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Molecular Epidemiology of Cancer: The Future is Now

By Robert A. Sikes, PhD

Deciphering what makes a normal cell become a tumor cell has been a central theme of cancer research for the last 25 years or more. The central assumption has been that cancer is the result of stable, heritable changes in gene/protein expression or function. Traditional research methods focused on the development of some altered but measurable characteristic of the tumor cell, like invasiveness, that was then used to isolate the protein(s) (e.g., integrins, extracellular matrix, and proteases) involved in that behavior. These genes were typically cloned after the protein was purified and a function for the protein was ascribed. Reverse genetics, a term coined to illustrate the acquisition of a nucleic acid sequence prior to a functional determination or isolation of a protein, has changed the way we search for meaningful changes in gene expression for cancer and other genetic disorders.

Background

So, where did it all start and where is it going? Before the advent of polymerase chain reaction (PCR), the cloning of differentially expressed genes required some form of subtractive or differential screening procedure. This process was expensive, time consuming, usually radioactive, and resulted in the cloning of a large number of falsely positive sequences. In the post-PCR/human genome project era, several rapid and efficient methods for cloning differentially expressed sequences have been developed. The PCR-based approaches include representational display analysis (RDA), differential display reverse transcription PCR (DD-PCR)[1] and serial analysis of gene expression (SAGE).[2] In the middle is CuraGen Corporation's statistically driven DNA Oligonucleotide-direct screening procedure.[3] The technology of high throughput DNA sequencing and associated developments in computer informatics, developed largely for the human genome project, has allowed for the rapid and cost-effective analysis of large numbers of randomly acquired cDNA clones or expressed sequence tags (ESTs).[4,5] The informatics has facilitated the examination of the novelty of those ESTs, allowed for rapid chromosomal assignation, a determination of the likelihood of tissue-specific

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expression, and the development of large microarrays of cDNAs and ESTs[6,7] for the comparison of mRNA expression between two closely related conditions.

Since mRNAs are polyadenylated they can be reverse transcribed efficiently using oligo d(T)N primers. DD-PCR[1] provides specificity to the reverse transcription reaction by the addition of two more bases, oligo d(T)N-XY, onto the reverse transcription primer (so that only a subpopulation of the mRNAs are amplified in any given reaction). Given that there are only three possible choices for the penultimate base X and four possibilities for the last base Y, it should be clear that a single oligo d(T) primer constructed in this manner will only prime one-twelfth of the total mRNA in any given sample. The use of additional, short primers for the 5'-end then further increase the specificity of the product formed during the PCR phase of the reaction. By using combinations of different 5'-primers with the 12 different 3'-anchors, one can efficiently amplify representative species from most of the expressed mRNAs. These products are then resolved in reducing polyacrylamide gels excised, cloned, and sequenced. Initially, this technique gave product ranging between 100-400 bp. Improved DD-PCR techniques now yield nearly complete cDNAs in single runs. We found that to avoid artifacts (false-positive signals), the test samples need to be closely related and the acquired clones' differential expression needs to be confirmed by additional experiments, like RNA blot or western blot if an antibody is available.[8]

Serial Analysis of Gene Expression

SAGE is based on the statistical representation of short oligonucleotides in an mRNA sequence.[2] Velculescu and colleagues found that an oligonucleotide of 9 bp contains enough information to specify an mRNA species. The mRNAs are biotin-oligo d(T) primed, followed by binding to streptavidin beads and separation into two fractions, using different linkers, A and B, on each fraction. These are then cut with another restriction enzyme, blunted, and the two fractions are ligated together. PCR using primers to A and B are then used to amplify all the sequences between A and B. The PCR product is cut with the anchoring enzyme, and the ditags are isolated, concatenated, and cloned. These concatenated or multiples of "ditags" are now ready for direct sequencing. The efficiency is basically one of informatics; that is, since the ditags are in a linear array, many can be sequenced from one concatenated clone to yield information about several expressed mRNAs. The other benefit from SAGE is that the information acquired is quantitative as well as qualitative. In other words, information about the relative abundance of an expressed sequence is given, as well as sequence/gene identity.

High Throughput Application

The human genome project created the need to acquire a lot of DNA sequence quickly—so-called high throughput application. Some investigators, like Liew and associates, invested in this new technology to examine the complexity of mRNA expression in developing organs.[4] In the fetal heart, Liew et al found that almost 47.4% of 3500 ESTs were previously undescribed or uncharacterized. Recently, Nelson and colleagues repeated this type of random cDNA selection and sequencing from the normal human prostate.[5] They examined 1168 cDNA clones to get a profile of prostate gene expression. They were able to detect the presence of prostate-specific genes like prostate specific antigen, human glandular kallikrein 2, prostate specific membrane antigen, and prostatic acid phosphatase. Likewise they found 30% matched only to other ESTs and that 6% were previously undescribed genes. Therefore, about 36% of the ESTs expressed in human prostate tissues have no known function or protein product. Nelson et al, therefore, have laid the groundwork to determine which of these genes' expression changes in prostate cancer.

