

Breast Cancer Genes: When the Sequence Is Not Enough

Few cancer genes are more notorious than the genes that cause familial breast cancer—BRCA1 and BRCA2. The New York Times described the cloning of BRCA1 in 1994 as “a genetic trophy so ferociously coveted and loudly heralded that it had taken on a near-mythic aura,” but cautioned that since the gene was unexpectedly large, it might take at least a year before a diagnostic test could be developed from it. Fifteen years later, there are indeed genetic tests to evaluate the risk of breast and ovarian cancer in women who possess one of several known mutations. There are, however, even more variants for which the risks are not yet understood. Shyam Sharan, Ph.D., Senior Investigator and Head of the Genetics of Cancer Susceptibility Section in CCR’s Mouse Genetics Cancer Program, understands the difficulties of studying these genes better than many. As a Postdoctoral Fellow, he got caught in the race to understand the BRCA genes by cloning their mouse homologues. That initial sprint turned into a marathon, and although it is far from over, the recently tenured Sharan appears exhilarated by the milestones he has recently passed.

In the four-year period after Mary Claire King’s groundbreaking identification of a region of human chromosome 17 linked to familial breast cancer, *BRCA1* and *BRCA2* were cloned in humans and mice, and their function was linked to DNA repair. This pace was a source of optimism for the field, intense competitive pressure for the scientists involved, and occasional humor. “I went to a Keystone meeting on breast cancer and gave a talk,” remembered Sharan. “After me, Thomas Ludwig also gave a talk about a *Brca2* knockout mouse, and as a joke, I got an award for winning the *Brca2* race by 15 minutes.” However, identifying the genes turned out to be only a first step in both understanding their role in tumorigenesis and predicting which mutations would be oncogenic.

In particular, two puzzles from that time have continued to drive Sharan’s research. The first led from the observation that the known mutations did not seem to cluster into any “hot spots” but were distributed throughout

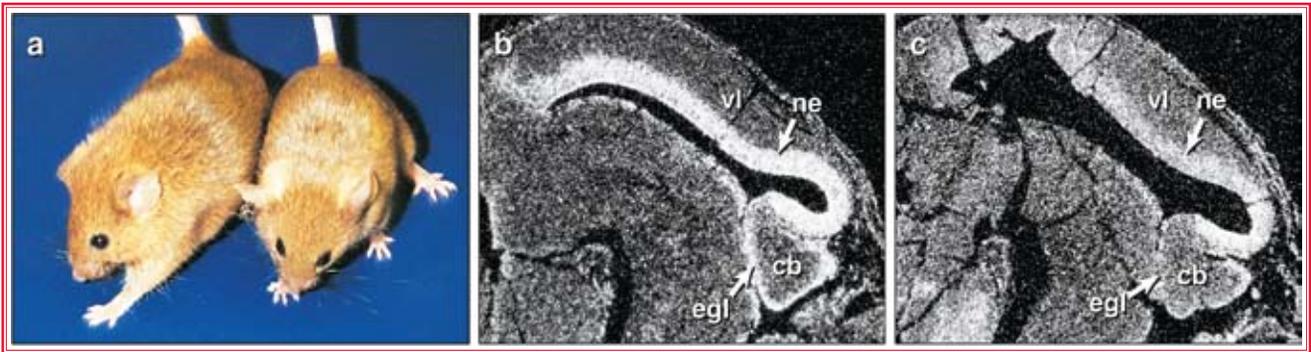


Shyam Sharan, Ph.D.

the gene, suggesting that all regions of the protein were equally important for tumor suppression. With 1,863 amino acids and 3,148 amino acids respectively, *BRCA1* and *BRCA2* are huge proteins (for comparison, hemoglobin, which carries oxygen in the

blood, has 574 amino acids). Sharan and others had identified associations of these proteins with DNA repair, but that was far from a complete functional explanation of these complex proteins. What did the rest of these proteins do, and how

(Photo: R. Bieri)



(Image: S. Sharan, CCR)

Human *BRCA1* is fully functional in mice, and its expression mirrors the mouse *Brca1* gene. In panel a, the *Brca1* mutant mouse (right) rescued by the human *BRCA1* BAC transgene appears indistinguishable from its wild type littermate (left). Panels b and c show an expression analysis of the human *BRCA1* transgene (panel b) and endogenous *Brca1* (panel c) in the brain of a 13.5-day mouse embryo. High level of expression was observed in the neuroepithelium (ne) of the ventricular layer (vl) and the external germinal layer (egl) of the cerebellum (cb), as shown by the arrows.

