Building a better understanding of prostate cancer

Looking at thousands of genes at once may help quell this common and deadly disease

BY WILLIAM WELLS

GENERAL NORMAN SCHWARZKOPF, SENATOR BOB DOLE, AND singer/actor Harry Belafonte have survived it. Celebrities Frank Zappa, Telly Savalas, and Timothy Leary have succumbed to it. The disease that will kill an estimated 37,000 American men this year—almost equal to the number of women dying from breast cancer—is prostate cancer. It is the most common cancer to afflict nonsmoking men.

Men don't like talking about their prostate, a walnut-sized gland that produces the milky fluid to carry sperm during ejaculation. And anyway, they figure that prostate cancer is a disease of older men (more than 80% of cases are in men over 65 years old), and one that progresses slowly. The scientific world has not been immune to such thoughts, and the result has been inactivity and a stunning lack of data. In 1987, many researchers resisted holding a consensus conference on prostate cancer at the National Cancer Institute (NCI; Bethesda, MD) because there weren't enough data to talk about. By 1996, matters hadn't improved much, and many patients started basing their treatment decisions on a cover article in *Fortune* magazine. The author, Andy Grove, CEO of Intel, had been so flabbergasted by the lack of clear directives in the lay press or even the scientific literature that he had done his own comparative literature search. "It's snuck up on people," said Marston Linehan, chief of urologic surgery at the NCI. "We've been saying for years that it's so important, but most people have only just realized it."

The U.S. Congress has certainly woken up. The Omnibus Appropriations bill for 1999 states that "spending for prostate cancer research over the years has not kept sufficient pace with the scientific opportunities and the proportion of the male population who are afflicted with the disease." Congress directed the National Institutes of Health (NIH) to produce a plan to remedy the situation.

From the NIH report (*1*), released in June 1999, one thing is clear: If curing prostate cancer involves devising acronyms, the NIH has the problem well under control. The agency has formed more working groups and consortia than seems humanly possible.

And yet within the mountain of governmental verbiage is buried a great deal of scien-

