SNP to it–moving arrays into clinical use

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Feature Story

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Single nucleotide polymorphism, or SNP, arrays may be the newest molecular sleuth poised for a prime-time position in the unfolding oncology drama of "what is it and how can we best treat it?"

Looking at DNA obtained from a tumor, the test can provide a genomewide assessment of copy number, similar to a conventional karyotype but at much higher resolution and with superimposed loss-of-heterozygosity, or LOH, status. The platform works well on paraffin-embedded tissues, is relatively inexpensive, has a short turnaround time, and is QC manufactured. And the results are readily interpretable, making it amenable to routine clinical use.

"Physicians use copy number and LOH status to make diagnoses, prognoses, and therapy decisions, and SNP array karyotypes provide this information at an unprecedented level of resolution and breadth," says Jill Hagenkord, MD, a molecular genetic pathology fellow at the University of Pittsburgh Medical Center.

SNP arrays provide a high-resolution "virtual karyotype" that shows the characteristic chromosomal signatures of various tumors, whether the tumors are classic textbook cases or morphologically ambiguous tumors that defy diagnosis under the microscope.

Being able to identify the ambiguous tumors is important because, Dr. Hagenkord says, tumors don't always read the textbook about what they are supposed to look like. Morphological outliers exist for all cancer subtypes. For example, five percent to 10 percent of renal tumors don't fit into any category based on their microscopic appearance. "And there is often a great deal of inter- and intra-observer variability in morphologic diagnoses," Dr. Hagenkord says.

The SNP array platform can also help answer one of the more common questions: Is a tumor really benign or malignant? says Federico A. Monzon, MD, medical director of molecular diagnostics at The Methodist Hospital, Houston. Many times pathologists making morphological diagnoses will report that they favor a benign diagnosis but there's a chance the tumor looks benign but will behave in a malignant fashion, he notes, so the oncologist can decide how to proceed with that caveat in mind.

Dr. Monzon and his team at Methodist Hospital are doing validation studies now with the aim of moving SNP arrays for classifying renal tumors into clinical use "relatively soon," he says. They also are studying the use of SNP arrays to guide renal cancer prognosis and therapy. And

Dr. Hagenkord, who has accepted a position as director of molecular pathology and clinical genomics at Creighton University, Omaha, plans to bring up the platform there this fall for glial tumors, renal tumors, and chronic lymphocytic leukemia. “Those are the ones we will test first because the translational research and assay development is most mature,” she says, though the assay applies to all human tumors.

In a pilot study performed at the University of Pittsburgh and reported in the February 2008 issue of Modern Pathology (Monzon FA, et al. Feb. 1, 2008, Epub ahead of print), Dr. Monzon, Dr. Hagenkord, and others found the SNP array karyotype could correctly categorize classic cases of renal epithelial tumors that had been subclassified already based on their morphology. The researchers also reported in the article how the assay identified three morphologically challenging tumors that genitourinary pathology subspecialists couldn’t diagnose microscopically.

In phase two of this study, which focused solely on morphologically difficult cases, Drs. Monzon and Hagenkord found the SNP array karyotype could subclassify 95 percent of 21 tumors based on their characteristic chromosomal gains or losses. The SNP array karyotype also differentiated benign renal tumors (oncocytomas) from malignant tumors. Oncocytomas sometimes masquerade under the microscope as malignancies (and vice versa), presenting a challenge for pathologists and oncologists.

In the study, Dr. Hagenkord says, if a tumor had a pattern of genomic lesions that matched oncocytoma, “then we said the molecular signature for the tumor is benign, even though it was originally reported as a renal carcinoma.”

What about the one challenging case that the SNP array could not classify? It showed chromosomal patterns that didn’t fit any currently known renal tumor subtypes, Dr. Monzon says. It may represent a new subtype of renal cancer, he surmises. In ongoing studies at Methodist Hospital, he and his colleagues are accruing data to see if the same chromosomal pattern pops up again.

