

Genome-wide Analysis of Epigenetic Modification Yields New Clues to Malignant Glioma Diagnosis and Treatment

The Swedish Medical Center's Anup Madan and MIT's Noam Shomron discuss new insights into the epigenetic regulation of the molecular pathways behind malignant glioma

By Stacey Ryder

SEATTLE, October 15, 2007—In the first genome-wide analysis of histone modification and promoter methylation in malignant gliomas, researchers at the University of Iowa and Seattle's Institute for Systems Biology have identified two genes, BEX1 and BEX2, that are epigenetically silenced in glioblastoma multiforme (GBM), a particularly deadly and incurable cancer.

The team, led by Anup Madan, a

research scientist at the Swedish Medical Center and the Institute for Systems Biology, used microarrays that examine more than 25,000 promoter regions in the human genome to map specific histone modifications and methylation patterns that promote tumor suppressor function.

The group used Human Promoter 1.0 Arrays in combination with other approaches, such as computerized brain

mapping and adenoviral-mediated re-expression of silenced genes, to examine genes that are abnormally silenced by promoter methylation or histone tail modifications in glioblastoma multiforme. By identifying these epigenetically silenced genes involved in tumor development, the researchers aim to gain a better understanding of the mechanisms underlying malignant glioma growth. Their goal is to be able to diagnose patients, determine the optimal treatment regimen without performing invasive surgery and ultimately to identify targets for new therapeutics to treat the disease.

"We hypothesized that our global investigation would yield additional sensitive and robust markers for disease diagnosis and prognosis," Madan said. "We think they will also provide biological insight into pathways that lead to malignant states."

Madan recently spoke to Noam Shomron, a postdoctoral fellow in Christopher Burge's lab at MIT, about bringing people and data together from different sources in order to gain new insights into the diagnosis and treatment of patients with malignant gliomas.

The two discussed:

- Importance and challenges of looking at epigenetic silencing in malignant glioma
- Tumor suppressor genes uncovered in the study
- Using patient-centered genomic medicine to diagnose and treat glioma patients



Anup Madan, Ph.D.,

is director of the Neurogenomics Research Lab at Swedish Medical Center. He has an affiliated appointment as Senior Research Scientist at the Institute for Systems Biology, Seattle, and as an assistant professor of neurosurgery at the University of Iowa Carver College of Medicine. He received his Ph.D. from the Tata Institute of Fundamental Research in Mumbai, India, and performed postdoctoral research in Dr. Leroy Hood's laboratory at the University of Washington. Dr. Madan works closely with Dr. Greg Foltz, studying the role of epigenetic mechanisms in glioblastoma multiforme (GBM).

Noam Shomron, Ph.D.,

is a postdoctoral associate in Christopher Burge's laboratory at the Massachusetts Institute of Technology. He received his Ph.D. from Tel Aviv University in Israel. His research focuses on post-transcriptional mechanisms of RNA regulation including pre-mRNA splicing and regulation by microRNAs.



A multi-pronged approach for studying malignant glioma

Shomron: What led you to perform the first genome-wide analysis of epigenetic silencing of tumor suppressor genes in malignant glioma?

Madan: Malignant glioma is the most aggressive form of brain tumor in adults. It remains an incurable and rapidly fatal disease. Prognosis for GBM patients is very poor, with only 10 percent of patients living two years after diagnosis.

We need new approaches to treat this cancer. Recently, promoter hypermethylation and epigenetic silencing of the DNA repair gene, MGMT, were identified as markers in a subset of patients with significantly improved survival at two years in response to combined chemotherapy and radiation. It has also been shown that several other genes, critical for cell proliferation and tumor progression, are silenced in association with promoter hypermethylation in malignant glioma.

Despite these recent findings, the genome-wide role and functional significance of individually silenced genes has not been extensively studied. We hypothesized that our global investigation would yield additional sensitive and robust markers for disease diagnosis and prognosis. We think they will also pro-

vide biological insight into pathways that lead to malignant states.

This was the motivation behind developing a global screen for epigenetically regulated genes. We have since identified several genes with potential tumor suppressor function in malignant glioma.