Research in my laboratory has taken a similar approach to genes isolated from the prostate progenitor, the urogenital sinus. We have examined 728 randomly

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pulled cDNA clones from a murine urogenital sinus library that account for a maximum of 678 unique cDNAs. We have observed a similar frequency of unique or EST-matching cDNAs as described above. When we screen these sequences against the RNA expression by LNCaP, an androgen-sensitive prostate cancer cell line as compared to the RNA expression by C4-2, an LNCaP-derived androgen-independent prostate cancer cell line, there are only 34 candidate genes whose expression profile changes. This represents less than 5% of the cDNAs sequenced. Since the LNCaP-C4-2 cell lines approximate the progression of prostate cancer from androgen sensitive, non-metastatic to androgen independent and highly metastatic, it is possible that these cDNAs correspond to proteins that are involved in prostate cancer progression.

Multigene Microarray Filters

Another direct result of these large-scale sequencing projects is the acquisition of many cDNA or PCR clones encoding both known and unknown genes. The most recent application of this material has been the development of multigene microarray filters for use in hybridization based experiments to determine differential expression of a gene in almost any given system.[9] The filters, or DNA microchips, have been robotically spotted with small aliquots of cDNA or PCR fragments corresponding to as many as 10,000 individual genes or ESTs,[6,9] and there is promise of larger arrays to come. The goal is to be able to quickly and efficiently screen the estimated 100,000 genes expressed by humans to determine what factors and timing influence their expression. The filters usually include control genes as landmarks/reference points so that the signals that change can be easily correlated with a gene spot or clone identity. Positive clones can then be ordered from the source for the investigator's use. Many filter-based microarrays are now commercially available from companies like Clontech or the IMAGE consortium. Caution must be exercised because these systems still have a low level of false-positive signals for differential expression and confirmation by other techniques is still recommended.

Molecular Epidemiology

The end result of these efforts is molecular epidemiology. Biomedical researchers can rapidly screen the expression changes that occur between normal and diseased tissues. The genes expression changes that occur between these two states can then be rigorously studied to determine their role in the disease development and progression and their potential use as diagnostic/prognostic markers, or they may be developed into therapeutic targets. The increasing knowledge of the human genome and the increasing density of DNA microarrays will eventually give us the ability to acquire a complete molecular snapshot of a diseased tissue. These technologies represent a promise to provide new tools for physicians to help guide therapeutic options and to provide new therapies. ❖

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Nuclear Matrix Proteins and Cancer

By Badrinath R. Konety, MD, and Robert H. Getzenberg, PhD

Cellular hallmarks of the transformed phenotype include abnormal nuclear shape, altered patterns of chromatic condensation, and the presence of abnormal nucleoli. Nuclear structural alterations are so prevalent in cancer cells that they are commonly used as a pathological marker of transformation for many types of cancer. Nuclear shape is thought to reflect the internal nuclear

structure and processes and is determined, at least in part, by the nuclear matrix.[1] The nuclear matrix has been demonstrated to play a central role in the regulation of important cellular processes such as DNA replication and transcription.[2] The nuclear matrix is the framework or scaffolding of the nucleus and consists of the peripheral lamins and pore complexes, an internal ribonucleic protein network, and residual nucleoli.[3] The nuclear matrix consists of approximately 10% of the nuclear proteins and is virtually devoid of lipids, DNA, and histones.[4]

The structural components of the nucleus are known to have a central role in the specific topological organization of DNA. DNA in the nucleus is not randomly organized, and although only approximately 10% of the DNA actually encodes proteins, only specific genes are positioned in a manner that permits the expression of both housekeeping and cell type specific genes. Nuclear structure is, therefore, involved in both this topological organization of DNA and the functional aspects that coincide with this organization.

The majority of known nuclear matrix proteins (NMPs) are common to all cell types and physiologic states. In addition, some NMPs appear to be unique to certain cell types or states, and NMPs have been shown to serve as a "fingerprint" of cell or tissue types. Mitogenic stimulation and the induction of differentiation have been demonstrated to alter the composition of nuclear matrix proteins and structure.[5,6] Differences in NMP composition have been found in a number of human tumors, including prostate,[7,8] kidney,[9] breast,[10] colon,[11] head and neck,[12] and bladder.[13]

Identification of Prostate Specific NMPs

Previously, we investigated the nuclear matrix protein composition in a rat model of prostate cancer. We initially examined the NMPs of the normal rat prostate in comparison with rat prostate adenocarcinoma lines from the Dunning R3327 rat model of prostate cancer. We demonstrated that the NMP composition of transformed cell lines differed significantly from their tissue of origin, and while these transformed prostate cell lines were almost entirely composed of a common set of NMPs, there were differences that would distinguish cell lines of different degrees of the transformed phenotype from one another.[7] We have been successful at sequencing as well as raising antibodies against several of the prostate cancer specific NMPs and those that were able to differentiate metastatic and non-metastatic tumors. These prostate cancer specific NMPs are being developed as potential diagnostic/prognostic markers for prostate cancer.