Findings from the phase two study, which Dr. Monzon presented at this year’s meeting of the U.S. and Canadian Academy of Pathology, showed that immunohistochemistry testing could identify the renal tumor subtypes in these morphologically difficult cases only nine percent of the time. Fluorescence in situ hybridization, or FISH, couldn’t classify any of them. Dr. Monzon says the FISH-related finding may be attributed to the fact that most renal cell cancers have chromosomal losses. “In our experience, FISH is not very good in detecting chromosomal losses,” he says, because sectioning of the cell nuclei causes a lot of normal cells to show such losses. (Other experienced groups can achieve good performance with FISH, he acknowledges, but the difficulty in detecting monosomies using interphase FISH is a known limitation of that procedure.)

The SNP array not only outperformed IHC and FISH in this study, but also its cost-effectiveness ratio (using the 10K 2.0 SNP Affymetrix Gene-Chip Mapping Array) was “dramatically better” than that of IHC or FISH, Dr. Hagenkord says. A 10K 2.0 Affymetrix SNP array is under $300 for arrays and reagents plus technologist time and overhead. The turnaround time for the test is about three to five days in a laboratory without a second shift. And the assay is reimbursable based on currently available CPT codes, she adds.

SNP arrays were originally designed for linkage analysis and whole genome association studies, since SNPs are variant nucleotides that can be used as genomic markers. However, Drs. Hagenkord and Monzon are not using the SNP array that way. Instead, they are determining the copy number and genotype of each SNP on the array and then using computational tools to line up the SNPs in chromosomal order to create a high-resolution picture of the tumor genome.

The Affymetrix SNP array used in the University of Pittsburgh study on renal tumors has

10,000 probes on it, which works well for renal tumors because the genetic lesions are large and thus easy to see, Dr. Monzon says. Higher-density arrays are available.

Describing the assay in simple terms, Dr. Hagenkord says she takes the genomic DNA from the tumor, fragments and labels it, and hybridizes it to the array. Each probe on the array is precisely 25 nucleotides long, and in the middle of the probe is a SNP. Each of the little DNA fragments in the tumor sample sticks to its complementary probe on the array, and if the tumor has the common nucleotide for the SNP in the probe, it will result in a strong fluorescent signal at that probe. If the tumor has the less common nucleotide at that SNP, it will not fluoresce, she explains. “This allows us to determine the genotype at each SNP, and the strength of the fluorescence relative to normal allows us to determine copy number.”

Then, by knowing the physical address of each SNP probe on the array and the physical location of each SNP in the genome, computational tools can be used to line up the SNPs in chromosomal order, thereby generating an ultra-high-resolution virtual karyotype providing the genome-wide copy number and LOH status.

“In humans, a copy number of two is always normal since we are diploid. We get one copy of a chromosome from our moms and one from our dads. In tumors, the number of copies of the chromosomes—or pieces of the chromosomes—changes,” Dr. Hagenkord says. For example, for clear cell renal carcinoma, loss of the p arm of chromosome three is the disease-defining genetic lesion. “On the SNP array karyotype for renal clear cell carcinomas, several contiguous SNPs located on the p arm of chromosome three show a copy number of one, while the other autosomal SNPs in the genome would show a copy number of two.” (Dr. Hagenkord notes that the SNP array can be performed without having the patient’s normal tissue as a reference because the software finds a “best fit reference group” and copy number.) In addition to the loss of 3p, the virtual karyotype would also show LOH of 3p. The software determines LOH likelihood for a region by looking at the genotypes for each consecutive SNP along the genome.

“Generically,” she explains, “each allele at a given SNP is assigned an A or B, so a heterozygote would have an AB genotype and a homozygote an AA or a BB genotype at a specific SNP.” About 30 percent of the SNPs on the array can be expected to be heterozygous. “If the person’s renal tumor has lost one copy of 3p, say the paternal copy, then there would be no AB genotype calls in all of the SNPs on 3p, since only the maternal genotype is present, indicating a loss of heterozygosity in the tumor—that is, a loss of AB calls.” The virtual karyotyping computer algorithm lines the SNPs up in chromosomal order and looks at what the neighboring SNPs are doing with respect to genotype, Dr. Hagenkord says. “And if 200 consecutive SNPs all have a genotype of AA or BB—that is, no AB—then that region of the genome shows a high likelihood of LOH. So, for the loss of 3p in the renal clear cell tumor, you would see a copy number of one and LOH for the p arm of chromosome three.”

Sometimes it’s known that the LOH involves a particular tumor suppressor gene, though ultimately “that information is not necessary if we know that every time we see LOH at a certain location it has a validated clinical correlation. The LOH itself can be a useful biomarker without knowing the underlying causative gene,” Dr. Hagenkord says.