What did the rest of these proteins do, and how did far-flung mutations contribute to tumorigenesis?

did far-flung mutations contribute to tumorigenesis? The second puzzle stemmed from the seeming paradox that eliminating either protein from mammary cells resulted in cancerous proliferation, whereas disrupting them in embryonic mouse cells resulted in a failure to proliferate and develop. How could a gene involved in something as basic to the cell as DNA integrity cause opposite effects in different cell types?

Sense through Missense

When Sharan came to NCI, he wanted to use mouse genetics to study the functions of the BRCA genes. He knew that most of the identified mutations in BRCA genes came from tumor samples, and it was therefore not surprising that they resulted in tumorigenesis, but Sharan wanted to be able to mutate regions of interest in these genes systematically to study their effects. However, there was a small problem—the mouse and human *BRCA1* genes are only about 60 percent homologous, which means that, in mice, the human gene of interest is already mutated by 40 percent. Nevertheless, Sharan decided to introduce the human *BRCA1* gene into mice. And not

just the gene, but the entire 200,000 base-pair length of human DNA that comprised all of the regulatory elements as well as the gene itself.

“It was kind of risky,” commented Sharan, noting that for the experiment to succeed, the mouse cells would need to contain the necessary cellular machinery to properly regulate the human elements, which was by no means clear. Indeed, a paper that came out just as they were making the first mice examined the regulatory elements in a 2,000 base-pair region of the mouse and human genes without finding any obvious conservation between the species. “But we wanted to express the gene at physiological levels and not hook it to a promoter that would overexpress it...and it actually paid off.” The human DNA was able to completely mimic—or rescue—the missing mouse *Brca1*. Most exciting, the expression pattern of the human gene in these mice was exactly the same as the normal mouse gene, which is expressed ubiquitously in early development and then downregulated in cells that begin to differentiate.

However, the goal was to study mutations introduced into the *BRCA1* gene. With mouse model in hand, the investigators’ next hurdle to overcome was to be able to make targeted point mutations in a large genetic sequence before creating the mouse. Here, Sharan had the help of his colleagues down the hall—Neal Copeland, Ph.D., Nancy Jenkins, Ph.D., and Don Court, Ph.D.—who had recently developed just the recombineering technology he needed to adapt into his own system (see “Science in Singapore: Aiming High for Biomedical Research,” page 26).

“As we started to make mutations, we quickly learned two lessons,” explained Sharan. The first was that mutations that were supposed to be deleterious based on their location in highly conserved (and hence arguably important from an evolutionary standpoint) regions of the gene often had no effect on the mice. Even biochemical data showing disrupted protein-protein interactions of the mutated *BRCA1* could not predict an abnormal phenotype in the mice. The second thing they learned was that several of the deleterious mutations were a result of altered splicing of the gene, effectively knocking it out completely. So, it was impossible to simply look at the amino acid sequence and predict the impact of a single mutation. Every mutation had to be studied individually.

“You can imagine how this could impact my career and my postdoc’s career. Making mice with no phenotypes is not exactly exciting.” The researchers tried everything they could think of to show the effects of their mutations—they aged the mice, made fibroblast cultures from them, and studied them biochemically. And yet, they still found that many of their mutations had no obvious phenotype. They needed to find a better way to screen mutations and to know that what they were looking at were important clues to *BRCA1* function and not just a difference between mice and men.