In a similar fashion, we have also analyzed the NMP composition of human prostate tumor and normal human prostate tissue.[8] We compared the NMP patterns for fresh prostate, benign prostatic hyperplasia (BPH), and prostate cancer from 21 men undergoing surgery for clinically localized prostate cancer or BPH. We identified, by molecular weight and isoelectric point, 14 different proteins that were consistently present or absent among the various tissues. One protein (PC-1), a Mr 56,000 protein with an isoelectric point of 6.58, appeared in all of 14 nuclear matrix preparations from different prostate cancer patient specimens and was not detected in normal prostate (0 of 13) or BPH (0 of 14).

Identification of Bladder Cancer Specific NMPs

The NMP composition of normal and tumor (transitional cell carcinoma) bladder tissue has also been analyzed.[13] In the initial study, tumors of various grades and stages were used. Only tumor samples that could be clearly identified by the pathologist as containing approximately pure populations of the stated tumor grade were utilized. Normal bladder tissue samples that were obtained from organ donors were also analyzed. The NMPs were extracted and separated by high resolution two-dimensional gel electrophoresis. All tumors were found to express differences in their nuclear matrix composition when compared with the nuclear matrix composition of the matched normal tissue from the same bladder. There are several notable differences in nuclear matrix composition of the bladder tumor when compared to the normal tissue. We have identified six proteins (BLCA-1 to BLCA-6) that are present in all of the tumors and are absent in the adjacent normal tissue and three proteins (BLNL-1 to BLNL-3) that are found in all of the normal bladder tissue samples and are missing in the tumor samples. These differences appear to be unique to bladder cancer in that the molecular weights and isoelectric points of the proteins do not appear to correspond to those proteins previously reported to be different in prostate and breast cancers.[13] We have also developed an immunoassay which can be used to detect the presence of one of the bladder-specific NMPs, BLCA-4, in the urine of patients with bladder cancer. This NMP is absent from the urine of normal individuals. We are in the process of developing an NMP-based diagnostic test for bladder cancer.

A diagnostic test for bladder cancer based on the detection of NMPs in the urine is currently available. The NMP22 test (Matritech Inc., Newton, MA) detects the presence of nuclear mitotic apparatus protein (NUMA), which is present in most dividing cells during mitosis. This protein however, is not specific for bladder cancer. The test has been used to detect bladder cancer in patients being monitored for recurrent disease after therapy for bladder cancer.[14] While the test is more sensitive than urine cytology (the routine diagnostic test used to

detect bladder cancer), it does not appear to have consistent specificity.

Identification of Renal Cancer Specific NMPs

Using a strategy similar to that employed in prostate and bladder cancer, NMPs unique to renal cell carcinoma have also been identified.[9] Using matched normal and tumor kidney tissue obtained from 17 patients undergoing radical nephrectomy, five NMPs exclusive to renal cancer were identified. One NMP was found only in all the normal kidney samples examined. All of these NMPs were found to be different from those detected in other tumors. Further studies are necessary to determine if any of these NMPs can be used as diagnostic or prognostic markers for renal cell carcinoma.

The relative success of the NMP22 test suggests that nuclear matrix proteins can be used for the diagnosis of tumors by identifying the proteins in body fluids such as urine and serum. Similar but more sensitive and specific diagnostic tests are under development for cancers of the prostate, bladder, and kidney and are expected to become available in the near future. *(Dr. Getzenberg is Director of Research, Prostate and Urologic Cancer Center, University of Pittsburgh Cancer Institute.)* ❖

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Apoptotic Dysregulation in Primary and Metastatic Breast Cancer

By P. Mojica, MD, and E.M. Mora, MD, MS

The process of programmed cell death or apoptosis plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms. The mechanism is activated when a signal and/or insult, such as viruses, toxins, irradiation, and reactive oxygen species, damage DNA. This process is initiated and completed in an orderly manner through the activation and synthesis of gene products necessary for cellular self-destruction. Developments in the apoptosis field over the past few years have provided a new perspective on how cell populations are normally maintained at equilibrium and have revealed how defects in cell death regulation can also contribute to the development of malignancy.

Apoptic-Cascade Classifications

The apoptotic cascade has been classified in three different groups of proteins. The first is the tumor necrosis factor (TNF) family, which includes membrane-bound proteins that send signals through second messengers to the nucleus for apoptosis to occur. Second, is the cysteine aspartate-specific proteases (caspases) family, which is free in the cytosol and act as intermediates in

the apoptotic cascade. Finally, we have the Bcl-2 family of proteins, which are mitochondria-associated proteins that are intermediate between intracellular signaling from the nucleus and the caspase family.

