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By Monica Heger

Single-cell sequencing is quickly entering the clinic with initial applications in cancer and pre-implantation genetic diagnosis and screening, researchers reported this week at the Beyond the Genome conference in San Francisco, Calif., which was sponsored by *Genome Biology* and *Genome Medicine*.

Within the field of pre-implantation genetic diagnosis and screening, BGI is already using single-cell sequencing to screen for aneuploidies prior to *in vitro* fertilization, and a team from Peking University is testing both single-cell transcriptome sequencing and single-cell whole genome sequencing for applications in IVF.

Meantime, a team from Harvard University has demonstrated through single-cell sequencing that circulating tumor cells from lung cancer patients show unique copy number variation profiles, while another group from Cold Spring Harbor Laboratory has tested single-cell sequencing methods in prostate cancer patients to monitor response to treatment and identify biomarkers and drug targets.

Reproductive health

BGI's Fei Gao said that BGI has been testing a method published earlier this year in *PLoS One* for detecting copy number variants from single-cell, low-pass, whole-genome sequencing on couples undergoing *in vitro* fertilization.

In August, the first IVF baby that was sequenced before implantation was born healthy, he said, and since then more than 20 healthy babies have been born healthy following pre-IVF single-cell sequencing to screen for aneuploidies and large copy number variants.

Gao said that the BGI team first tested several kits for whole-genome amplification including ones that used multiple displacement amplification, degenerate oligonucleotide primed PCR,

and a technique known as MALBAC developed by Sunney Xie's group at Harvard University.

While each had its advantages, Gao said the team ultimately chose to go with a DOP-PCR kit developed by Sigma Aldrich.

Following the *PLoS One* proof-of-concept study, the team tested the method on 38 trophectoderm samples — the outermost layer of cells from which the trophoblast differentiates.

Gao said the team analyzed the samples for chromosomal aneuploidies and large copy number variants, and showed that the results were concordant with microarrays.

Next, they conducted a study of 41 couples that were undergoing IVF either because they were carriers of chromosomal abnormalities or had already had repeated miscarriages.

From those 41 couples, the team biopsied and sequenced 150 blastocysts. While 71 were identified as euploid, 25 had chromosomal aberrations, 40 had imbalanced structural aberrations, and 14 had both chromosomal and structural aberrations.

The sequencing test enabled the physician to choose only euploid blastocysts for implantation, Gao said.

He said that the next steps include doing targeted single-cell sequencing of blastocysts to screen for monogenic diseases and to do HLA typing. For this, he said BGI is using targeted capture and an MDA-based whole-genome amplification kit from Qiagen, which demonstrated the highest coverage of the WGA kits BGI tested. High coverage is especially important when looking for point mutations as opposed to copy number variants, Gao said. Additionally, he said the team also does low-pass whole-genome sequencing of the single cells because allele dropout is still a problem with the targeted approach.

Separately, a team from Peking University is testing single-cell whole-genome sequencing using Xie's MALBAC technique, published in *Science* last year (*IS 1/2/2013*).

Fuchou Tang, an assistant professor at Peking University's Biodynamic Optical Imaging Center, said this week that his group is testing the technique on the 1st and 2nd polar bodies — by-products of the IVF process from which chromosomal numbers in the female pronucleus can be deduced.

The advantage of sequencing the polar bodies, as opposed to cells from the blastomere, is that there is no risk in harming a potentially viable embryo.

Tang's group has been collaborating with Xie's group, who presented at this year's Advances in Genome Biology and Technology meeting in Marco Island, Fla.

At the meeting, Xie said that in a pilot of six female donors, the technique could correctly infer embryo aneuploidy by sequencing to 0.1-fold depth (*CSN 2/27/2013*).

Since then, Tang's group has demonstrated that sequencing depth can be as low as 0.03-fold to accurately call aneuploidies, and he is now testing the technique to call point mutations that cause Mendelian disease.

For this study, he is increasing sequencing depth to 1-fold and pooling data from 11 oocytes

from the same donor. "Since we are trying to identify Mendelian inherited mutations, we can use linkage," he said. Not all SNPs will be detected at the lower sequencing depth, but some will, and those can be used to deduce the SNP at the locations of interest.

In a preliminary test, Tang demonstrated that the technique could detect a mutation in the CFTR gene, which causes cystic fibrosis.

Circulating tumor cells

Aside from IVF applications, researchers are looking to single-cell sequencing to aid in cancer prognostics, diagnostics, and disease monitoring. Harvard's Xie has been using MALBAC to look at circulating tumor cells in lung cancer patients.