Embryonic Stem Cells Tell All

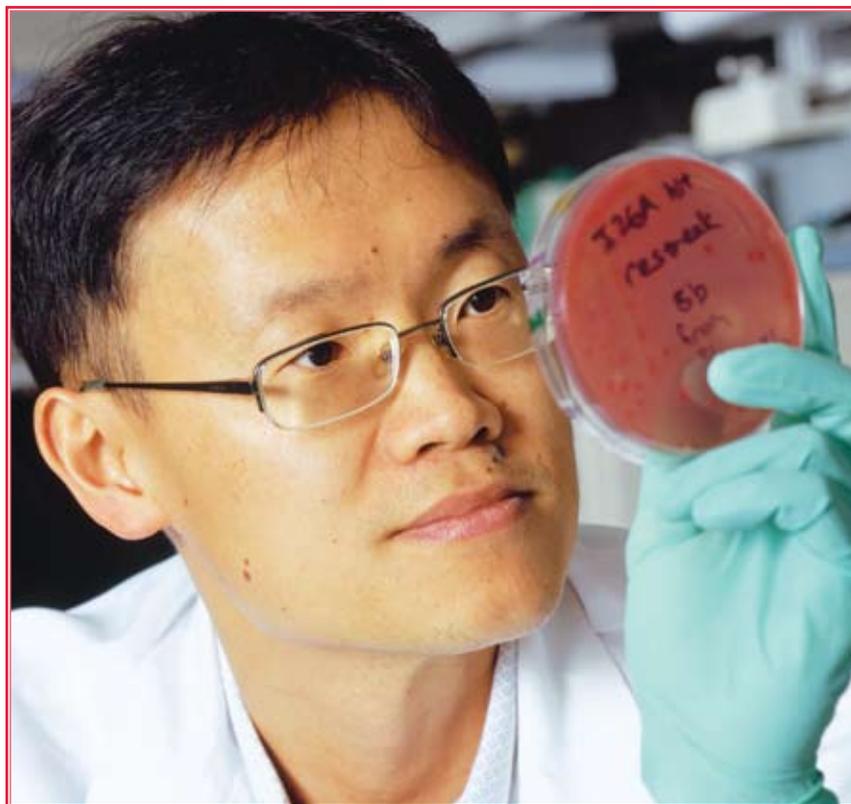
To generate *Brca1* or *Brca2* knockout mice, the first step was to make mouse embryonic stem (ES) cells in which one copy of the gene is disrupted by gene targeting technology. While they were waiting for the

mice, Sharan, at that time a Postdoctoral Fellow in the laboratory of Allan Bradley, Ph.D., at the Baylor College of Medicine in Houston, thought a relatively easy and straightforward next step would be to make mouse ES cells with both copies of the gene missing in order to study the resulting defects. The problem was that he just could not get ES cells that were missing both copies of the gene to survive.

"I wasn't doing anything with these cells," recalled Sharan, "when I one day realized that they could be a powerful system." He recognized that if he made the remaining mouse copy conditional—so that it could be deleted at will—and added in the human gene sequence, the ES cells would only have one human *BRCA* gene remaining once he deleted the second mouse copy. How the cells behaved with only the human *BRCA* gene with or without mutations could tell them a great deal about the individual mutations. If the mutation was neutral, then the cells should survive; if it was deleterious, the cells would die. And with any luck, there would be a range of phenotypes, depending on the specific mutations, that would not affect survival per se but would affect DNA repair or other cellular functions when tested.

Sharan and his Postdoctoral Fellow, Sergey Kuznetsov, Ph.D., first tested founder mutations of *BRCA2*—those highly specific mutations found in families that have remained relatively genetically isolated—that are strongly linked to breast cancer. As expected, the ES cells did not survive. Then they tried mutations or variants that are frequently found in the general population and are, therefore, thought to be neutral. The ES cells appeared normal. Finally, they tried mutations that they thought might be deleterious based on the available literature. As they had found in their mouse models, the majority did not show an effect in their cell-based assay. "We were kind of depressed," Sharan explained candidly. "We thought that the assay might not be sensitive enough."

He then decided to contact Myriad Genetics, the company that first cloned and patented the human *BRCA1* and *BRCA2* genes to develop genetic tests. Myriad Genetics had a database of human mutations from breast tumors



(Photo: R. Bezer)

Postdoctoral Fellow Suhwan Chang, Ph.D.

that included data on whether or not the specific mutation was genetically associated with cancer in families and whether other deleterious mutations were found in the gene that might instead be responsible.

"And that was one of the best days," said Sharan, smiling at the memory. "In every single case, our data matched with the family segregation or linkage data. Where we found it to be neutral, the family data showed it was not segregating with the disease or, in some cases, they found additional deleterious mutations."