The TNF family of proteins comprehends many different receptors. They interact and coordinate reactions from the environment to the nucleus in an orderly fashion through a signal transduction cascade. One such receptor is CD 95 (also called Apo-1 or Fas receptor), which plays a key role in apoptosis in lymphocytes and other cells.[1] This receptor contains a ligand, Fas-L, that interacts with a protein-tyrosine phosphatase (Fap-1)[2] and protein tyrosine kinase,[3] which activates apoptosis by second messenger mechanisms inside the cell. Other, downstream targets of CD 95 also appear to include FADD/MORT-1, caspase 8, caspase 3, and ICE-like protease, which act on other cytoplasmic components that morphologically demonstrate apoptosis.[1] In MCF-7 breast cancer cell line, the estrogen receptor plays a key role in the regulation of the apoptotic cascade. The apoptotic mechanism is inactivated upon estrogen stimulation, which leads to increased levels of Bcl-2 mRNA and increased survival.[4] Like CD 95 and estrogen receptor, there are other cell-surface receptors with similar roles that interact with second messengers. What type of receptor participates in this cascade depends on the cell type. In this way, external stimuli contribute to tumorigenesis and apoptotic regulation.

The caspase family of proteins plays a key role in the effector pathway to apoptosis. Ten different proteins in this family have been identified. All caspases are synthesized as an inactive precursor. These proteins are activated when cleavage at specific aspartate residues is followed by assembly into heterotetramers, which results in the active forms of the enzyme.[1] These are activated by the liberation of cytochrome C and apoptotic initiation factor (AIF) from the mitochondria upon Bcl-2 family activation or during TNF receptor family activation. Also, the caspase family can be inactivated by the Bcl-xl protein in a tertiary complex with caspase-9 and APAF-1.[5] This is the common pathway by which extracellular and intracellular stimulation converge to one end-effector mechanism that ends with the fragmentation of DNA. One of the final substrates of this cascade is poly-ADP-ribose-polymerase (PARP), which is associated with nuclear DNA fragmentation leading to apoptosis. There is a strong correlation between the rate of internucleosomal DNA fragmentation and poly-(ADP-ribosyl)ation of histone H1 during apoptosis, which suggest that PARP activation increases the susceptibility of chromatin to cellular nuclease(s) and DNA degradation.[1,6] In fact, inhibition of this end-effector cascade results in increase survival of tumor cells which in turn, promotes tumorigenesis.

The Bcl-2 family of proteins is a critical regulator of cell death pathway. This family of proteins is localized in the mitochondria. To date, 14 cellular homologs in this family have been identified, including the pro-apoptotic proteins (Bax, Bcl-xs, Bad, Bak, Bik, and Bid) and anti-apoptotic proteins (Bcl-2, Bcl-xl, Mcl-1, A1/Bfl-1, Bcl-W, Nr-13, and Ced-9).[6] These proteins interact with each other, forming heterodimers that regulate the fate of the cell. Essentially, the pro-apoptotic proteins of this family mediate apoptosis by increasing the plasma membrane permeability, reactive oxygen species, and liberation of cytochrome C and AIF to the cytosol, which are strong caspases activators.[7,8] The anti-apoptotic proteins interfere with these events and block the apoptotic cascade.

Mediators of Tumorigenesis

Studies have shown that cells of normal breast tissue pass through apoptotic events during the menstrual cycle.[4] This process takes place during the peak levels of estrogen and progesterone stimulation. It is during this time that dysregulation of apoptosis can lead to tumor transformation and proliferation. Studies using breast cancer cell lines have been used as a primary model system in vitro and in vivo to examine apoptotic dysregulation and its relation to the pathogenesis of cancer.[4] Some studies demonstrate that the ratio between levels of pro-apoptotic proteins and anti-apoptotic proteins correlates with tumor formation. Bargou and associates found that strong expression of Bax was observed in normal breast tissue;[9] in contrast, only weak expression was found in its malignant counterpart. This correlates with prolonged survival and decreased apoptosis. Also, the anti-apoptotic proteins of the Bcl-2 family have been implicated in the pathogenesis of tumorigenesis in breast tissue. Bcl-xl protein was overexpressed in invasive breast cancers when compared with adjacent breast tissue.[10] Other mediators of tumorigenesis are the tyrosine kinases, epidermal growth factor, and Her-2, which are overexpressed in up to one-third of breast cancers. Their overexpression had been associated with poor clinical outcome.[4]

