

## Researchers Develop Improved Molecular Classification System for Rare Childhood Cancer

Dr. Timothy Triche, of Childrens Hospital Los Angeles talks about using gene expression profiles to provide safer, more effective treatments

By Stacey Ryder

**Los Angeles, March 31, 2006** — Researchers at the Childrens Hospital Los Angeles and the University of Southern California (USC) Keck School of Medicine have discovered gene expression and DNA polymorphism profiles that more accurately diagnose and predict outcome in the most common malignant soft tissue tumor in children and young adults, rhabdomyosarcoma (RMS). The team, led by Timothy Triche has classified RMS into three subtypes and expects the results of its study to help doctors provide safer

and more effective treatment regimens for nearly 25 percent of patients with these tumors.

“I don’t think anybody is going to claim that expression signatures offer an alternative to conventional diagnostic methods,” said Triche, professor of pathology and pediatrics at Childrens Hospital Los Angeles. “But when used in conjunction with conventional diagnostics, they are remarkably precise and reveal new information not available by any other method.”

In Triche’s laboratory, researchers are also using the microarray data they have generated to develop potential therapeutics. Knowledge of tumor-specific genes can be used to develop targeted therapies such as siRNA to treat tumor cells and spare normal tissue. They are currently testing this technology in mice using targeted nanoparticles to deliver the siRNA and conventional anti-cancer agents. The ultimate goal is to develop FDA-approved, nanoparticle-based targeted therapeutics for humans, but this will require a lengthy validation and

**Timothy Triche** is a professor of

pathology and pediatrics at the Childrens Hospital

Los Angeles. His laboratory is focused on the

molecular genetics and developmental biology of

childhood sarcomas, particularly Ewing’s sarcoma,

rhabdomyosarcoma and osteosarcoma. Triche has

made extensive use of Affymetrix microarrays,

particularly the Human Genome U133 array set,

to define cancer signatures to more accurately

diagnose and effectively treat childhood sarcomas.



approval process. For now, Triche's work focuses on developing methods for choosing optimal selections of genes to target the nanoparticles to specific tumors.

Triche's work focuses on several types of childhood sarcomas, including Ewing's sarcoma, RMS, and osteosarcoma. He recently spoke to AMB Editor, Tommy Broudy, about the cancer research being performed in his lab, covering everything from collecting patient samples to determining the right treatment for individual children with cancer. They discussed:

- How to make accurate diagnoses and treatment decisions for different tumor types
- Genetrix software for data analysis and the merits of creating 'metagenes'
- Signature validation and follow up in large-scale studies
- Identifying drug targets and developing siRNA-based therapies

### **Making accurate diagnoses and adjusting treatments for different types of tumors**

**Broudy:** What are some of the examples of prognostic and diagnostic markers you've identified and are testing through functional studies?

**Triche:** The longest standing project and the best example is clearly the work we've been doing in sarcoma since 1999, under the auspices of a Director's Challenge Grant from the National Cancer Institute (NCI). The original goal of that project was to determine whether a subset of genes, or a so-called expression profile or signature, could be a powerful adjunct or even an alternative to conventional forms of diagnosis. When you correlate diagnosis with clinical factors, such as outcome on a clinical therapeutic protocol, you can estimate prognosis for new patients before they are treated, and thus better choose so-called 'risk-adapted' therapy.

Ultimately, we are hoping that this will lead to better choices of targeted therapies in development right now,

## **“Microarray-based tools are spectacularly useful for diagnostic classification.”**

based on the particular idiosyncrasies of the tumor.

The conventional pathology workup for childhood RMS is pretty straightforward. Patients are biopsied and the tissue is submitted for conventional pathologic diagnosis. First, we ask is it RMS? A conventional histologic classification is made and that usually suffices for most patients. That's where maybe about 10 percent of the diagnoses disagree; agreement among pathologists as to histologic class is somewhat subjective and less than perfect.

Interestingly, we have found that expression profiles are more objective determinants of this diagnosis than histologic criteria, even when the latter are augmented with immunohistochemistry for two or three muscle markers. Further, these markers only classify the tumor as RMS, and make no distinction between sub-classes with differing prognosis. We need to develop precise diagnostic criteria for the overall diagnosis of RMS, and for prognostic classes within that disease.