Kuznetsov and Sharan continued to develop their assay and tested several additional mutations. In every case, their results matched the outcome predicted from human data. "For a long time, we knew that the assay was working—we were convinced—but we only had cases in which the cells would die completely or they would be normal," explained Kuznetsov. To dissect the functions of these genes, they needed to find mutations that were not so severe that they would cause ES cell death but that could be measured as abnormalities in the cells. Eventually, they found some

examples of such intermediate mutations, but the most memorable example came while the paper they eventually published in *Nature Medicine* in July 2008 was still under review.

These same techniques could be applied to many diseases.

One of the mutations they had studied resulted in a single amino acid substitution of tryptophan for arginine, which they found to be deleterious. A reviewer of the paper was aware of a human variant at this same nucleotide that would change the arginine into a glutamine and, according to the literature, be neutral. "In principle, we could have argued that the phenotype would be different [depending on the exact mutation]," said Kuznetsov,

but the researchers instead took it as a challenge. Could they assay a specific mutation in the three months allowed by the journal for resubmission? The answer turned out to be more interesting than either expected. The cells survived, as predicted by the reviewer, but they had subtle defects depending on the drug researchers used to challenge the cells. However, when they went back to the paper proposing that this mutation was neutral, the researchers realized that it was actually a borderline case according to the scale used by the authors. Furthermore, they were able to show through structural modeling that although the second mutation did not disrupt the conformation nearly as much as the first mutation they had tried, it did not leave the protein undisturbed, arguing for the possibility of subtle functional defects. The resubmitted paper was accepted without further ado.

Sharan and his Research Fellow, Suhwan Chang, Ph.D., have now developed a similar assay for mutations

in *BRCA1*—work that faced its own unique challenges—that is published in the October 2009 issue of *The Journal of Clinical Investigation*. The assays are licensed through NCI and are available as a research tool for clinical scientists interested in characterizing additional human variants of the BRCA genes. Sharan is hopeful that in a world of increasingly available genetic testing, his assays will ultimately help inform the risk of disease. “Our approach is not just limited to *BRCA1* and 2. As long as there is a phenotype that can be studied at the cellular level, these same techniques could be applied to many diseases.”

Variations on a Theme

Although they believe they now have the tools to test any mutation in BRCA genes, Sharan’s goal is not to catalog BRCA mutations. “Understanding why the mutations are deleterious—that’s what I wanted to do, and it’s taken me 10 years to get there.”

Sharan is grateful for the support he has received to allow his research to mature. “This took a long time. In most cases, you get more support once things are published and if it’s in a good journal...so I feel extremely grateful to Bob Wiltout [Director of CCR] who gave me more resources before our work was recognized by others.”

Sharan is particularly interested in the intermediate phenotypes that they have discovered through their assays—mutations in *BRCA1* and *BRCA2* that show subtle signs of chromosomal instability and other cellular abnormalities. In addition to further testing in ES cells, Sharan and his team are planning to return to making mice in order to analyze their most interesting mutations in a whole animal. Chang is focused on *BRCA1*, whose amino acid sequence reveals multiple functional domains. “It is a really interesting project to study because it’s been more than 10 years since *BRCA1* was discovered and people have put a lot of effort into studying it, but we still don’t know its function.”

Sharan also wants to return to the second half of the puzzles that have driven him from his postdoctoral work to his current position—namely, why BRCA mutations have different effects depending on the cell type. “We now have a very simple system—we have ES cells that are dying. What genes are there that make the cell die instead of survive?” His plan is to use the ES cells with altered BRCA as a screen to find other genes that are involved in BRCA-dependent survival. With no shortage of new avenues to pursue, Sharan is aware of the need to stay focused. “Do it right, and slow but steady will win the race.”

To learn more about Dr. Sharan’s research, please visit his CCR Web site at <http://ccr.cancer.gov/staff/staff.asp?profileid=5567>.



(Image: S. Sharan, CCR)

Betty K. Martin, Suhwan Chang, Ph.D., and Kajal Biswas, Ph.D., (left to right, back row) and Dr. Shyam Sharan, Ph.D., and Susan Lynn North work together to pursue the functions of the *BRCA1* genes.