Dysregulation of apoptosis is not only implicated in the tumorigenesis of breast tissue, but also has important implications in treatment response. One of the proposed mechanisms by which apoptotic dysregulation contributes to decreased sensitivity to chemotherapy is that the resulting increase in cell survival provides the cell with time to repair any damage done by chemotherapeutic agents. Overexpression of anti-apoptotic proteins, like Bcl-2 or Bcl-xl, has been shown to result in reduced

sensitivity to the inhibitory effects of chemotherapy.[11] These findings also support the fact that not only the absolute number of anti-apoptotic proteins, but also the balance between pro-apoptotic and anti-apoptotic proteins determines the final fate of the cell. In fact, a decreased ratio of pro-apoptotic to anti-apoptotic proteins had been correlated with a decreased sensitivity to various chemotherapeutic agents. Manipulation of the expression of the apoptotic proteins in vitro has changed the sensitivity of some cancer cell lines to chemotherapy. One example is the overexpression of the pro-apoptotic protein Bcl-xs in the MCF-7 cell line, which is associated with increased sensitivity to chemotherapy-induced apoptosis.[12] Also, upregulation of the death-promoting gene Bax in breast cancer cells sensitizes the cells to drug-induced apoptosis.[13] In the future, apoptotic manipulation by increasing the pro-apoptotic proteins or decreasing the anti-apoptotic proteins may give new targets for adjuvant therapy in cancer patients by increasing the sensitivity to chemotherapy drugs.

Possible Role of Bcl-xl

In our laboratory, we have studied the role of apoptotic dysregulation and metastatic potential of breast cancer cells. Comparing metastatic breast cancer cell lines from pleura and bone, we found that the protein Bcl-xl was overexpressed in the bone-metastatic cell line compared to the pleural-metastatic cell lines MDA-231 and MCF-7. Other Bcl-2 family proteins (Bcl-2, Bcl-xs, Bad, and Bak) did not show any significant difference in their expression in cancer cell lines. This finding suggests that the anti-apoptotic protein Bcl-xl may play an important role in the mechanism of metastases. Recent studies by Olopade and colleagues has shown that overexpression of Bcl-xl is associated with high tumor grade and nodal metastases in breast cancer cell lines and primary untreated breast carcinoma.[10] This suggests that Bcl-xl may have a key role in the progression from tumorigenesis to metastases. We still need to delineate the mechanism by which Bcl-xl contributes to breast cancer metastases to bone. Future experiments will focus on the manipulation of the apoptotic cascade.

In summary, studies have shown that apoptosis not only plays an important role in the normal development of breast tissue during the menstrual cycle, but also is intimately related in the process of tumorigenesis and metastatic potential in breast cancer. Several studies suggest that important components of the apoptotic cascade are differentially expressd between normal, primary, and metastatic malignant cells. This difference contributes to the aggresiveness of the tumor, making it more resistant to current chemotherapy, which sometimes can be indicative of poor clinical outcome. The current challenge is to identify the apoptotic mechanism, activators, and inhibitors that are important in the pathogenesis of breast cancer, since their identification could provide opportunities for the development of new preventive and therapeutic approaches. Also, the identification of important mechanisms in the progression of tumorigenesis has the potential to define metastases and apoptotic markers that can serve as prognostic or predictive factors in breast cancer diagnosis and treatment. *(Dr. Majica and Dr. Mora, University of Puerto Rico, Medical Sciences Campus, School of Medicine, Department of Surgery. San Juan, Puerto Rico.)* ❖

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p16, The Cyclin-Dependent Kinase Inhibitor in Prostate Cancer

By Cheryl T. Lee, and Carlos Cordon-Cardo

The ink4a gene maps to the 9p21 region, and was initially described as encoding a 148 amino acid protein termed p16. The p16 protein exclusively associates with Cdk4 and Cdk6, inhibiting their complexation with D-type cyclins, and the consequent phosphorylation of the product encoded by the retinoblastoma gene (RB), *pRB*. This interaction contributes to cell cycle arrest. In prostate cancer, the role of p16 has not been well elucidated, though analyses utilizing microsatellite markers in the vicinity of the *INK4A* gene have revealed loss of heterozygosity in a subset of primary and metastatic prostate tumors. However, unlike other primary tumors, p16 inactivation, either through deletions, mutations, or through promoter methylation, appears to be an infrequent event in prostate cancer. Nevertheless, we have recently observed that a subset of prostate cancer patients demonstrates overexpression of p16. Moreover, these patients have a poorer clinical course. The mechanism for this association has not been fully elucidated, although androgen depletion and/or alterations in the RB pathway may contribute to the inactivation of p16-mediated tumor suppressor activities.

p16

The *INK4A* gene maps to the short arm of chromosome 9 (9p21) and was initially described as encoding a protein of Mr 15,845, termed p16.[1,2] The p16 protein forms binary complexes exclusively with Cdk4 and Cdk6, inhibiting their kinase activity and subsequent pRB phosphorylation during the G1 phase of the cell cycle.[1,3] Additional complexity results from the presence of a second *INK4A* product termed $p19^{ARF}$.[4] The $p19^{ARF}$ protein has recently been shown to interact with mdm2 and to block mdm2-induced p53 degradation and transactivational silencing.[5] The two products, p16 and p19ARF, share exons 2 and 3 of the *INK4A* gene, but have distinct promoters and exon 1 units: exon 1α (p16) and exon 1β ($p19^{ARF}$). The *INK4A*-α gene encodes p16 and is mutated in a wide variety of tumor cell lines and certain primary tumors.[2] In addition, methylation of the 5' CpG island of the exon 1α promoter region is a frequent mechanism of p16 inactivation in primary tumors.[6]