Microarray-based tools are spectacularly useful for diagnostic classification. I don't think anybody claims that they are an alternative to conventional means, but when you use them in conjunction with conventional diagnostics, they are remarkably precise.

We rather exhaustively analyzed these cancer signatures and found a small set of genes, sometimes as few as 10, that accurately predict diagnosis and even prognosis. We validated many of these genes using immunohistochemistry on tissue microarrays containing 111 tumors on each slide.

We found that the microarray data were very predictive of what we find at the protein level.

The implications of this work are surprising. Reclassification is likely to affect upwards of 25 percent of all these soft tissue sarcomas. That may mean

that patients will be treated on protocols more tailored to the expected outcome based on the data from these studies.

The other surprise in this work was that histology does not always predict diagnosis as we conventionally understand it. Based on these expression criteria, we have come to realize that a subgroup of RMS, termed 'alveolar rhabdomyosarcoma', is not conventional 'alveolar', despite its histologic appearance.

Conventionally, alveolar histology is associated with greater risk. Most of these tumors have a characteristic gene translocation that fuses a PAX gene with the FKHR or FOXO1 gene, creating the so-called PAX-FKHR fusion gene. To date, all alveolar patients are assumed to have similar risk, regardless of their translocation status. We now know, based on our gene expression work, that translocation negative tumors have a unique cancer signature indistinguishable from the more common and better risk group, termed embryonal, and very different from the translocation-positive alveolar tumors. We also know that they have a very different clinical behavior, similar to the embryonal tumors. This discordance between histology, fusion gene status, and outcome occurs in 25 to 35 percent of alveolar cases.

We must now decide how to manage this finding when assigning patients to risk-based therapy, where embryonal vs. alveolar histology is a known risk factor. Are we going to stay with the old criteria and put that child on a high-risk protocol, when in fact he may be a low-risk patient? If his tumor is translocation negative with alveolar histology, we now know from having done hundreds of these cases that the tumor will have a profile that looks like the low-risk category, embryonal RMS. Unless there are other factors like widespread disease or the tumor is located in a bad anatomic site, it's considered a low-risk disease. The patient is put on less-aggressive

“We are already using gene expression profiles to assist in the initial diagnosis of these sarcomas.”

therapy, he has fewer complications and actually has a better outcome survival outcome—in the order of 80 percent or better. The obvious question is whether such patients should be assigned to the more favorable arm of these protocols, all other things being equal? We think so, but making the transition from research to medical practice requires proof beyond what we know now. That is in fact the purpose of our new award from the NCI, which is intended to validate these diagnostic and prognostic signatures in prospective studies over the next five years.

#### Data analysis using Genetrix

**Broudy:** What tools do you typically use for primary data analysis to mine your array data?

**Triche:** We’ve been using a suite of software tools called Genetrix. It’s designed specifically for analyzing genomic data like this. At its heart, it is a statistical analytic engine using all the standardized statistical tests like multiple T-testing, ANOVA, Kaplan-Meier survival analysis, and Cox proportional hazard analysis, for example, but applied to gene expression data as complex predictors of diagnostic class and outcome.

I should note that we developed this tool, starting in 1999 when we first began studying these sarcomas, because at that time there was no gene expression software that also incorporated clinical biostatistical data. We were extremely fortunate to have Jonathan Buckley join our effort. He is an M.D./Ph.D. with years of clinical biostatistical experience in clinical trials who also happened to create biostatistical software. When I approached him with our then-unique need for such a tool adapted to complex genomic datasets, he agreed to assume the responsibility for creating the appropriate software, which he did, and continues to do to the present. Currently, for example, he has adapted the software to handle all 1.4 million data points from a