Virtual karyotypes can, however, pinpoint the status of known tumor suppressor genes or oncogenes, and they can be used also as a discovery tool to identify new genes involved in oncogenesis. LOH of tumor suppressor genes is a biologically important mechanism, according to the Knudson two-hit hypothesis for how cancer develops. As that theory goes, Dr. Monzon says, the first hit is usually a mutation that occurs in the gene. “After the first hit, you have a mutated gene and a normal gene,” he says.

The second hit usually is a deletion in which a random mutation causes the person to lose the normal gene. Sometimes a carcinogen, such as smoking, a virus, or radiation, causes that to happen, Dr. Monzon says—“anything that causes a cell to make an error when copying DNA

can lead to that kind of change." When the latter occurs, the person is left with only one gene copy, which is mutated and doesn't work or works aberrantly, leading to cancer. If that aberrant gene duplicates itself, creating what is known as "copy-neutral" LOH, the person has two copies of an abnormal gene, both from the same parent.

Identifying copy-neutral LOH is critically important in oncology applications, Dr. Hagenkord says, given that the literature shows 20 percent to 80 percent of the LOH in human tumors is copy-neutral LOH, depending on the tumor. And SNP arrays are the only genomewide platform that can detect copy-neutral LOH. "Copy-neutral LOH is missed by any copy-number-only platform," she says, such as FISH, conventional cytogenetics, or array comparative genomic hybridization (array CGH).

While renal tumors tend to have less copy-neutral LOH compared with other types of tumors, the pilot study that Drs. Monzon and Hagenkord did at the University of Pittsburgh did find three tumors with copy-neutral LOH lesions that identified the tumor subtype. Using FISH or array CGH would not have detected these lesions, Dr. Hagenkord says.

It's now known that a high percentage of LOH is copy-neutral LOH, and the SNP array is a "good tool" for detecting copy-neutral LOH clinically, she says.

SNP arrays aren't all things to all cancers. While the test can easily identify chromosomal gains/ losses and LOH associated with a tumor type, it can't detect chromosomal inversions where a piece of DNA gets flipped upside down but remains in the same place, Dr. Hagenkord says. Nor can it identify translocations, which occur when two parts of two chromosomes exchange places.

In addition, even the most advanced SNP array algorithm currently can only detect the abnormal clone if the tumor has at least 20 cancer cells for every 80 normal cells.

FISH and PCR can detect translocations but not inversions, "but only if you know what you are looking for ahead of time," Dr. Hagenkord says. "Conventional cytogenetics, where you look at chromosomes under a microscope, can identify translocations and inversions, but only if they are large. And you cannot do it on formalin-fixed, paraffin-embedded tissue."

In renal cell tumors and many others, most of the chromosomal changes identifying subtypes are not a result of translocation, so SNP array karyotyping would suffice for routine clinical use. In some tumors, especially soft tissue and hematologic malignancies, translocations are important, and FISH, PCR, or conventional karyotyping should be used to detect translocations, if needed. "Copy number and LOH can also be clinically relevant in these tumor types, so the SNP array could add vital information, too, in addition to the translocation. What is appropriate is going to be different for each tumor type," Dr. Hagenkord says.

Research using SNP arrays to identify chromosomal lesions associated with various tumors is growing in volume. In addition, a wealth of literature exists on chromosomal lesions and tumor prognosis, Dr. Monzon says, though much of it is based on other techniques, such as PCR, FISH, and conventional karyotyping. Even so, the SNP array platform will help people use that information clinically for various types of tumors, he says.

For example, Dr. Monzon and Raymond Tubbs, DO, of the Cleveland Clinic, are planning a research project using SNP arrays to identify HER2 amplification in breast cancer tumors.

Dr. Hagenkord believes the SNP array platform could be used for HER2 testing in equivocal immunohistochemistry cases. "If you see a big spike in the virtual karyotype designating focal amplification of [the HER2] gene, it would confirm whether the person is amplified at HER2. A lot of borderline cases may be resolved with the SNP platform and you could also see the entire genome for copy number changes, not just the HER2 gene, as well as assess LOH. So

one assay could yield much more than just HER2 copy number." That work has not yet been done, she adds.