p16 and Prostate Cancer

The precise role of p16 in prostate cancer development and progression is not well understood. Unlike other primary tumors and cell lines, p16 inactivation, either through deletions, mutations, or promoter methylation, appears to be an infrequent event in prostate cancer.[7-12] Homozygous deletions of the *INK4A*-α gene do not occur in six of the prostate cancer cell lines available, including LNCaP, PC3, DU145, TSU-Pr1, PPC-1, and DuPro-1[8,9,11,13] However, a missense mutation has been reported for DU145 cells.[9,11] As stated above, it is known that the transcription of the *INK4A*-α gene can be inhibited by promoter methylation. In PC3, TSU-Pr1, and DuPro-1 cells, lack of p16 mRNA expression has been associated with methylation of the promoter region of the *INK4A* exon 1α, effectively inactivating p16.[11,13] Induction of p16 mRNA was subsequently accomplished by treating these cells with 5-Aza-2'-deoxycytidine, a demethylating agent acting through inhibition of 5-methyltransferase. In contrast, LNCaP cells were reported to have an unmethylated *INK4A* exon 1α and expressed a p16 mRNA product.[11,13,14]

In primary prostate tumors, mutations and deletions of the *INK4A*-α gene are also infrequent, with alterations reported in 0-6% of cases.[7-10,12] However, microsatellite analyses utilizing markers in the vicinity of the INK4A gene, have revealed loss of heterozygosity in a subset of 12 of 60 (20%) primary and 13 of 28 (46%) metastatic prostate tumors.[11] The significance of this finding is not clear, as the 9p21 locus is quite complex, producing at least three tumor suppressor genes: 1) the *INK4A*-α product, p16; 2) the *INK4A*-β product, $p19^{ARF}$; and 3) the *INK4B* product, p15, another cyclin-dependent kinase inhibitor. Unlike prostate cancer cell lines, promoter methylation appears to be an uncommon event; DNA extracted from non-microdissected primary tumors revealed methylation in only 5-12% of cases.[11,12]

p16 Expression

We have recently reported the patterns of p16 expression in normal and malignant tissues, including 88 primary tumors. This study was conducted using in situ hybridization and immunohistochemistry assays in order to determine the status of the *INK4A* exon 1α

transcripts and levels of p16 protein, respectively.[15] Associations between altered p16 phenotypes and clinicopathologic variables were also studied to further define their potential implications in prostate cancer. Clinicopathologic variables included pre-treatment prostate-specific antigen (PSA) level, Gleason grade, pathologic stage, hormonal status, and biochemical (PSA) relapse after surgery.

We found that the levels of p16 expression and INK4A exon 1α transcripts in normal prostate and benign hyperplastic tissues were undetectable. However, two distinct p16 phenotypes were observed in primary prostatic adenocarcinomas. Most tumors were found to have undetectable or very low levels of p16 protein expression (Group A, 57% of cases). This was associated with low levels or absence of INK4A exon 1α transcripts. Another group of tumors showed elevated p16 protein expression (Group B, 43%), which was consistently associated with increased *INK4A* exon 1α transcripts. Based on these results, we concluded that upregulation of the *INK4A*-α gene led to p16 protein overexpression. Overexpression of p16 was associated with a higher pre-treatment PSA level (P = 0.018), the use of neoadjuvant androgen ablation (P = 0.001), and a sooner time-to-PSA relapse after radical prostatectomy (P = 0.002). A trending association of p16 overexpression with higher pathologic stage was also observed (P = 0.087). These data suggest that p16 overexpression is associated with tumor recurrence and a poor clinical course in prostate cancer patients. In support of this postulate, another study dealing with prostate cancer has also reported an association between p16 overexpression and poor outcome, as related to biochemical failure.[16]

The expression data demonstrate that normal prostate tissue and most primary tumors have undetectable levels of p16, both at the protein and transcript levels.[15] This negative phenotype could not be explained by molecular analyses, since the *INK4A*-α gene is infrequently altered in prostate cancer. As a matter of fact, it can be deduced that the negative phenotype observed in most primary tumors corresponds to the normal physiologic state of p16. This is further supported by our observation that prostate cancer patients with low-to-undetectable p16 have a less aggressive behavior.[15] This is not the case in other neoplastic diseases, since it has been reported that certain tumors with diminished p16 protein levels, such as non-small cell lung cancer, lymphoma and melanoma, tend to have a more aggressive clinical course.[17-19]

The upregulation of the *INK4A*-α gene, resulting in the overexpression of p16 protein, may develop through different mechanisms. An association between increased p16 transcript and protein levels occurs in tumor cell lines and certain primary neoplasms that lack functional pRB.[1,20] Moreover, p16-mediated inhibition of cell cycle progression appears to be dependent upon functional pRB. These data support an association between p16 and pRB, where absence of functional pRB limits p16 activity and possibly promotes *INK4A*-α upregulation. Alternatively, enhanced activation of the *INK4A*-α gene may occur. E2F1, a direct activator of the *INK4A* exon 1β promoter, does not directly activate *INK4A*-α transcription.[22] However, evidence does exist for an indirect effect, as E2F1 overexpression has been reported to markedly increase p16 transcripts and p16-related cyclin-dependent kinase inhibitor activity. Overexpression of cyclin D1 and/or Cdk4 may also influence p16 expression, through a compensatory feedback loop where deregulation of cyclin D/Cdk4 complexes results in increased levels of p16 protein.[24,25] In summary, it appears that an altered RB pathway could trigger p16 overexpression in certain tumors.