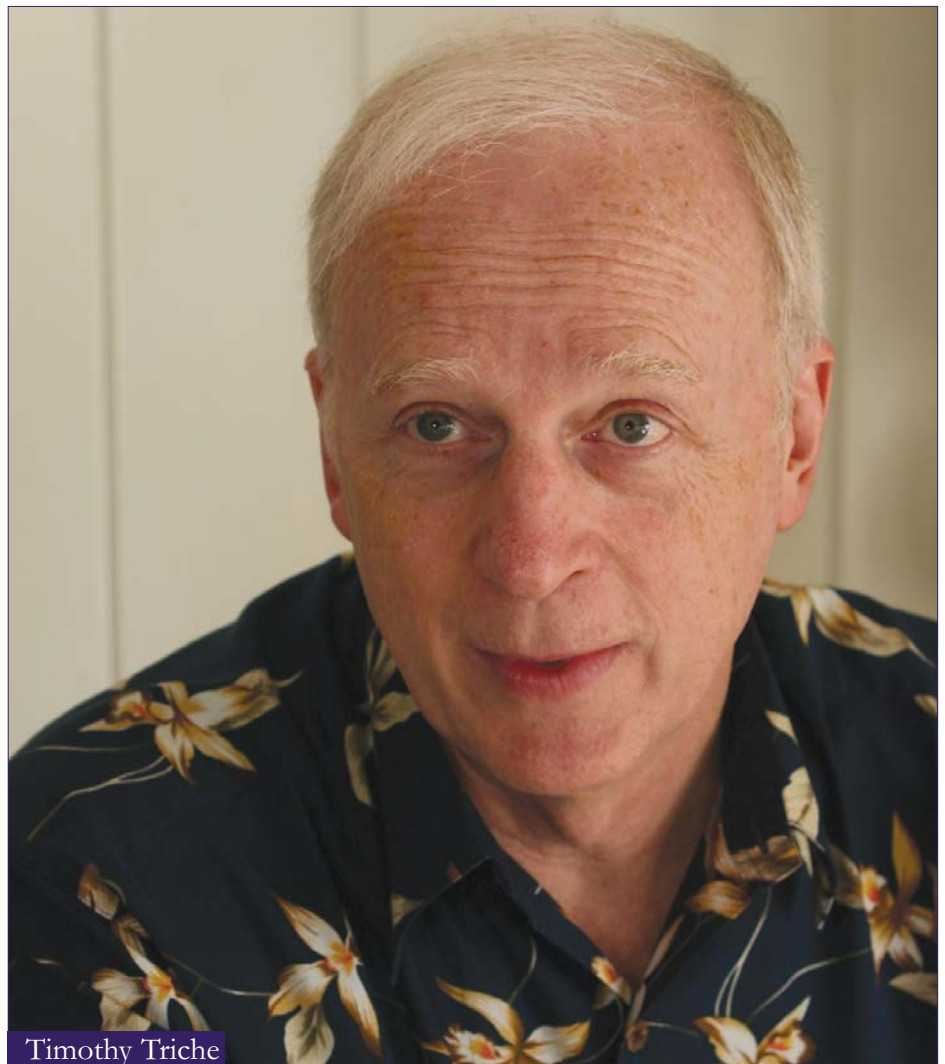
Human Exon array, or all 500,000 polymorphism values from a 500K single nucleotide polymorphism (SNP) chip, and apply the same statistical testing to these massive datasets. No small accomplishment, and very important to our work.

One of the reasons we prefer Genetrix is that Jonathan has continually adapted the software to meet our needs. For example, the extraction of the classifier gene lists, rank ordered by P-value, is typically done thousands of times, as is the clustering, in a reiterative fashion. This has led to rather stable gene expression profiles, as determined when we subsequently test them in test sets and validation sets. This routine, which Jonathan calls ‘metaclustering,’

has become a primary tool in our work. Likewise, the Genescreen tool he created, which compares gene expression value vs. outcome for all genes as a group has allowed us to readily identify important prognosis genes that might have therapeutic value in the future.