Illustration by Curtis Parker

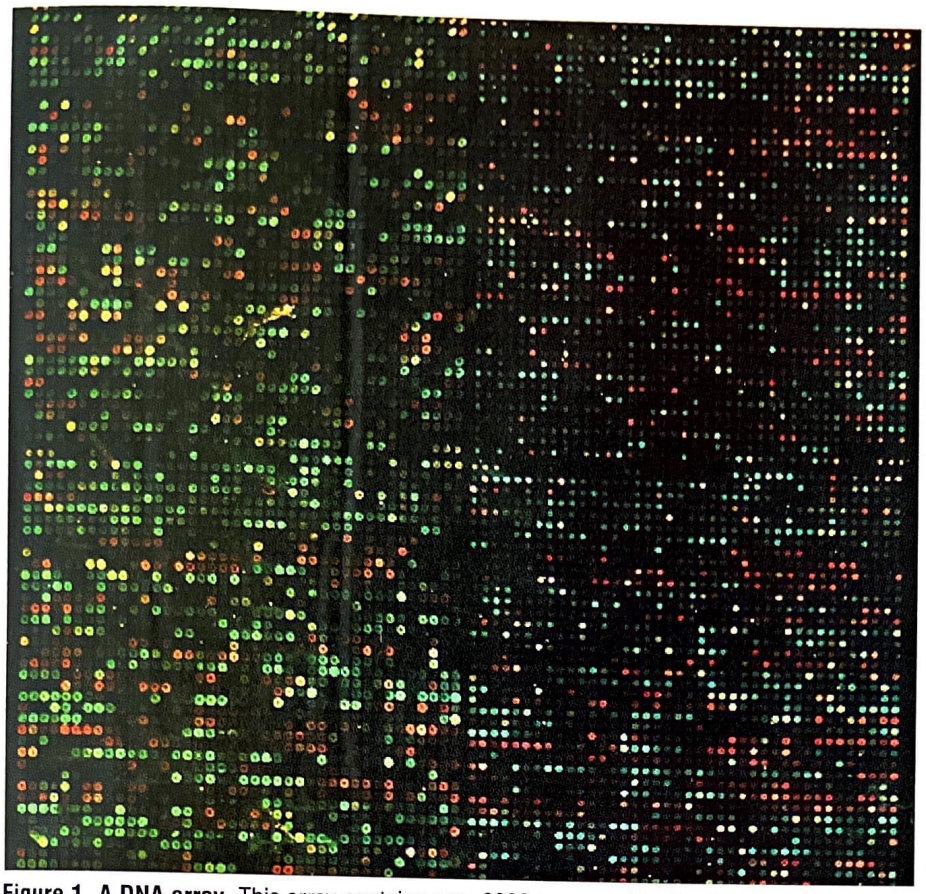


Figure 1. A DNA array. This array contains over 6000 genes—virtually all the genes of the baker's yeast *Saccharomyces cervisiae*—with one gene per spot. Gene activity in cells is measured by collecting mRNA from the cells, converting it to cDNA, and labeling it with either a red dye (e.g., for cells grown in glucose) or a green dye (e.g., for cells grown without glucose). The mixture of cDNAs is then added to the chip, and each cDNA is allowed to stick to the spot that has its corresponding gene. In our example, the red spots represent genes that are turned on in glucose, green spots represent genes that are turned off in glucose, and yellow spots (red plus green) represent genes that do not change their output in this experiment.

tific excitement. The amount of money the government has earmarked for prostate cancer won't hurt—an increase in funding from $114 million in 1998 to $420 million in 2003, plus $60 million from the Department of Defense—but the incremental progress following Richard Nixon's 1971 declaration of "War on Cancer" shows that governmental will and money are not always enough. This time around, however, there is a revolution in genetics that promises to prize apart the workings of cancer. A confluence of DNA sequence information and the new technology of DNA arrays is providing prostate cancer researchers with more leads than they can follow. "This is a great time to devise novel strategies to treat prostate cancer," said the NCI's Michael Emmert-Buck. "The explosion in knowledge is just staggering."

Sequencing ad nauseam

That explosion in knowledge starts with a very tedious activity indeed. Emmert-Buck is part of the Cancer Genome Anatomy Project (CGAP). Since its inception at the end of 1996, CGAP has been churning out DNA sequences with the goal of producing a molecular picture of cancer cells. The idea of random large-scale sequencing of expressed genes was initiated by Craig Venter at the NIH. Venter now directs the Celera Genomics Corporation (Rockville, MD), which has pledged to sequence the entire human genome by the end of 2001.

When Venter started his sequencing efforts at the NIH, there were so few genes sequenced that any sequence was a good sequence. Now, however, CGAP wants to be more focused. The NIH team sequences only genes that are converted into protein, or expressed, in cancerous cells or in the normal cells from which they are derived. As the sequences pile up by the hundreds of thousands, the activity of the cancer cell becomes clearer. "We'd like to be able to look at the complete genetic anatomy of a cancer—not just a single gene at a time but hundreds of genes at a time," said the NCI's Robert Strausberg, who directs the CGAP project. "That would let us determine the most informative changes in a cancer."

To create a useful gene list, the CGAP team must first isolate only those cells it wants to study. Any tissue, including a tumor, is a complicated mass of nerve cells, blood vessels, immune cells, connective tissue, and cancer cells. Emmert-Buck developed a procedure called laser-capture microdissection to pick out the cancer cells and their normal counterparts (*2*). He identifies the cancer cells by histology under a microscope, then pushes a button to trigger a laser pulse. The laser hits a clear film lying on top of the cells, thus fusing the film to the cells. The film is bonded to a vial cap, and so the attached cells can immediately be processed for RNA extraction and cDNA library construction. To view an image of this technique, visit www.ncbi.nlm.nih.gov/CGAP and click on "Methodology", then click on "Laser Capture Microdissection".

