Upon viral infection, assessing the host nasal epigenome

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August 2023—Analyzing the nasal epigenome can shed light on viral infections, strain differences, and potentially infection severity, and for influenza B in particular the results are striking.

The epigenome describes modifications to the genome that don't affect the DNA sequence but determine whether genes are switched on or off where and when they are needed.

In a plenary presentation last November at the Association for Molecular Pathology meeting, Elin Grundberg, PhD, the Roberta D. Harding and William F. Bradley Jr. endowed chair in genomic research, Genomic Medicine Center, Children's Mercy Research Institute, Children's Mercy Kansas City, reported the results of her group's recent efforts in understanding epigenome perturbation in infectious respiratory disease using DNA obtained from nasal swabs.

Dr. Grundberg and colleagues at Children's Mercy use next-generation sequencing technologies to understand how environmental triggers and genetic predisposition affect the functional genome and disease risk.

While links are commonly made between genetic variation across populations and disease outcome, and a statistical link can be made between a genetic marker and disease risk, "it's sometimes challenging to understand the molecular mechanism and biological consequences of this association," she said. Epidemiology studies have also been able to link variables such as diet and smoking to disease outcome. "The challenge with all of these is we don't have the molecular pathways," said Dr. Grundberg, who is also associate professor of pediatrics, University of Missouri–Kansas City School of Medicine, and research associate professor of pathology, University of Kansas School of Medicine.

"What's become clear and successful across multiple studies is that we can use an intermediate trait, such as a cellular phenotype, to link genetics to epigenomic function assessments to improve our understanding of the biological mechanism to the disease association," and then find the cause of these diseases, she said.

DNA methylation, a chemical modification to the DNA that can be used to predict regulatory element presence and activity, is the most commonly used epigenetic trait. "It allows us to gain additional insight into biological pathways linked to our disease trait of interest," Dr. Grundberg said. DNA methylation variation is region dependent. Promoter regions are larger, mostly invariable, unmethylated, and often close to the transcription start sites. Enhancer-like regulatory regions, on the other hand, are more variable when assessed across individuals and have intermediate cytosine methylation and low CpG density.

"My lab spent a fair amount of time assessing and trying to improve the way we measure DNA methylation genomewide," Dr. Grundberg said. The two most common approaches to methylome investigations—microarray and whole genome bisulfite sequencing—are limited by biased content (microarray) and high cost (sequencing), she said. Her laboratory focused on how to retain the high resolution and single CpG resolution of next-generation sequencing.

"So we designed an approach," methylC-capture sequencing, "where we simply filter out anything we don't think is a variable and then enrich regions overlapping regulatory elements, like we do in exome sequencing, for instance," Dr. Grundberg said. Her group at McGill University worked with Roche NimbleGen to design this capture approach using specific probes to target each epigenome of interest. "We designed probes and targets toward disease we're interested in or the tissue we're sampling with" (Allum F, et al. *Nat Commun.* 2015;6:7211; Allum F, et al. *Nat Commun.* 2019;10[1]:1209).

Dr. Grundberg's laboratory team at Children's Mercy Research Institute hypothesized that since viral infections involve functional associations of the host-gene expression machinery, infection severity could also be linked to

regulatory changes marked by epigenetic modifications.

“We set up a study where we leveraged the nasal mucosal samples” used clinically and kept for research, Dr. Grundberg said, noting their focus was children but they’ve done the same in adults. The appeal of nasal swabs was based on the ready availability of the samples and their being “at that time a relatively untouched biospecimen type in terms of understanding epigenome perturbations.”

Dr. Grundberg (above) and her colleagues at Children’s Mercy use NGS technologies to understand how environmental triggers and genetic predisposition affect the functional genome and disease risk. [Photo by Photo courtesy of Children’s Mercy Kansas City]

They focused on genomewide assessments of respiratory viral infections and their link to infection status and severity. Their aim, she said—they’re not there yet—is to do epigenome analysis in the host and link that to the deep clinical information they have from the samples and then perform sophisticated pathway and network analysis. Although their study samples were small in quantity and limited in terms of useful molecular and cellular phenotypes, Dr. Grundberg’s group was also able to perform protein and single-cell analysis on the salvage nasal mucosa samples.

In their first project, in the 2018–2019 viral season, Dr. Grundberg said her group was less interested in the interindividual variation within infections and more focused on viral type. They limited the sample group to infants under age six months to focus on the primary infections pre-vaccination.