**Broudy:** What about visualization tools?

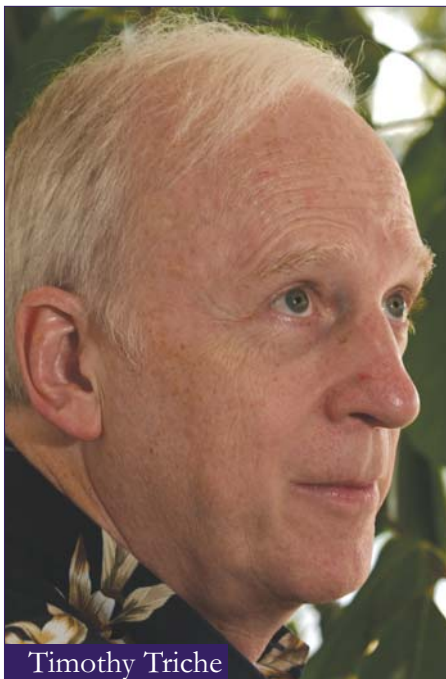
**Triche:** Genetrix has all of the standard visualization tools such as principal component analysis, multi-dimensional scaling, hierarchical clustering with or without expectation maximization, support vector machines, similarity matrices, scatter analyses, and meta-clustering, which uses these tools to define class in a more robust, repetitive manner. We use them all. We’ve taken the approach that all these tools are useful, but none is perfect. We like to compare results from different methods and choose those methods and gene lists that appear to be the most reproducible.



Timothy Triche

More and more, we are using MetaCluster which creates a metagene. A metagene is a group of genes that act as a cancer signature. For example, you can set an arbitrary cutoff rate based on P-values and then test the power of that group to predict important parameters, such as prognosis. The group of genes is treated as a single gene predictor, and incorporates both up- and down-regulated genes. We have found this approach to be very powerful.

Will we miss some important genes? Probably. Will we miss a lot? I doubt it. Will we miss the most important ones? I would be very surprised if we did. We all recognize these are imperfect tools, but the pleasant surprise is that biologically important genes are being identified by virtually all groups using these tools for purposes such as these. Too much has been made of the lack of correspondence between the gene list from one study when compared to another. People forget that many factors determine whether a specific gene will be included in a given list. Typically the most important genes will appear in most if not all lists. That is the real importance of these studies. We were pleased to see that a European group validated most of the genes we found in RMS in their recent publication, for example. This couldn't have happened if the results weren't grounded in biologic reality.



Timothy Triche

**Broudy:** How do you go from a large set of microarray data to a double-digit signature?

**Triche:** If we start off with the U133A chip that contains about 22,000 genes, we typically find less than 10,000 expressed at significant levels. From that group of expressed genes, many classification tools will extract dozens to a few hundred genes significantly associated with a class or outcome. We typically use a cutoff of  $p < 0.001$ . When we use a reiterative testing algorithm like meta-clustering, the number is further reduced, typically to double digits. Depending on the patient dataset (size, complexity, accurate class distinction, and so forth), this number may drop further. In one prognostic analysis, we found little difference between 50 genes and as few as 10. Further analysis on the same dataset, and on separate datasets tests using leave-n-out analysis tests the reproducibility of those genes to make a distinction such as a diagnosis or prognosis.

#### Signature validation

**Broudy:** How do you go about validating your signature?

**Triche:** We have done quantitative polymerase chain reaction (QPCR) with sequence validation, tissue immunohistochemistry on individual tumors and tissue microarrays (TMAs) on over a hundred cases at a time. We identify the most reproducible genes from the expression signatures, find appropriate antibodies from commercial vendors whenever possible, validate the antibodies on Western blots as well as on fresh frozen and paraffin-embedded tissue, and analyze completely unrelated cases from a different source to determine that the proteins are present in these cases. So far, we have found a good correlation with the Affymetrix data using quantitative real-time PCR and histochemistry data from the 3-way comparison.

We are also interested in adapting proteomics methods for our purposes. These mass spectrometry methods offer more quantitation and would free us from the need to identify and validate antibodies, which are themselves imperfect

tools for protein identification. However, the TMAs are all paraffin-embedded material, which is not suitable for mass spectrometry based methods of protein detection, so we must still rely on less available fresh or frozen tissue for this purpose.

**Broudy:** You're planning an even larger study supported by the Strategic Partnering to Evaluate Cancer Signatures [SPECS] grant to validate your findings. What's the scale of this validation study?

**Triche:** We are expecting to analyze every available case of sarcoma in children, adolescents, and young adults who are treated on Children's Oncology Group (COG) protocols over the next five years. We estimate this will be about 2,500 specimens over the five-year course of the SPECS grant. In every case, we will compare our cancer signatures with clinical parameters like diagnosis, response to therapy, outcome, age, stage, site, and other well-characterized risk factors. The goal is of course to prove whether these cancer signatures can be utilized in the management of these patients in order to improve outcome while minimizing long-term treatment effects.