The SNP array platform can also be used to monitor tumor progression by identifying whether a tumor is accumulating additional chromosomal lesions over time. This is done now by conventional cytogenetics, FISH panels, or both, for several tumor types.

For example, chronic lymphocytic leukemia, or CLL, progresses in some people but remains indolent in others. "Using the SNP array karyotype, we can detect the same prognostically significant lesions that are currently detected by the standard FISH panel for CLL, but at a fraction of the cost," Dr. Hagenkord says. And the SNP array shows the entire genome rather than just those regions that the FISH probes target. (See above for a SNP array showing CLL progression.)

Could SNP arrays eventually trump morphology for diagnosing solid tumors, especially for difficult cases? To answer that question, Dr. Hagenkord points out that in he-mato-pathology, molecular diagnosis of chromosomal lesions in leukemias and lymphomas already often supersedes the morphology if the two are in conflict.

Yet, she concedes, it remains to be decided which will win in the realm of solid tumors. "We feel very confident, however, about our data," Dr. Hagenkord adds, noting that others have done basic research using SNP arrays and have validated the findings. She thus believes that if a tumor reported as benign by microscopy is morphologically "wishy washy," the SNP array call will end up having more weight than the morphology. And molecular diagnostics will come to carry a lot of clout when clinicians decide how to treat people with solid tumors, she predicts.

"That's the direction in which solid tumors are going," she continues, "and SNP arrays can help bring solid tumors into the molecular age."

The larger question, in her view, is whether that molecular age will be one that pathologists bring to fruition, or "will it grow up in cytogenetics by default?"

In fact, she points out that several genetics laboratories are already offering array CGH testing for conventional genetic applications, which is conceptually similar to SNP array karyotyping. And it won't require a big leap for such labs to do the SNP array testing as demand for it grows in oncology.

In Dr. Hagenkord's view, pathologists have a better knowledge base, in general, to analyze SNP array virtual karyotypes from tumors because pathologists have been trained "to think like a tumor." And pathologists have a visual image in their heads about what tumor specimens consist of—"for example, some tumors have a lot of normal stroma and blood vessels mixed in with their abnormal [cells], whereas others do not." And the ratio of tumor to normal tissue has to be taken into account when setting analysis parameters for SNP arrays.

Analyzing virtual karyotype data for tumors requires not only an understanding of tumor biology and morphology, "but also the science of the platform and the assumptions underlying every single step in every single algorithm used to generate a single virtual karyotype," Dr. Hagenkord says. And not all assumptions hold true for every tumor. You have to recognize red flags or know when to make adjustments, or both, she says.

Dr. Monzon, too, believes that pathologists "should do as much as possible to retain clinical testing with SNP arrays within the pathology space." CAP president Jared Schwartz, MD, PhD, who is known to beat the drum warning pathologists will miss "a big boat" if they don't understand and bring new molecular tools into their labs, says the new SNP array platform is "creating a lot of excitement." Dr. Schwartz views the SNP array technology as holding a lot of promise—the key word being promise. "We are really poor at predicting how new

technology will develop and be used over time," he says.

As a case in point, expression arrays, which generated much hoopla when they first became available, have yet to make it into routine clinical use. Dr. Hagenkord says that's because, in part, expression arrays fail to meet many of the criteria necessary for a platform to enter routine clinical use, such as being relatively inexpensive and reimbursable, having a rapid turnaround time, being easy to interpret by the average MD, and providing clear clinical utility that cannot be obtained by another method already in routine use. "The copy number arrays actually satisfy these criteria," she says, "and that is why they entered routine clinical use before the expression arrays. Most pathologists are not demanding that expression arrays be used routinely because it is not clear that this will make their daily work easier, and they feel too removed from the data, and confused by it, and if the expression level of a certain transcript is clinically important, there are generally cheaper, faster ways to ascertain it, such as IHC or PCR."

In fact, Dr. Hagenkord finds that when she tells pathologists that she is working with SNP arrays, they hear the word "array" and sometimes tune her out. She has to explain how SNP arrays are different from expression arrays and that virtual karyotypes could actually make their daily sign-out easier.

But she is betting that pathologists won't be tuning out SNP arrays anymore. "The platform's move into oncology is the next tidal wave in molecular diagnostics," she boldly predicts, "and it's just starting to curl upward."

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