QnAs with Sangeeta N. Bhatia

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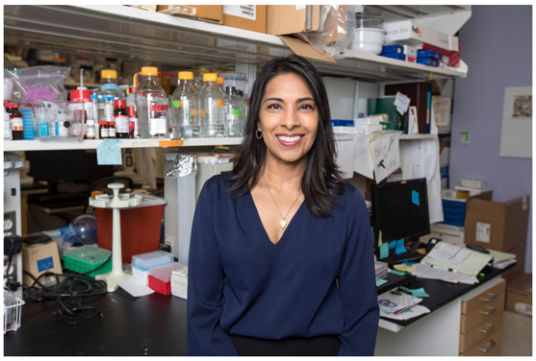
More than 2% of the human genome is claimed by genes encoding proteases, or protein-degrading enzymes. A diverse family of more than 550 members, human proteases perform a raft of functions in cells, from recycling damaged proteins to regulating signaling and growth. So it follows that proteases have been implicated in a range of human diseases, particularly cancer. Despite the appeal of proteases as therapeutic targets, few protease inhibitors have gained approval for cancer treatment, largely because the enzymes are indispensable for myriad cellular functions and targeting individual proteases is no small task. In recent years, however, proteases have become a growing focus of interest as diagnostic targets in human diseases. Moreover, preclinical findings from assorted studies suggest that, early setbacks notwithstanding, proteases can be suborned to activate or augment other approaches to combat cancer, including immunotherapies. Sangeeta N. Bhatia, a biomedical researcher at Massachusetts Institute of Technology, a Howard Hughes Medical Institute Investigator, and a member of the National Academy of Sciences, National Academy of Engineering, and National Academy of Medicine, has spent years trying to unlock the latent potential of proteases for disease diagnosis, classification, and management. At a recent Wyss Institute symposium on Next-Generation Diagnostics at Harvard Medical School, Bhatia, an associate faculty at the institute, expounded on the promise of proteases as diagnostic tools for a range of indications, including cancer. Bhatia shares some of her recent findings with PNAS.



Sangeeta N. Bhatia. Image courtesy of Howard Hughes Medical Institute/Scott Eisen.

PNAS: You have long been interested in proteases as diagnostic and therapeutic targets in cancer, and your recent work is focused on lung cancer. Can you explain the unmet medical need?

Bhatia: Lung cancer is the most common cause of cancer-related death. Survival rates are over 10-fold higher for patients with localized disease at the time of initial diagnosis, but the majority of cases found today have already spread to distant sites. This has led to a significant interest in early detection of lung cancer. In the [United States], low-dose CT is now recommended for high-risk patients (i.e., with extensive smoking histories), but it can be difficult to distinguish between benign and malignant nodules radiographically. Some have described the identification of so many pulmonary nodules that require invasive follow-up as a "nodule epidemic." Against this backdrop, we have been interested in developing tools that might help noninvasively identify malignant lesions. Eventually, we are interested in finding ways to replace tissue biopsies and other invasive procedures with molecular assays, not only for lung cancer but for chronic liver diseases such as nonalcoholic steatohepatitis (1).

PNAS: How can proteases be used to distinguish different types of lung cancer?

Bhatia: Proteases have long been implicated in cancer pathogenesis. They are involved in multiple stages of cancer: growth, survival, the angiogenic switch, invasion, metastasis, and interactions with the immune system. Perhaps the most well-known of the cancer-associated proteases are the type IV collagenases, such as matrix metalloproteinase 2 and 9, which are required for cancer cells to break out of the basement membrane and spread. As a family, these enzymes represent a rich trove of diagnostic targets for cancer. But a longstanding challenge in the field has been that the biology is driven by local enzyme activity in the complex tumor microenvironment. Proteases exist in the context of endogenous inhibitors and many are

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membrane-bound or matrix-bound. Furthermore, any one protease signal is unlikely to have diagnostic value, given clinical sensitivity and specificity requirements. Thus, we are taking a multiplexed approach to simultaneously monitoring the activity of multiple proteases using probes that can traffic directly into tissue.

PNAS: How did you design and formulate the multiplex protease probes for lung cancer?

Bhatia: As a starting point for lung cancer, we went to the Cancer Genome Atlas [a publicly accessible molecular catalog of genetic changes in more than 20,000 primary samples of 33 cancer types and matched normal samples] and identified the top 20 dysregulated proteases in lung cancer. Next, we identified peptides that are susceptible to cleavage by these proteases and coupled these peptide substrates onto nanocarriers that can be administered systemically. The resultant probes are designed not to be cleared by the kidneys, which represent an approximately 5-nm filter, until the peptides have been cleaved, producing a nondegradable synthetic reporter. When we administer the probes systemically, some of them enter into the tumor where they can interact with local proteases, but a large fraction of the particles is filtered out by the liver and the spleen. In order to assess the potential for increasing the signal-to-noise ratio in the context of lung cancer, we have explored local delivery into the pulmonary compartment using intratracheal administration in mouse models. We are now working on a nebulizer formulation, in which the sensors can simply be inhaled.

