA-mediated knockdown of adhesion molecules, particularly *PODXL*, partially or completely reversed the *in vitro* tumor cell aggregation induced by the loss *ST6GAL1*. PODXL protein expression was also higher in CTC clusters than in single CTCs isolated from patients' blood. Moreover, *PODXL* knockdown or administration of anti-PODXL neutralizing antibody strongly inhibited *in vivo* tumor cell clustering and effectively blocked metastatic seeding after intravenous injection of both ST6WT and ST6KO cells into mice. Of note, the effectiveness of anti-PODXL in treating metastatic PDX models resistant to PAX chemotherapy was evaluated. In an experimental metastasis setup, pretreatment of dissociated ST6WT PDX tumor cells with PAX and subsequent inoculation into mice showed that anti-PODXL treatment significantly inhibited PAX-promoted metastatic seeding to the lungs. Furthermore, anti-PODXL administration concurrent to PAX treatment in spontaneous metastatic PDX models effectively blocked lung colonization with a dramatic reduction in CTC clusters.

Overall, this study highlights the potential of PODXL as a target for mitigating breast cancer metastasis in response to PAX therapy. Dashzeveg and colleagues provide insights into sialylation in the context of CTC clustering and metastatic seeding. Despite the evidence presented highlighting innovative targeting approaches to inhibit cluster-mediated seeding, there remain a few lingering unresolved questions. The authors examined glycosylation in homotypic CTC clusters, but the sialylation levels in heterotypic clusters involving both tumor and nontumor cells, such as neutrophils (8), remain unexplored. Similarly, factors regulating the dynamics of sialylation in tumor cells are not well understood, suggesting that future research efforts should also focus on this aspect. Another important characteristic to take into consideration is the diurnal rhythmicity of CTC intravasation rates, previously demonstrated in patients with breast cancer and mouse models (9). Bearing these findings in mind, it would be intriguing to determine if sialylation is also influenced by systemic changes, such as those governed by the circadian clock. Last but not least, it will be interesting to explore if this phenomenon is TNBC-specific, or if it extends to additional cancer types. Continued investigation into these questions holds promise for the development of new targeted therapies for aggressive cancers.

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