**Broudy:** Are your samples paraffin embedded?

**Triche:** No. For about 50 percent of the 2500 patients I mentioned, we're getting fresh tissue. That's one of the reasons why I would like to consider using the human exon arrays as opposed to the U133 series. The exon arrays use a random-primed PCR protocol to generate the target, as opposed to the reverse transcriptase method used on all prior arrays. I think there is a reasonable chance that the human exon array would be usable in paraffin-embedded material. We are currently learning to use these arrays and cope with the massive data import and analysis challenge of about 1.5 million data points per array. The last six months have been challenging, but now that we have good data for comparison, we are impressed with the quality of data from these arrays, and the relative independence of the data from RNA quality. We are hoping that useful data might be obtained from paraffin-

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embedded, formalin-fixed tissue, but haven't done the studies yet to determine whether this is a realistic expectation. If worse comes to worse, we will do multiplex QPCR to determine expression values for our signature genes.

I should note that the other reason why I want to go to the human exon array is that the starting sample requirement is about 1/150th of that for the U133. We currently use 100 ng of total RNA, as opposed to 5 µg for the U133 series. The implications for our studies are immense. Tissue is limited from these tumors, and we calculate that a single frozen section, such as obtained by the pathologist at the local institution at the time of initial diagnosis, would be sufficient to do these studies. Even a tumor imprint may suffice. This would also allow us to use methods like laser capture technology to avoid non-tumor tissue to generate our expression profiles. It also means we can ensure the best quality control; we can photograph the section before extracting the RNA, so we have a permanent record of what was profiled.

You get one chance at this. The original biopsy is probably smaller than your thumb tip. Once you use up that tissue it's gone forever. The number of possible uses increases over time, but the available material does not. The less we need to use, the more cases we will be able to study.

#### Signature database

**Broudy:** Where will you store the microarray data collected from these samples?

**Triche:** In addition to central submission to the NCI caArray database, all this data will go into a COG-based data archive. That will mean that the genomic data can be married in the analysis phase with all the clinical covariate data. This is

perhaps one of the most exciting parts of this study. It is not common that one can compare genomic data with a vast amount of well-organized, constantly updated clinical data such as is available through COG. I suspect that is one of the reasons we have been successfully funded. Many people believe the COG model of a single comprehensive clinical database on every patient entered onto any COG protocol is a unique resource. Over 30 years of data are linked to these tissues, and more are added each day. It's a remarkable resource for cancer biology and treatment studies.

The other point I should make is that all six of the SPECS study groups are working together to compare their data, virtually all of which is on the Affymetrix platform, as well as proteomics data from one group and oligo array data from another. All six groups will generate Affymetrix-based microarray data. At a recent meeting of the SPECS PIs, we were able to arrange for the NCI to facilitate central data archiving and sharing among the groups. We believe this will enable us to compare important genes that predict clinical behavior, for example, across all six cancer types being studied by these groups. The six types are lung, breast, and prostate cancer, and lymphoma, leukemia, and of course our sarcoma studies. The chance to compare gene signatures across most of the most common cancers is obviously an important goal of SPECS.

#### Identifying drug targets and developing siRNA-based therapies

**Broudy:** How are you identifying potential drug targets from your array data?

**Triche:** They're preliminarily identified by expression arrays, but we need to

validate them. If the targets are really biologically significant, then we should be able to manipulate them in our in vitro model systems and observe at least some biologic effects. We have done this with several genes and plan to do a great deal more. We have created mouse models of RMS, Ewing's sarcoma, and osteosarcoma using human tumor cell lines and have genetically engineered these lines in some cases to enable studies of gene targeting on biologic behavior in the mouse models. The most common models we use are orthotopic xenografts and metastatic models. These usually follow the in vitro validation studies where we use the same cell lines in culture.

In a typical experiment, we might take a gene that is overexpressed in an important group of tumors and suppress it with siRNA. If we see an effect, we might target that gene in a mouse model using nanoparticle delivery of the siRNA, such as we have published. For genes that are suppressed, we utilize inducible expression models to control the up-regulation of the gene of interest, also as we have published. However, the time and effort required to create and test these models is not trivial, so choice of the most important genes becomes imperative. That is where the analytic component of patient material becomes critically important.