The CGAP group has used the prostate tissue from five patients to construct 12 microdissection-based libraries. The list of genes expressed in the prostate has grown to more than 6500, based on over 29,000 sequences. The critical genes are those that are turned on or off as prostate cancer develops. A DNA array is the perfect technology for defining those changes (*3–5*). The full set of genes is arrayed on a glass slide, and cDNA from

Michael Milken's money

The 1980s were good to Michael Milken. Junk bonds fueled his accumulation of a fortune—$550 million in one year alone. But as the decade came to a close, it all came crashing down. In 1989, Milken was fined $1.1 billion for securities fraud and sentenced to jail. The day after his release, in 1993, Milken was diagnosed with metastatic prostate cancer. He was 46.

Within a month of his diagnosis, Milken set up the Association for the Cure of Cancer of the Prostate, known commonly as CaP CURE. To date, the group has dispensed $65 million in research grants and trial funding, and it has recruited patients for Leroy Hood's studies (see main story) by going on *Larry King Live*. Some have argued with Milken's emphasis on late-stage rather than basic research, but he has been credited with prompting the NIH to set up a similar trials-funding system called Rapid Access to Intervention Development (RAID). Milken was put on testosterone-blocking therapy in 1993, and his cancer is still in remission.

normal prostate tissue (labeled red) or cancerous prostate tissue (labeled green) is added to the slide in a DNA hybridization reaction.

After hybridization and detection of the signals, the genes can be divided into three groups. The majority of the genes are expressed in both normal and cancerous tissues, and so the combined signal is yellow (red plus green). Genes that light up as predominantly red are candidate tumor suppressors. These genes are turned off as cancer develops, and they could potentially be replaced with gene therapy. Genes that light up as green, however, are candidate tumor promoters. These genes are turned on as the cancer develops, and their protein products are potential drug targets. See Figure 1 for an example of a DNA array.

The theory is simple enough, but the reality is that hundreds of genes change expression levels at least twofold. Prioritizing the results has become the next big challenge. For the moment, with such a rich pool of genes to choose from, Emmert-Buck is taking a simple approach. "We've been skimming off the top, looking at the changes that are drastic," he said.

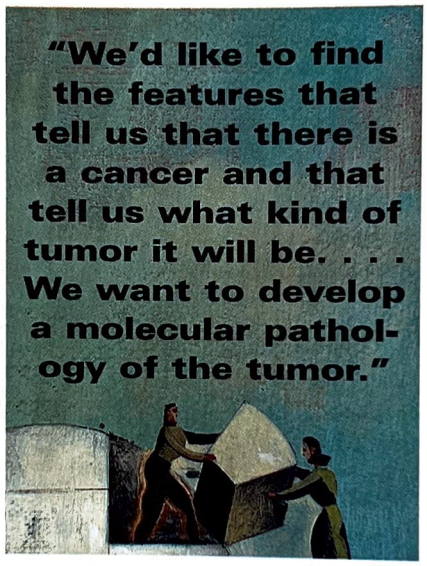
Candidate targets for imaging agents include any cancer-specific cell-surface proteins. Secreted proteins may be useful in a blood test for cancer detection. In choosing a therapeutic target, there are other desirable attributes: a proposed enzymatic activity, possible exposure to the cell surface, and prostate-specificity, which would allow an all-out attack without the concern of side effects. The most attractive candidates are those that have been well characterized in other contexts but not previously implicated in prostate cancer.

Functional testing can narrow down the pool of candidate tumor promoters. The CGAP team is injecting hundreds of genes into tissue culture cells and then transferring those cells into mice. Most genes will disappear as the cells die, but a few genes will drive the cells to form tumors. The researchers will extract the tumors and identify the inserted genes that they contain.

