Tumor microenvironment of metastasis (TMEM): a novel tissue-based assay for metastatic risk in breast cancer

“Observations from intravital imaging in animal models suggest specific relationships between tumor cells and their microenvironment that are crucial to establishing the level of metastatic risk.”

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Over the past 50 years, while death rates from cardiac and cerebrovascular disease have dramatically declined in the USA, cancer-related death rates have remained almost constant. Cancer now ranks as a close second to heart disease as a leading cause of death, with breast cancer being the most prevalent tumor in women. The current lifetime probability of a woman in the USA developing breast cancer is one in eight, with over 40,000 breast cancer deaths occurring last year in the USA alone. The principal cause of death in breast cancer patients is metastatic disease, and the treatment for patients considered at risk of metastasis is chemotherapy. However, determination of metastatic risk has remained imprecise. Oncologists initiate therapies based on prognostic factors that have evolved through correlation with clinical outcome over time. In this editorial, we review the origins of traditional prognostic indicators and suggest an alternative approach to the assessment of metastatic risk, based on direct observations of tumor cell intravasation and hematogenous spread in animal models.

The traditional prognostic factors in breast cancer are embedded in the tumor, node, metastasis (TNM) staging that forms the basis of every surgical pathology report on a breast cancer case [1]. They reflect characteristics of the tumor and its ability to spread, including the size and differentiation of the tumor, axillary lymph-node status and presence or absence of lymphovascular invasion. The assumption is that a bigger, more poorly differentiated tumor that has already spread to the lymph nodes will be more likely to have invaded blood vessels and

gained access to the systemic circulation. These assumptions are rooted in the linear progression model of metastasis, which asserts that tumor cells undergo successive rounds of mutation and selection, resulting in a stepwise accumulation of genetic and epigenetic alterations [2]. The end point is a final clonal expansion that is fully malignant and linked to tumor size. In this model of metastasis, it is only during the late stages of tumorigenesis that rare cells acquire the ability to metastasize. In the last two decades, this list of prognostic factors has been expanded to include specific biological properties of the tumor cells, such as hormone receptors and HER-2 neu status. However, the starting point for each of these factors remains the tumor itself. In more recent years, other models of metastasis have emerged that consider more than the characteristics of the tumor cells. One of these, the ‘tumor microenvironment invasion model’, includes a role for the tumor microenvironment and has benefited from observations obtained by intravital imaging [3].

Intravital imaging provides the opportunity to directly observe interactions between tumor cells and the surrounding microenvironment. In rodent models, one can generate spontaneous malignant breast tumors through mammary epithelial-restricted expression of the *PyMT* oncogene, driven by a mouse mammary tumor virus (MMTV) promoter. These animals can also be constructed so that different cell types, including tumor cells, endothelial cells or macrophages, constitutively express green fluorescent protein (GFP), making them visible in the multiphoton microscope [4]. Double cell imaging is

also possible. For example, if the interaction of interest is between tumor cells and macrophages, the tumor cells can be labeled with GFP, and macrophages in the same animal can be visualized after tail vein injection of Texas red dextran, which the macrophages will phagocytose. A similar approach can be utilized to look at GFP-labeled endothelial cells and Texas red dextran-labeled macrophages. Finally, either GFP-labeled tumor cells or GFP-labeled macrophages can be visualized in relation to a vascular space that has been injected with Texas red dextran. Thus, it is possible to look at the relationships and interactions between tumor cells, endothelial cells and macrophages.

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Using these techniques, intravital multiphoton imaging of rat and mouse mammary tumors has demonstrated that invasive carcinoma cells polarize, move towards and invade blood vessels. Furthermore, this is a process of relay chemotaxis, in which both tumor cells and macrophages participate in a paracrine loop involving macrophage-derived EGF and carcinoma cell-derived colony-stimulating factor-1 [5]. Time-lapse imaging of mice with GFP-expressing tumor cells and macrophages that have ingested Texas red dextran demonstrates that tumor cells migrate towards vessel-associated macrophages and intravasate only where perivascular macrophages are present. If the same experiment is conducted in a macrophage-deficient *Csf1^{op}/Csf1^{op}/PyMT* mouse, not only is there a sixfold decrease in the density of perivascular macrophages, there is a marked 16-fold decrease in the number of viable circulating tumor cells and, hence, the number of lung metastases [4].