PNAS: What have your experiments using these probes in mouse models revealed?

Bhatia: We are excited about the results in mouse models, with the recognition that this represents only a subset of human cancers. Together with our colleague Tyler Jacks, we have used a 14-probe panel administered directly into the lungs of genetically engineered mice. Barcoded protease cleavage products enter into the urine, and these are analyzed using mass spectrometry. We are able to detect tumors as early as 7.5 weeks after disease initiation, which compares favorably with the sensitivity of imaging based on micro-CT scans and cell-free circulating DNA, albeit in this very specific mouse model (2). The diagnostic accuracy stems from the multiplex monitoring of many proteases and the use of machine learning to classify the tumors. Bearing in mind the caveat that these are animal studies, we were able to demonstrate 80% sensitivity and perfect specificity.

PNAS: Are you planning to test and refine these findings in the clinic?

Bhatia: This technology is advancing to the clinic through a start-up called Glympse Bio, which I cofounded with Gabriel Kwong at Georgia Tech [Bhatia has a financial stake in the start-up]. The start-up has oncology and nononcology programs in its pipeline. They are beginning first-in-human studies with a multiplex protease panel for nonalcoholic steatohepatitis. I'm excited to see how this plays out in patients; you can do all of the experiments you want in animal models, but you're not going to really learn about both the power and limitations of a technology until you enter clinical trials.

PNAS: In a related but distinct application of protease-based diagnostics, you are using proteases to detect infectious agents like pneumonia bacteria in the lungs, using what you call a "breathalyzer" approach. Can you explain the underlying principle?

Bhatia: In the case of the pneumonia test, the aim is to monitor proteases, from both the microbial pathogens as well as those produced by the host. We looked at proteases associated with the infiltration of neutrophils and inflammatory processes as well as a bacterial protease that serves as a virulence factor. First, we showed that we can monitor not only the bacteria but also the inflammation associated with pneumonia and its reduction after successful antibiotic treatment (3). Having laid this groundwork, we became interested in developing a system for rapid disease monitoring at the point of care. This was the motivation for developing the breathalyzer test.

The breathalyzer test is similarly based on the concept of administering a probe that liberates a "synthetic biomarker," the creation of which is induced rather than being naturally shed by the body. The readout for the test is a volatile organic compound, and the idea is that patients would inhale the probe and exhale the volatile. Our preliminary study in an animal model of bacterial pneumonia showed a detectable bacterial signal in the breath within around 10 minutes.

PNAS: You have extended the use of the breathalyzer for lung disease monitoring beyond bacterial pneumonia.

Bhatia: Right. We also applied the breathalyzer to a rare genetic disease called α-1 antitrypsin deficiency, which affects the lungs and liver. α-1 Stems from a reduction of a protease inhibitor, and a current treatment is a systemic administration of the inhibitor. We used the breathalyzer to determine how long the inhibitor is effective in the lungs once administered. We think of this as a tool to monitor pharmacodynamics of therapy.

PNAS: What is the translational potential of these findings?

Bhatia: These are early days, but we recently received funding from the Gates Foundation for the pneumonia work in collaboration with Purvesh Khatri at Stanford University. We are working on a multiplex protease panel that can differentiate the host response to bacteria versus viruses, and the hope is to develop a test that can help clinicians rapidly distinguish between bacterial and viral pneumonia at the point of care, in

order to help decide at the bedside whether to put the patient on antibiotics.

PNAS: You have also shown that in-tissue analysis of proteases can be used to gain mechanistic insights into so-called conditional therapeutics, which are activated upon protease cleavage. Can you explain the purpose and principle of this work?

Bhatia: One of the main challenges faced by the community of researchers working on conditionally activated therapeutics, such as immune cell activators or protease-activated antibodies, is to identify peptide "linkers" that are selectively cleaved at the site of disease. Because proteases are promiscuous enzymes, peptides may be cleaved by a number of proteases, so it is important to know exactly where cleavage events occur. In the case of tumor microenvironments, we developed an in situ zymography tool to visualize protease activity within a tissue section (4). The tool turns the synthetic biomarkers into a sort of "tissue paint," in which the probes fluorescently label tissues that express active proteases. At this stage, this is a discovery tool that could be used to provide mechanistic insights into tumor biology and inform the design of conditional therapeutics.

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2 J. Kirkpatrick *et al.*, (2018) Noninvasive lung cancer detection via pulmonary protease profiling. bioRxiv https://www.biorxiv.org/content/10.1101/495259v1 (posted 13 December 2018).

3 C. G. Buss, J. S. Dudani, R. T. K. Akana, H. E. Fleming, S. N. Bhatia, Protease activity sensors noninvasively classify bacterial infections and antibiotic responses. *EBioMedicine* **38**, 248–256 (2018).

4 E. J. Kwon, J. S. Dudani, S. N. Bhatia, Ultrasensitive tumour-penetrating nanosensors of protease activity. *Nat. Biomed. Eng.* **1**, 54 (2017).