Commentary

Breast Cancer

The Matrix Is the Message

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Pathologists characterize many aspects of breast cancer specimens but rarely pay much attention to the stroma. Whereas some tumors have more stroma than others, desmoplasia is only one of many properties that vary among individual ductal carcinomas. It seems logical that the pathology assessment should identify risk factors associated with rapid tumor progression; however, the pathologist focuses on issues that are more pragmatic: the extent of tumor, classification into histologic subtypes, and identification of properties that define therapeutic intervention, such as expression of estrogen receptor (ER) or HER2/neu. In this issue of *The American Journal of Pathology*, Conklin et al¹ have demonstrated a stromal signature that has a correlation with progression. Although the potential exists for this signature to be used for prognosis, the main value of their correlation is to identify two important areas for further investigation. First, the stromal signature indicates a biologically significant process because it predicts how the tumor will behave. Defining this process is the first step in learning how to control it. Second, the stromal signature may indicate a novel mechanism of tumor progression if it is independent of other prognostic markers.

Multiphoton Microscopy

The new study is based on research from the Keely laboratory^{2–4} and others^{5–7} who used multiphoton microscopy to examine the structure of collagen fibers in tumors. Multiphoton microscopy (or two-photon microscopy) utilizes lasers that generate pulses of light with extremely high photon density in the near-infrared wavelength range of 700 to 1300 nm.^{8–11} For fluorescence imaging, the photon density is so high that near-simultaneous absorption of two photons by a fluorophore can occur, resulting in activation that is equivalent to absorption of a single photon with twice the energy. For exam-

ple, with the laser tuned to 920 nm, simultaneous absorption of two photons is equivalent to absorption of a single 460-nm photon that can thus activate green fluorescent protein, which has a broad absorption peak around 500 nm. The nonlinear dependence of this effect on the square of photon density makes multiphoton fluorescence microscopy confocal in nature. It is also more efficient than ordinary fluorescence microscopy; there is less bleaching of fluorophores out of the plane of focus and deeper penetration into tissues because of the reduced scattering of near-infrared photons.

To image unstained collagen fibers in the tumor, Conklin et al¹ used second harmonic generation, an additional but less well known advantage of multiphoton imaging. In second harmonic generation, periodic structures that are not centrosymmetric—eg, collagen fibers—can act like frequency doubling crystals. In essence, two photons of one frequency enter the fiber, and one photon with exactly half the frequency leaves it. These second harmonic signals are mostly emitted in the same direction as the excitation light, but some are scattered backward and detected by the microscope used to deliver the excitation signal. The second harmonic signal is dependent on the presence of appropriately polarized structures including collagen fibers, myosin, and even microtubules.

The second harmonic signal for collagen fibers is detectable in paraffin sections of formalin-fixed tissue. Thus Conklin et al¹ examined a standard breast cancer tissue microarray. The orientation of the collagen fibers was determined relative to the tumor cell masses to generate what the authors term the “tumor-associated collagen

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Figure 1. Stromal patterns resembling TACS-2 and TACS-3 in invasive ductal cancers with dense fibrous stroma. **A:** The TACS-2-like stroma organizes concentrically around the cancer cell groups, suggesting that the tumor cells have controlled its formation. **B:** Some tumor cells are interspersed between bundles of dense collagen; other tumor cells disrupt the bundles. Consistent with TACS-3, this pattern suggests invasion into a pre-formed stroma. Scale bars = 50 μ m.

signature” or TACS.⁴ Different TACS categories correspond to various ways that the extracellular matrix can be organized with respect to the tumor cells. Originally these categories were defined by studying mammary tumor development in mouse models.⁴ TACS-1 arises first in early tumors with increased numbers of curved, apparently relaxed collagen fibers around the tumor. TACS-2 develops as the tumor grows larger. The surrounding fibers become straight and parallel to the surface of the tumor, probably reflecting stretching of the fibers due to the expansion of the tumor. TACS-3 reflects a significant reorganization of the matrix, so that straight matrix fibers now lead directly into the tumor cell mass. In TACS-3, the fibers can act as pathways along which cells can crawl, as has been seen in multiphoton imaging of metastatic tumors.¹²