Cellular Stress may Trigger Overexpression

Cellular stress produced by altered androgen levels may also trigger p16 overexpression. In prostate cancer patients treated with neoadjuvant androgen ablation prior to radical prostatectomy, overexpression of p16 protein was observed in 71% of hormone-treated vs. 26% of hormone-naïve patients (P = 0.001).[15] These data suggest that p16 expression may be enhanced by androgen depletion. Androgens are known to modulate the expression of other cyclin-dependent kinases, such as p27/KIP1 and p21/WAF1.[26] In addition, it has been reported that the presence of androgens triggers downregulation of p16 in LNCaP cells—a finding consistent with the observation that p16 overexpression occurs at a greater frequency in cases of androgen ablation.[27]

The successful transduction of p16 using adenoviral vectors has demonstrated a reduction in the viability of prostate cancer cells in vitro, as well as a decrease in the growth of prostate tumor implants in nude mice. PC3 cells transfected with a p16 expression vector underwent a 70% reduction in cell number when compared with parental and control vector-transfected PC3 cells.[28] Similarly, when PPC-1 tumor cells implanted into nude mice were treated with a p16 expression vector, a reduction in tumor size and longer animal survival time were demonstrated.

Clinical Significance

The clinical significance of p16 in prostate cancer is not yet elucidated. Point mutations or deletions are infrequent causes of p16 inactivation. However, p16 overexpression appears to represent an altered phenotype,

which identifies a subgroup of patients with a higher likelihood of post-surgical recurrence. Though p16 acts as a negative cell cycle regulator, specific mechanisms may contribute to its altered expression, overcoming p16-mediated tumor suppressor activities. Future studies are necessary to better understand the mechanism of p16 overexpression relative to androgen depletion. Clinical trials aimed at transducing wild-type tumor suppressor genes, such as *p53* and *p16*, are being pursued. It is too early to foresee the impact of such novel therapies in patient management. As in any emerging field, obstacles impose limitations that are usually overcome with experience and advances in technology. *(Dr. Lee is Fellow, Urology Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY; Dr. Cordon-Cardo is Director, Division of Molecular Pathology, Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY.)* ❖

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Funding News

Several non-governmental organizations offer research support grants that are either provided to support work on a broad range of cancer-related subjects or are directed at specific types of cancer or patient populations. While a few funds are generally structured to support "all comers," the majority are intended for a specific target, such a postdoctoral fellow or junior faculty pursuing a project on a specific subject. The following is a brief list of some of the available sources of funding which we will continue to expand in future issues of *Cancer Research Alert.*

The Lymphoma Research Foundation of America (LRFA) was founded seven years ago with a commitment to funding clinical and basic science lymphoma research at top universities and cancer centers across the nation. It is hoped that breakthroughs in lymphoma treatment will offer the key to understanding and curing other forms of cancer. **Research grants** are awarded yearly and cover the period July 1-June 30. These fellowship awards provide funding for third-year researchers to encourage young investigators to pursue a career in lymphoma research. To date, the Foundation has funded 58 projects.

The grant applications consist of a detailed description of the research project, its goals and relevance, plus letters of endorsement from the applicant's research supervisor. Applications are carefully reviewed by a scientific review board and judged on scientific merit and their potential to improve our understanding of lymphoma and its treatments. Highly technical projects are sent to independent lymphoma experts for peer review.

Grant applications for funding year 2000-2001 will be available in August, 1999. To receive additional information or to receive LRFA's fellowship guidelines, send a request by e-mail to LRFA@aol.com or by fax to the Foundation at (310) 204-7043. Further information is available at www.lymphoma.org.

The American Philosophical Society (APS) awards a limited number of Clinical Investigator Fellowships for research in clinical medicine, including the fields of internal medicine, neurology, and pediatrics. This award is intended to support patient-oriented research.

APS fellowships are awarded to those who have had a MD/PhD degree for less than six years. This is generally intended to be the first post-clinical fellowship, but each case will be decided on its individual merits. Preference is generally given to candidates who have not more than two years of postdoctoral training and research. Applicants must expect to perform their research at an institution in the United States under the supervision of a scientific adviser, and essentially 100% of the fellow's time will be devoted to research. Additional salary may be granted by the institution at which the fellow is located, from another fellowship, or from a similar award during the tenure of the fellowship. Candidates are to be nominated by their department chairman and, as a general rule, no more than one fellowship will be awarded to a given institution in the same year of competition.