Shomron: Your study incorporated computerized mapping of malignancies, tissue acquisition, microarray analysis, promoter methylation, epidemiological studies and functional characteristics of candidate tumor suppressor genes. What were the most challenging issues in creating and using this multifaceted platform?

Madan: The most challenging issue in creating this platform was bringing all these people with different expertise from different institutions together. It was very difficult to establish the institutional agreements and approvals from Institutional Review Boards, but once we got those established, it was pretty smooth sailing from that point onward.

Rather than taking genes that are differentially expressed by computerized mapping or immunohistochemistry and looking for those genes on our microarray, we first identified the genes and then looked at them on other platforms. That made it much easier to perform these kinds of studies.

Investigating “hypothetical genes”

Shomron: What are the key genes you have identified?

Madan: We found several genes that were already known to be epigenetically regulated in malignant glioma. Initially, we looked at DKK1 and other epigenetically regulated genes. But when we performed the network analysis, we found the majority of genes don't have any function assigned to them. Those genes are not associated or reflected in various pathway analysis programs. We decided to look at these hypothetical genes, which are generally ignored, and see whether they have potential tumor suppressor function or not. These studies will map these genes into known pathways or uncover new pathways that contribute to tumorigenesis.

Shomron: When you say “hypothetical,” you mean they are not named or do not have substantial EST evidence?

Madan: Yes. Basically, some of these are predicted genes. For example, they have names like c1orf124. That means this is a computationally predicted gene on human chromosome 1. Others have names like KIAA1235. In this case there is some EST sequence information, which shows that they exist, but nothing is known about their function.

We are only interested in coding genes at this point in time. Our microarray screen did not look for non-coding genes. So we took all the genes that were up-regulated in response to epigenetic inhibition by pharmacological drugs and looked for histone modifications.

Whenever you do a microarray screen, you find a large number of activated genes. Some of those are activated by direct action of drugs and some are activated by indirect action of drugs, due to the activation or repression of transcriptional factors.

In our study, it was important for us to first dissect the genes that were activated by direct drug action from those activated by indirect action. So we performed a lot of chip assays as well as bisulfite sequencing to identify a subset of genes that are likely to be activated by direct action of the drugs. This can be a tedious job on a genome-wide scale.

Therefore, we used promoter microarrays to identify genes whose promoter regions show desired change in the histone tail modifications after drug treatment.

Finally, we cloned all the genes into expression vectors and evaluated tumor suppressor function by looking for the ability to suppress the growth of malignant cell lines and mouse models.

Shomron: Does that make your study unique in the world of high-throughput epigenetic analysis?

Madan: It does. Another aspect that differentiates our study from others is that we wanted to be as closely related to the patients as possible and therefore used cell lines that were derived from patient tumor samples. Wherever you have immortalized cell lines, they have been passaged through several generations. As a result, a lot of promoter methylation changes take place. If you study those promoters in patients, you don't find them to be hypermethylated compared to non-tumor samples. On the other hand, the immortalized cell lines are easier to work with.

So we investigated both immortalized cell lines and patient-derived cell lines in order to study the effect of epigenetic inhibition on both. This approach greatly increased the fidelity of our results.

Surprising results

Shomron: What proportion of the epigenetically regulated genes are non-tumor suppressor genes?

Madan: That's an interesting question. When we screened 40 hypothetical genes, we were surprised to find only 10 of them had the ability to suppress growth of malignant cell lines. Although the other 30 genes are epigenetically regulated, they may not have a tumor suppressor function. It is possible that these genes alter the expression of other genes with important roles.

Shomron: Was this the most surprising result of your study?

Madan: Yes. In addition, what we found surprising was the role of histone modification. Our studies found that histone modification plays a more dominant role than promoter hypermethylation.

We expected it to be the other way around, so that was a surprise.

Shomron: What criteria did you use to select tumor suppressor genes for further analysis?

Madan: When we looked for tumor suppressor genes, we found that the genes first have to be up-regulated, not only in the immortalized cell lines but also in the 10 patient-derived cell lines, by HDAC inhibition or DNA methyltransferase inhibition. Once we identified those genes, we looked for differential histone marks or differential promoter hypermethylation associated with drug treatments. After we applied those criteria, we narrowed our gene list to a manageable level.