They collected 10 samples across seven respiratory viruses and a noninfected cohort, pooled the samples, and performed high-resolution whole genome bisulfite sequencing per individual.

“We can use the methylation landscape to create footprints and inform about regulatory activity, so we were able to do the same here for the first time using the nasal samples and creating first a landscape or a map of regulatory elements in these nasal samples,” she said. They saw the same patterns seen in other tissue samples—“roughly 60,000 enhancer elements that we can capture and 20,000 promoters.”

Given this was a new tissue type they hadn’t worked with previously, they wanted to see the underlying cell types they were capturing. “So we were using high-throughput histone modification data that had been generated from various consortia to overlap that with our data,” Dr. Grundberg said. A fair amount—20 percent of their regulatory

elements—appear to be immune specific, but, as expected, they identified epithelial-specific elements too.

How did the regulatory element activities differ based on infection type? They found that 42 percent are unique to a viral or nonviral pool. “The most commonly unique condition was those that were hypomethylated, an activation of a regulatory element, which made more sense than having a repression.”

The influenza B samples provided the first evidence that one of their viral types was an outlier. They “showed a striking number of unique regulatory elements compared with others,” Dr. Grundberg said, referring to the hypo- and hypermethylated regions. Of the 42 percent of regions that seemed to be specific to a viral or nonviral pool, 80 percent of those were seen just in influenza B. “So it was a little exciting that the first analysis of its kind showed a signature like this.”

They then looked at the single-site CpG levels and measured about 20 million of them across the 10 viral sample types. Next they assessed the correlation distances using hierarchical clustering. “Again, we noticed that the influenza B samples continued to be an outlier in terms of similarities of the epigenome landscape.”

Dr. Grundberg’s group addressed the challenge of how to validate the findings working with these salvage samples stored in freezers or refrigerators by connecting with Olink, a company performing high-resolution, highly sensitive protein analysis. “Because mRNAs are not intact, we could not use gene expression as a validation approach, so we explored protein measures instead. Olink was eager to work with us since the nasal swab was a new sample type for them.” Olink was able to use the very limited material it had of exactly the same samples her group had used for epigenome analysis. “We were using their NGS readout for about 1,600 proteins and did an unbiased protein analysis,” Dr. Grundberg said.

In this case, they used not pooled samples but individual samples to ensure they did not have a driver individual in their pools who might have been driving the influenza B signature. They used the full populations of hundreds of samples from the infants, and they “noticed a top and significant association where influenza B samples continued to be the strongest associations shown by cytokines and chemokines that are known to be the first responders in viral infections and attract the immune cells.”

When they went back to their DNA methylation data and looked at the magnitude, creating differentially methylated regions (DMR) and contrasting the negative controls, “the influenza B continued to be the largest epigenome landscape change,” at more than 14,000 DMRs, she said. Influenza A and parainfluenza were much lower in frequency.

They also assessed whether this change-up in the viral infection was an activation or a repression and whether it became hypo- or hypermethylated. They found a bimodal pattern. “We assumed that the majority will be hypomethylated, something that becomes activated,” Dr. Grundberg said, because if there is viral infection, “we need an activation of gene machinery.” While activation was a large component, “an interesting component was hypermethylation.”

In examining the hypermethylated regions, “we were intrigued by seeing how significantly enriched they were among immune regulatory elements,” Dr. Grundberg said. “So basically, something becomes suppressed or deactivated in the immune cells, which continues to be intriguing.”

Many different immune cells are present in the nasal mucosa. When they zoomed in and mapped it further, they were able to identify those regulatory elements that are specific to the myeloid lineage and specifically to the neutrophils and monocytes that become deactivated, she said.

To assess the robustness of the influenza B infection epigenome perturbation, Dr. Grundberg’s group returned to the same 2018–2019 viral season and redid the pooling strategy and age-matched samples, repeating it for viruses that showed the most striking effect. They validated the significant regions seen in their discovery, “and again influenza B was shown to be extremely robust,” she said. “We could validate 50 percent of our differentially methylated regions in an independent population.” The replication rate was consistent for activated or deactivated

regions. Other viral infections showed poor replication rates (influenza A, 12 percent; rhinovirus, three percent; human metapneumovirus, two percent), "potentially indicating we don't have a robust impact on the host epigenome by these viruses potentially linked to milder respiratory symptoms."