Stipends for the fellowship are $50,000 for the first year and $50,000 for the second year. The term of the fellowship is one year, with renewal for one year if satisfactory progress is demonstrated. Applications for first-year fellowships are due no later than September 1, and a written decision will be mailed to candidates in January. Foreign nationals who wish to apply may write directly to their scientific advisers and ask their advisers to contact the Society. Application forms are available at the APS website at www.amphilsoc.org or by writing to: Clinical Investigator Fellowship Committee, American Philosophical Society, 104 South Fifth Street, Philadelphia, PA 19106.

The Cancer Research Institute's (CRI) programs are designed to support basic and clinical research focused on the link between the immune system and cancer and developing immunological approaches to therapy. Traditionally, CRI has focused its funding on melanoma and prostate cancer, however, they recently have broadened the scope of funding to include grants

supporting research on gynecologic malignancies and AIDS. Last year, the Institute expanded its existing programs to offer funding to every level of investigator: institutional grants for predoctoral students, training grants for postdoctoral fellows, and investigator awards for junior and senior faculty. Awards support laboratory research in cancer immunology, provide seed money to support Phase I and II clinical trials of new cancer immunotherapies, and support projects for which funds have been specifically raised.

Through **CRI's predoctoral pathway,** universities and research centers are invited to apply for training grants establishing multi-year programs that support doctoral students interested in pursuing careers in cancer immunology. The grants provide the institution with $450,000 over a four-year period.

The Postdoctoral Fellowship Program fosters the training of qualified young immunologists worldwide. Fellowships are awarded for a period of two years but can be extended to a third if the fellow has demonstrated substantial research progress. As of July 1, 1998, the stipend for new fellows was raised to $32,000 for the first year, $34,000 for the second, and $36,000 for the third. A yearly allowance of $1,500 is paid to the host institution to help meet expenses for research supplies, travel to scientific meetings, and health insurance incurred on behalf of the fellow. In fiscal 1998, CRI awarded $3.8 million to support 36 postdoctoral fellows. In all, 104 fellows (57 men and 47 women representing 53 institutions in 4 countries) were supported this year.

The Investigator Award Program provides support ($50,000 a year for a period of four years) for 57 assistant professors undertaking their first independent investigations. In 1998, the program was restructured to include awards in both basic and tumor immunology.

An eight-person panel of the Scientific Advisory Council selects recipients based on the applicant's entire body of research. In fiscal 1998, the Institute awarded $1.2 million to support six new investigators.

CRI's Preclinical Research Grants program, begun last year, funds both immunological and cancer immunological research in human cancers and in animal models. Grants provide $100,000 a year for three years. Six grants totaling $1.8 million dollars were awarded in 1998.

Funds for this program are awarded through the Institute's prostate cancer and melanoma initiatives. The deadline for Prostate Cancer Preclinical Grants is April 15 and for Melanoma Preclinical Grants is September 1. The Institute may also invite applications on other topics.

In 1992, CRI inaugurated its first formal program to support clinical trials (carefully controlled patient studies) of cancer immunotherapies. Since then, it has awarded funds to support 19 Phase I and Phase II (early-stage) trials. In 1998, the Institute's Clinical Trials Program was reevaluated to provide increased funding. These grants now provide $150,000 a year for three years. In fiscal 1998, CRI awarded $1,050,000 to support five clinical trials. Applicants may apply for **Clinical Trials Grants** through the Institute's prostate cancer and melanoma initiatives. The deadline for Prostate Cancer Clinical Trials Grants is April 15 and the deadline for Melanoma Clinical Trials Grants is September 1. Proposals in other areas may be invited at certain times.

Other programs sponsored by CRI, including grants for research on AIDS, breast cancer, and other malignancies, will be discussed in future issues of *Cancer Research Alert.* Further information is available at the CRI website: www.cancerresearch.org. ❖

CME Questions

1. **The field of research that seeks to determine a gene's sequence before its protein product has been identified or assigned a function has been termed:**
   a. clonal genetics.
   b. reverse genetics.
   c. mendelian genetics.
   d. hereditary genetics.

2. **Nuclear matrix proteins:**
   a. are uniformly expressed in all cell types and tissues.
   b. provide a scaffolding for specific topological organization of nuclear DNA.
   c. differ in composition in various human tumors.
   d. Answers b and c

3. **Apoptotic cascades have been shown to involve all of the following patterns *except*:**
   a. caspases.
   b. tumor necrosis factor.
   c. MCF-7.
   d. Bcl-2.

4. **The protein product of the p16 gene:**
   a. complexes with Cdk4 and Cdk6.
   b. inhibits complex formation of Cdk4 and Cdk6 with D-type cyclines.
   c. inhibits phosphorylation of the RB protein.
   d. All of the above

In Future Issues: Biomonitoring of Cancer