This motile subpopulation of tumor cells can be collected and the gene-expression profile of these cells has been called the ‘invasion signature’ [6,7]. Overall, these cells are nonproliferating, nonapoptotic and chemotactic to EGF-receptor ligands. The increased cell motility and invasion in these cells is the result of alterations in the expression of cytoskeletal regulatory proteins. A key regulatory protein in actin polymerization

that is part of the invasion signature and is upregulated in invasive tumor cells is Mena. Mena regulates cell movement by its ability to protect actin filaments from capping proteins during polymerization and is part of the minimum motility machine responsible for amoeboid cell movement [5,7]. Since both the stimulatory and inhibitory parts of these pathways are coordinately upregulated, actin polymerization occurs as a transient phenomenon. There is a loss of polarity and the cell undergoes a transition to aggressive amoeboid movement. This plasticity permits the greatest responsiveness to directional chemotactic signals [8].

These observations are consonant with a tumor microenvironment invasion model for breast cancer metastasis that incorporates a role for the microenvironment via macrophage signaling to tumor cells [9]. This microenvironment may result, in part, from stable, sequential genetic changes, but the microenvironment itself can induce transient alterations in gene expression in the tumor cells, which in turn drive marked, but transient, cell migration and intravasation. According to this model, invasion and micro-metastases can occur early and repetitively [3]. This would suggest that metastatic risk could be assessed early in tumor development.

To test this hypothesis, we asked how we might apply the observations in animal models to our surgical resection specimens of breast cancer. Intravital imaging shows tumor cells moving, with macrophage assistance, into vessels. Since immunohistochemistry provides us with a way to visualize cell types, we developed a triple immunostain whereby we can simultaneously visualize vessels (CD31), macrophages (CD68) and invasive motile tumor cells (Mena) [10]. The tumor microenvironment for metastasis (TMEM) was defined as the direct apposition of an invasive Mena-expressing tumor cell and a perivascular macrophage. Scoring a series of well, moderate and poorly differentiated tumors, we found that well-differentiated tumors had a significantly lower TMEM count than moderate and poorly differentiated tumors. This result was encouraging because, in general, well-differentiated tumors do not metastasize. In this series, we also found that macrophage density, or microvessel density alone, did not correlate with grade.

As our next step, we designed a case-control study of 30 matched pairs of metastatic and nonmetastatic cases, focusing exclusively on moderate and poorly differentiated tumors. Except for the presence or absence of

metastasis, the pairs were matched for the traditional prognostic variables: size, differentiation, lymphovascular invasion and lymph node status. A minimum follow-up time of 5 years was established and the follow-up time for the nonmetastatic member of the pair was at least as long as its metastatic counterpart. The triple immunostain was applied and TMEM scores were generated.

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The result was that the median score for the metastatic cohort was more than double the median score of the nonmetastatic group, a finding that was highly significant ($p = 0.00006$) [10]. For each ten-unit increase in TMEM, the odds of metastasis almost doubled. This estimate was robust, even after separate adjustment for tumor grade, size, lymphovascular invasion and lymph node status. The result supports the conclusion that TMEM may be a new independent prognostic factor.

In conclusion, assessment of metastatic risk continues to be problematic for the oncologist treating breast cancer. Observations from intravital imaging in animal models suggest specific relationships between tumor cells and their microenvironment that are crucial to establishing the level of metastatic risk. This relationship among cell types can be identified in human breast cancer samples and is called TMEM. Determination of TMEM score in human formalin-fixed paraffin-embedded tissue uses a novel assay that may be able to provide a reliable assessment of metastatic risk at the time of presentation with breast cancer. Better assessment of metastatic risk at the time of presentation will allow greater customization of treatment and may prevent both overtreatment and undertreatment of disease.

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