Circulating tumor cells are believed to be indicative of metastasis, which "accounts for 90 percent of cancer mortality," Xie said. "We need single-cell techniques to tackle this problem," particularly because cancer is so heterogeneous, and even more so after it metastasizes.

In a proof-of-concept study, Xie used MALBAC to do single-cell exome sequencing and in some cases whole-genome sequencing as well, of eight circulating tumor cells from one patient. He also sequenced the patients' primary and metastatic tumor and compared the mutational profiles from each.

Looking at just one CTC, he identified recurrent mutations to EGFR in the primary tumor, metastatic tumor, and CTC. Additionally, there were mutations to PIK3CA, RB1, and TP53 in both the metastatic tumor and the CTC but not the primary tumor. However, this pattern did not hold true across all the CTCs. When he looked across all eight CTCs, different mutations were present, and not every CTC had the EGFR mutation.

The picture was very different when he looked at copy number variants. "The CNVs are quite reproducible among the CTCs," Xie said. All eight CTCs "from one patient have identical CNVs, and the [CNV] patterns are the same in the metastatic tumor, but different from the primary tumor."

To ensure that the cells he was examining were actually CTCs and not simply cells from the metastatic tumor, he verified that the CTCs had different SNV patterns from the metastatic tissue.

The results indicate that looking at CNV patterns in CTCs could be used to monitor metastasis, Xie said.

Next, he did a study of 11 patients with different subtypes of lung cancer from Beijing Cancer Hospital. The patients either had adenocarcinoma, adenocarcinoma that transitioned into small-cell lung cancer, or both adenocarcinoma and small-cell lung cancer.

Looking at the CNV profiles from single circulating tumor cells from each of the patients, he was able to identify distinct patterns from the three subtypes.

"This shows the promise of using CNV analysis of CTCs for the classification of cancer types," he said.

Additionally, looking at the CTCs throughout the patients' treatment regimen, he found that while point mutations changed depending on treatment and the numbers of CTCs dropped

during effective treatment, the CNV patterns stayed consistent, indicating that they could also be used to monitor patients' response to therapy.

Meanwhile, at Cold Spring Harbor Laboratory, James Hicks' laboratory is using a single-cell sequencing technique originally developed by Nicholas Navin called single-nucleus sequencing and later refined to Cell-Seq (*IS 5/18/2010* and *IS 5/15/2012*).

His group is using the technique to sequence CTCs, monitor cancer cell trafficking, and to study cancer stem cells.

Hicks said that the group is evaluating "CNVs as a means of assessing heterogeneity and is using copy number breakpoints as surrogates for SNVs.

"Each CNV is unique and can serve the purpose of a SNV in terms of following [tumor] lineage," he said.

He said his group has sequenced around 8,000 single cells and has been able to reduce the cost to around $25 to $30 per cell by multiplexing and sequencing at low coverage.

At this week's conference, Hicks highlighted work his lab has done in prostate cancer, which lends itself particularly well to single-cell sequencing techniques because there are already multiple drugs that target the circuitry important for the disease. "As long as we can follow [patients] in real time, we may be able to find biomarkers for sensitivity and ones that determine resistance," he said. Additionally, looking at CTCs in prostate cancer is especially important, because "there are up to hundreds of metastases through the body, mostly in bone, and you can't biopsy all of those."

Unlike Xie's team, which found unique CNV patterns in the CTCs of lung cancer patients, Hicks said that while there are recurrent genes with CNVs in prostate cancer, "each case has a unique combination of CNV biomarkers."

Nevertheless, he said that his group has already started to demonstrate that CNVs in CTCs can point to drug resistance. Highlighting one patient from a study of 18 subjects with progressive castrate-resistant prostate cancer, he said that by doing blood draws over the patient's course of treatment and sequencing CTCs, he was able to monitor changes to the patient's CNVs, including the development of an amplification to the androgen receptor gene that conferred resistance to the drug arbiraterone, marketed by Johnson & Johnson as Zytiga, which the patient eventually failed. The patient did not originally have the AR amplification, but developed it after taking the drug. Later blood draws showed significantly different CNV profiles, despite the fact that the actual cancer cells looked exactly the same.

Aside from continuing the studies of CTCs, Hicks said his lab is also working on improving the single-cell sequencing technique itself. Despite its utility, there are still problems with amplification bias in all of the methods. His group uses DOP-PCR for the amplification, low-pass whole-genome sequencing, and a binning approach to determine CNVs. Additionally, the team often performs deeper sequencing on a subset of cells and uses barcoding techniques to reduce the number of false positives.

Nevertheless, all the researchers agreed that the methods need improvement. Going forward, the major hurdle in translating the techniques into the clinic is that "there are high error rates in all the methods," said Xie. "None of them are good enough."

Monica Heger tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Monica Heger or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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