**Broudy:** From your metagene, are you able to pull out potentially metastatic genes that you want to knock down using siRNA?

**Triche:** Yes. We look at the metagene membership and then we start doing analyses that link these genes to biologic functions, such as pathway and Gene Ontology (GO) code analysis and so forth. Intuition is also a useful tool. If you see a gene that is known to promote cell motility or extracellular matrix degradation, as we often do, it is not a giant leap of faith to assume such genes enhance metastatic potential. The harder part is genes with no known function or the stated function in online databases is irrelevant to the question at hand. That is where tools that uncover such functions become very handy.

I should also note that we do not rely on parameters like fold change in expression values. On the contrary, we often look at something as small as a 40 percent change in expression and find strong association with clinical parameters like diagnosis or prognosis or treatment resistance. That's a lot less than the usual two-fold increase/decrease seen in the literature, and a lot of important genes are in that range including we believe, some primary targets such as transcription factors.

**Broudy:** Do you use arrays to look at the effects of siRNA to return gene expression values back to the baseline?

**Triche:** That's how we developed the therapeutic signatures to look at specific gene targets. So if you look at the June 2005 Cancer Research paper by Huelieskovan, it nicely demonstrates how we use the Affymetrix arrays to validate gene targets. The combination of induced gene expression, a time-course study for primary, secondary and tertiary targets, and confirmatory down-regulation of the induced gene using siRNA is a powerful method for analyzing gene function in these tumors.

**Broudy:** Are you using animal models?



Timothy Triche

**Triche:** We're doing more and more of that. We're using mouse models of common childhood sarcomas introduced by orthotopic injection for bulk tumor studies. We also developed a disseminated tumor model that mimics metastatic disease using tail vein injection of tumor cells. Both are relevant, the first as a model of primary tumor, and the second as a model of disseminated cancer, which is what kills most patients.

**Broudy:** How effective are siRNA treatments in mice?

**Triche:** Depending on whether we're going after metastases or primary tumor, the siRNA alone is highly efficacious in inhibiting formation of metastases. It prevented metastases in 80 percent of the animals in the study we published in the October 2005 issue of Cancer Research. In the case of large bulky tumors, you can see a 90 percent tumor volume reduction, but then it promptly grows back as soon as the siRNA treatment ceases. However, I should point out that we don't expect to use a single siRNA in any future clinical setting. One thing we have learned over time about cancer is that no single agent cures cancer. I doubt a single siRNA will either. That is where knowledge of these expression profiles becomes critical. They represent multiple potential therapeutic targets. We just need to figure out which ones are important.

**Broudy:** What are some of the primary targets that you have found or are following up right now?

**Triche:** For the alveolar RMS subset of tumors I've been talking about, one of those targets is transcription factor AP2 beta, (TFAP2B). We have demonstrated a near perfect correlation between the expression of AP2 beta and the presence of a functional expressed gene translocation. We would like to know if it also plays a role in the biologic behavior of this tumor. That is a subject of our ongoing studies at present, for example. P-cadherin is another one, and that is known to mediate cell-cell and cell-extracellular matrix interaction. There are unfortunately many others, too many to pursue, so one must make

painful choices. In the most obvious case, we have suppressed or expressed the primary suspects, the Ewing's or alveolar RMS fusion genes, and found a marked effect on both gene expression profile and biologic behavior. We also identified dozens of target effector genes. Now the challenge is to figure out which ones are important and target them accordingly.

It is important to not overlook the immediate practical value of these genes, however. We are already using gene expression profiles to assist in the initial diagnosis of these sarcomas. We find that they consistently fall within well-defined groups and some genes are critical to the clustering and thus the diagnosis. We are using multiple methods including arrays, PCR, and even immunohistochemistry to identify these genes as useful biomarkers of diagnostic or prognostic class. That we can do now. Understanding the biologic significance is a larger challenge, which will take time. For now, use of this gene expression data for diagnostic and prognostic profiling has emerged as an important and useful tool. Ultimately, we hope it will translate into better therapy as well.

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### Companies

■ Affymetrix Inc.  
<http://www.affymetrix.com>

### Organizations

■ Children's Hospital Los Angeles  
<http://childrenshospitalla.org>

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