TACS-3 Score As a Marker of Survival

Thus TACS-3 is a potential marker of a highly invasive tumor, in which the reorganization of the matrix around the tumor apparently reflects a dramatic increase in the ability to spread and metastasize. The results by Conklin et al¹ provide the first retrospective test of this hypothesis using a tissue microarray of 207 breast cancer patients with median clinical follow-up of 6 years. Several different ways of scoring TACS-3 in the tumor samples were compared (detailed in the legend to Figure 2 in the article by Conklin et al¹). We will focus here on the most successful scoring method, termed Score 1. After each tissue core was divided into 14 areas, the presence of TACS-3 structures was scored separately by three pathologists. A receiver operating curve analysis determined the optimum threshold and demonstrated that detection of only one or two TACS-3 structures in a tissue core was sufficient.

In univariate analysis, TACS-3 scoring showed a significant association with both disease-specific survival and disease-free interval, each with a hazard ratio of 3. To test whether the TACS-3 scoring might provide a novel independent biomarker, the authors then performed a multivariate analysis. A wide range of markers were evaluated including tumor grade, size, patient age, estrogen receptor, progesterone receptor (PR), HER-2, and node status, among others. Within this group, TACS-3 was an independent prognostic marker for both disease-spe-

cific survival and disease-free interval, together with PR, ER, node status, and tumor size. Notably, TACS-3 did not correlate with any other markers tested in the analysis. Given these promising results, a classification and regression tree analysis was then performed for predicting 10-year disease-specific survival. Importantly, the TACS-3 score provided valuable prognostic information for patients with large ER-positive tumors. Patients with TACS-3-positive tumors showed a 40% reduction in survival compared with those who had TACS-3-negative tumors.

The TACS-3 scoring shows significant correlation with survival in an important category of breast cancer patients—those with ER-positive tumors that are >1.35 cm. The challenge is to translate this correlation to the pathology laboratory. The use of multiphoton microscopy for research studies of human cancer tissues, including freshly biopsied tissues, has been reported by a number of groups.^{13–16} Our opinion is that this practice is currently impractical for standard histopathology laboratories because of the expense of multiphoton microscopes and the amount of time required for the analysis. However, with the development of inexpensive laser sources, multiphoton microscopy may be accessible in the future.¹⁷ In considering less expensive substitutes, it is not exactly clear how TACS-3 signals detected by multiphoton microscopy correspond to standard histopathology, but there are several options for analyzing stroma. These options include selective demonstration of collagen fibers by Sirius red or reticulum stains or visualization along with cellular detail in H&E and trichrome stains.

Final Reflections

We suggest that two distinct processes exist: TACS-2 reflects stromal formation and organization controlled by the tumor cells, an orderly phase of growth and maturation that follows initial stromal penetration by tumor cells. In contrast, TACS-3 reflects invasion and disruption of preformed stroma by tumor cells (Figure 1). It therefore seems essential to define the histologic correlates of the two-photon TACS-3 detection so that the signatures can be assessed within a standard pathology work-up. The data by Conklin et al¹ also imply a correlation with *stromal* expression of syndecan-1, a cell surface receptor that links extracellular matrix and cytoskeleton. Their de-

scription is a bit confusing, because an earlier article from this group characterized *tumor cell* expression of syndecan-1.¹⁸ The localization of syndecan-1 thus requires clarification, because immunohistochemical detection of syndecan-1 could prove to be an easily detected surrogate of TACS-3.

In summary, Conklin et al¹ provide an admirable example of studies that bridge mouse cancer models and human diagnostic pathology. However, application of TACS-3 as a histologic or prognostic biomarker requires translation into parameters that fit into the routine pathology work-up. From a biological perspective, TACS-3 may represent a specific type of matrix organization controlled by tumor cells or a result of aggressive invasion. These alternatives define a valuable investigation into fundamental mechanisms of tumor progression.

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