Further analysis of the influenza B hypomethylated signatures revealed links to epithelial-specific genes, Dr. Grundberg said. "We performed a gene ontology analysis and could identify expected genes that got activated," those in interferon (e.g. *CX3CL1, CX3CR1*), including the type three interferon-related genes (*IL 10RB*), as well as key antiviral-related genes (e.g. *ISG15, ISG20*). The most relevant and "extremely striking" link, she said, was to bronchial epithelial cells.

The group tried to assess other ways of replicating its findings that influenza B hypomethylated signatures are linked to upregulated genes in epithelial cells. "We've been doing protein analysis, methylation analysis, but we've also shown that we can use a similar type of sample for single-cell analysis. Here we need to be a little quicker when we do the analysis, work more closely with the clinical labs," she said.

They obtained influenza B-positive samples and controls from the same season, performed single-cell analysis on them, and "were excited to see that we can map a lot of these genes that have been shown to be specific to the nasal mucosa," Dr. Grundberg said. When they overlapped the patterns with their activated genes or regulatory elements for epigenome analysis, "they validated quite well."

The influenza B hypermethylation signatures were "not trivial," she said. The authors of a 2021 article used a comparable approach but focused on vaccinations and single-cell and epigenome assessments using blood (Wimmers F, et al. *Cell.* 2021;184[15]:3915–3935.e21). Their findings, she said, were similar to those of her group: a reduction in promising accessibility and activation but also a fair amount of deactivation. "They speculate that that could potentially be to avoid excess inflammatory host damage during late stages of infection. That's the leading hypothesis we see on the epigenome analysis as well."

But Dr. Grundberg and her colleagues were interested also in the age-dependent effect. They were working with infants so they repeated their analysis but expanded their age group up to three years. "We then started to see a completely different pattern—this clear methylation was only seen in the first few months of life," she said. Hypermethylation response to influenza B infection peaked between ages two and four months, declining until eight months, then leveling out likely due to vaccinations having been introduced.

Age association of viral infection then became "a side story" for the project, Dr. Grundberg said, so her group set up a supporting study to see how the nasal mucosa cell proportions change across ages. "We created a cell atlas across the lifespan where we focused on the healthy mucosa or being negative in the pathogen testing." Children's Mercy Kansas City conducts respiratory testing for employees, so they were able to expand the nasal swab sampling up to age 60. Their single-cell analysis on the extended age range of nasal samples confirmed the presence of "a rich epithelial and immune landscape," she said.

The immune cell occupancy in normal nasal mucosa declines with age when comparing children with adults. If looking only at children, it was higher in older children—peaking at age two—compared with infants. The results were in line with influenza being dangerous for the very young and very old.

While finalizing their analysis in spring 2020, Dr. Grundberg and colleagues incorporated COVID-19 samples. They repeated the epigenome analysis using nasal samples collected from children with relatively mild SARS-CoV-2 infection. "If we thought that influenza B had been an outlier, COVID-19 was in a different level," she said—reaching nearly 80,000 DMRs, a striking epigenome perturbation.

"We incorporated age in a similar way we did with influenza B associations, but as this was before any sort of COVID-19 vaccination was introduced, we could see a very different pattern," Dr. Grundberg said. All those assessed from age four weeks to 19 years had a similar effect in the epigenome, apart from one case that dropped and was clustered close to the negatives, "potentially an asymptomatic case," she said, noting the high volume of preoperative testing performed at the time. "Unfortunately, we don't have that clinical information."

Her group has set up new programs to study individual samples and perform targeted approaches, “given the significant cost of whole genome bisulfite sequencing,” she said. In one study of 60 adults in spring 2020 using salvage nasal samples, “we had relatively rich clinical information so we could distinguish those who are hospitalized versus those who are mild and do similar epigenetic assessments.” They found that *FUT4* hypermethylation—the CD15 marker of neutrophils—is linked to COVID-19 severity.

“We are excited that salvage nasal swab sampling has been successful” for high-resolution epigenome, proteome, and single-cell expression analysis, she concluded. In using the salvage samples, they can identify epigenetic signatures that seem to distinguish viral infections and potentially infection severity.

If these types of epigenome analyses are done across individuals, as shown in SARS-CoV-2 infection, she said, “we may be able to start to inform about markers for disease severity.”

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