Diagnostic patterns

Although treatment of prostate cancer is the ultimate goal, the most immediate impact of the DNA arrays is likely to be in diagnosis. Physicians and patients would be ecstatic if expression patterns could distinguish the normal from the cancerous, and the aggressive from the benign. Prostate-specific antigen (PSA) is the current standard for diagnosis. PSA is produced and secreted into the blood at low levels by the normal prostate and at high levels by cancerous prostate tissue. But PSA is also produced at high levels in prostates that are old, inflamed, or suffering from a condition called benign prostatic hyperplasia (BPH), which is, as its name suggests, benign.

Many PSA test results fall into the "borderline" range of 4–10 ng/mL, necessitating confirmation with expensive imaging tests and invasive biopsies. Even if there is a tumor, it may be better left alone. Without treatment, the disease will not progress for up to 70% of patients in early phases of the disease. This has led many countries to practice "watchful waiting", which really means "do nothing and hope for the best". Unfortunately, the unlucky 30% cannot be identified even by histological examination of biopsies. In the United States, standard practice dictates aggressive treatment involving surgical removal of the prostate or extensive radiation treatment. The treatments are expensive and involve substantial risks of incontinence and impotence.



"PSA doesn't even tell you that there's a tumor," said Strausberg. "We'd like to find the features that tell us that there is a cancer and that tell us what kind of tumor it will be—how aggressive, how responsive to drugs. We want to develop a molecular pathology of the tumor."

Useful patterns are guaranteed to emerge, according to Patrick Brown of Stanford University. Brown devised the concept of cDNA arrays (*4*). "When two biological samples have differences that are significant enough that we care about them, it is a sure thing that there will be corresponding differences in gene expression," he said. The NCI's Lance Liotta agrees. "We're going to find some things," he said. "It's just a matter of which ones and how many."

Once candidate patterns are identified and narrowed down to a few key genes, the validity and reproducibility of the changes will be tested in larger groups of patients using simpler techniques such as the polymerase chain reaction (PCR). The CGAP team has also developed tissue arrays, which consist of hundreds of tiny tissue samples embedded in paraffin, that are ready to be probed by hybridization reactions with one or a few markers.

If any given change in expression is reproducible, that will be sufficient for diagnostic purposes. The identity of the gene in question, the reason for the expression change, and the complex biology behind that reason do not matter for the change to be useful as a diagnostic.

Gene expression patterns will also streamline drug trials. Enrollment in trials can be limited to patients who have the expression pattern that predicts progress to metastatic disease. And the reversion of an expression profile to a pattern characteristic of a benign state could be an early indicator of treatment success.

Three-dimensional profiling

Comparing cancerous with noncancerous tissues sounds easy enough, but patient samples are messier than that. Both cancerous and noncancerous tissues are heterogeneous. Most patients have multiple independent tumors, only some of which will progress to form metastatic disease. And tissues that look noncancerous under the microscope may have started the progression toward cancer, may be inflamed, or may be reacting to proteins secreted by a nearby cancer. Even if the cells are free of the influence of disease, their expression pattern will vary with their location or function in the prostate.

Emmert-Buck's response to this complexity has been to construct a three-dimensional model of prostate cancer (*6*). He has removed groups of 1000–2000 cells from different regions of diseased prostates, then amplified and processed their mRNA for use on a DNA array. The result is an mRNA expression snapshot of many areas of the prostate. The expression data can be overlayed on histological views of the same tissue, and further information—the cells' protein complement and mutation history—may be added in the future.

One gene at a time

Prostate cancer clusters in families, suggesting that inherited mutations may control susceptibility. But this is not a simple genetic disorder. "Prostate cancer, compared to breast cancer, is so remarkably difficult," said Jeffrey Trent of the National Human Genome Research Institute (Bethesda, MD). The incidence of prostate cancer is so high that clusters can occur simply by chance, and there is no way of differentiating disease that arises sporadically or from different mutations. Finally, the late age of onset makes the construction of multiple-generation pedigrees almost impossible.

Despite these difficulties, large groups of geneticists have implicated regions named HPC1, HPCX, CAPB, and PCaP in inherited prostate cancer. The HPC1 region was defined in 1996 as a stretch of almost 20 million base pairs of DNA. The relevant gene in that region (and the other regions) is yet to be identified, although Trent said that he is "guardedly optimistic that this will be the year." Meanwhile, the genomics company Genset SA (Paris, France) has identified as many as three prostate-susceptibility genes, although it has not disclosed any details about them.