“Another aspect that differentiates our study from others is that we wanted to be as closely related to the patients as possible and therefore used cell lines that were derived from patient tumor samples.”

Then, we confirmed the expression of those genes in a much larger panel of tumor and non-tumor tissue samples by real-time PCR in order to verify that only those genes that met the criteria were selected for further studies.

Finally, we evaluated the ability of these genes to suppress growth of the two immortalized cell lines as well as the ability to suppress tumor growth in nude mice.

Future directions

Shomron: Are you planning to combine your data with additional high-throughput experimental data such as proteome screens or alternative transcription start sites, promoter usage or alternative splicing?

Madan: We are trying to do as much as possible. We are trying to integrate all of the available data into our studies and perform proteome analysis that we will eventually integrate. Changes in the transcriptome are not always reflected in changes in the proteome. So it is very

important to investigate proteome changes and combine that information with our data. Looking at data from these different types of studies may yield insight into the pathways that are perturbed in malignant glioma.

Shomron: What follow-up studies are you planning?

Madan: Now that we have found these genes, we want to get a better understanding of their function and the pathways they are associated with.

We are making retroviral vectors and expressing the genes in glioma cells. Then we perform the microarray studies to determine the secondary level of transcriptional activity modulated by these genes. This information is utilized to

map these unknown genes into known networks or identify new networks that are epigenetically regulated.

Shomron: How many genes did you pick for this study?

Madan: For our first pilot study, we have started to investigate three genes. Once we have some preliminary data, we will look at more genes. Two of the genes are BEX1 and BEX2. We have expressed these genes in glioma cells and identified the putative pathways that are perturbed.

Now we are going back and verifying the bioinformatic data being generated by blocking these pathways in order to understand how these genes act as tumor suppressors. We are also investigating whether there are additional features of the promoter regions, other than the epigenetic regulation, that control the differential expression of these genes in tumor cells.

We are trying to correlate promoter methylation with various therapies. In order to identify differential methylation

in promoters, we have performed bisulfite sequencing in 100 tumor tissue samples and 50 non-tumor tissue samples. We are trying to correlate the data with various treatments. Similar investigations are being performed with promoter regions of 100 other genes to see if we can identify additional diagnostic or prognostic markers.

Shomron: Do you feel you will be able to define a diagnostic signature for identifying glioblastoma multiforme?

Madan: I don't know whether it will be possible to identify diagnostic signatures. This is not a genetic disease. It does not run in families. So we don't really know what our target population will be. However, these studies will help classify patients into different therapeutic regimens. Most patients die within nine months, so the window of opportunity for therapeutic intervention is very small. If you give a patient therapy to which he does not respond, crucial time is lost. If we can identify which patients are likely to respond best to different therapies, these studies will be very helpful. Our aim right now is to be able to classify

patients based on methylation marks.

Shomron: Do you have an estimate for how many genes you would need to accurately profile glioma tissues?

Madan: It's hard to say how many genes will be required for accurate prediction or prognosis for any disease. My guess is that it will be at least 10 genes and we'll have to determine that they are differentially methylated.

A promoter will not be differentially methylated in 100 percent of cases. It might be methylated in 80, 70 or 60 percent of cases. So we can combine the results from six, seven or maybe 10 differentially methylated promoters and use that to predict response to therapy.

Shomron: And do you expect to follow up on these patients using microarrays during the therapy as well?

Madan: Yes. That's a unique part of our study. We are pursuing patient-centered genomic medicine. We will follow the patients through therapy. We collect blood samples to see if we can detect these methylation marks. It is a noninvasive procedure, so patients do not have to undergo surgery.

If a patient has to undergo a second surgery, we collect the tissue samples and run the microarrays again. That gives us an opportunity to see how these patients are responding to certain therapies and how the methylation markers are changing.

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Further Reading

■ Foltz G., *et al.* Genome-wide analysis of epigenetic silencing identifies BEX1 and BEX2 as candidate tumor suppressor genes

in malignant glioma. *Cancer Research*
66(13):6665-74 (2006).