Some groups have suggested that HPC1 does not even exist. Kathleen Cooney is part of the HPC1 team at the University of Michigan, Ann Arbor, and a believer in HPC1, but she said that "some of these regions may not pan out in larger studies. They are at the limit of our ability to detect."

The benefit of finding even a single gene may be great. "With the inherited genes, you are trying to get a handle on the fundamental triggers of the process," said the NCI's Emmert-Buck. The discovery of a gene may point to a pathway that was not otherwise implicated in cancer, and identify a group of individuals who should be monitored more closely or treated more aggressively.

The results will affect diagnostic methods and interpretations. "Right now, we take whatever looks grossly like tumor [for a biopsy]," said Emmert-Buck. "You may compare two men who have a dominant tumor focus and say they look identical, whereas in fact one has an aggressive growth elsewhere." The gene expression heterogeneity may be far greater than is predicted by histology alone. Imaging agents that identify the molecular differences will be vital to distinguish the most aggressive growth in a given prostate.

Prevention and late-stage treatment

Disease prevention is popular with cost-conscious health insurers, but unfortunately, the leads for prostate cancer prevention are scarce. Various studies have found possible correlations between a low incidence of prostate cancer and either a low-fat diet or high levels of sulforaphane (a chemical found in broccoli), selenium (an essential mineral), lycopenes (anti-oxidants found in tomatos), and genistein (an isoflavinoid in soy).

Testing these associations requires large controlled studies, but to see whether there might be an obvious molecular basis to the effects, James Brooks is using DNA arrays. Brooks, a urological surgeon at Stanford University, is working with Brown to determine which genes are turned on when prostate cells are treated with the potentially protective agents. Early results suggest, for example, that sulforaphane induces various Phase II detoxifying enzymes, including the family of glutathione *S*-transferases, which may protect the prostate from mutagenic chemicals (*7*).

Peter Nelson (University of Washington, Seattle) is looking at the other extreme of the disease. Prostate cancer grows slowly in the prostate, but once it spreads to bone it grows rapidly. Initially, blocking all testosterone production can combat this growth. The part of the prostate that the tumor normally grows out of is dependent on testosterone for its growth, and when the tumor first grows, it is also dependent on testosterone for growth. Thus, turning off testosterone production initially starves the tumor of this growth signal. But after 1–10 years of remission, the cells gain the ability to grow without testosterone, after which patients usually live less than 18 months.

There are clues that the testosterone-sensing pathway is still active in the final stages of the disease. It may be turned on by tiny, residual amounts of testosterone or by other signaling proteins that should not feed into the testosterone pathway. Nelson is defining the components of the testosterone pathway by adding testosterone to prostate cells and looking for the changes on a DNA array. The first set of induced genes includes PSA and several genes involved in fat metabolism, which may tie in with the suggestion that a low-fat diet leads to less prostate cancer.

Nelson is using custom-made arrays generated in Leroy Hood's department of molecular biotechnology at the University of Washington, Seattle. The prostate cancer project in that group is complementing the CGAP effort and has approximately 15,000 distinct sequences that are expressed in the prostate that it can use on DNA chips. For researchers with fewer resources, several companies are now producing chips, including the oligonucleotide chips made by Affymetrix Inc. (Palo Alto, CA), and a prostate cDNA chip made by Research Genetics, Inc. (Huntsville, AL).

As chips become less expensive and the NIH money is distributed, expression patterns may become a standard means of characterizing genomewide changes in cancer.

"This is a big deal," said David Botstein, a geneticist at Stanford who is working with Brown to disseminate the chip technology. "Everybody understands the opportunity here, and it's our responsibility to make the best of it."

For more information on cancer genomics

Visit www.ncbi.nlm.nih.gov/CGAP/.

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