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Commentary

Krüppel Cripples Prostate Cancer

KLF6 Progress and Prospects

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Prostate cancer is a leading cause of cancer death in men. In the U.S. there will be an estimated 198,000 new prostate cancer cases diagnosed and nearly 32,000 deaths this year.[1] Moreover, risk will increase substantially as the male population over the age of 65 nearly doubles in the next 25 years.[1] Thus, it has been estimated that one in eight men will be diagnosed with prostate cancer in their lifetime and that more than 1 million men now older than 50 will eventually die of prostate cancer in the U.S.[2,3] Not evident within these statistics is that certain populations – specifically elderly and African American males – may harbor even greater risk. For example, 20% of all cancer-related deaths in men aged greater than 75 years in the last decade was due to prostate cancer and regardless of age, black men had the highest prostate cancer incidence and death rates among ethnic groups.[4]

While risk factors for prostate cancer development, including age, race, and family history, have long been recognized, our understanding of the genetic basis of this complex and multi-factorial disease remains insufficiently clarified. The challenges of identifying and characterizing candidate prostate cancer susceptibility genes have been thoughtfully reviewed by several authors.[5–8] However, targeted approaches, including linkage analysis and positional gene cloning of hereditary prostate cancer genes, combined with high-throughput genomic analysis of sporadic tumor samples, have begun to reveal key molecular determinants.

In this issue of *The American Journal of Pathology*, Chen et al[9] present the first independent confirmation of a recently identified candidate prostate cancer tumor suppressor gene, Krüppel-like factor 6 (KLF6).[10] These collective findings represent an exciting turning point in the genetic analysis of prostate cancer and point to important new directions for further study. To put this discovery into proper biological context, it is instructive to first review other recent advances in the disease, including risks associated with aging, race, and heredity.

Prostate Cancer and Aging

Early autopsy studies clearly established that an increased prevalence of prostate cancer is significantly associated with age.[11,12] These investigators astutely distinguished between clinical and pathological incidences, implying that not all prostate cancers were clinically significant. The prevalence of both clinical and pathological prostate cancer increases exponentially after age 50 and faster than any other major cancer in this group. By age 80, approximately 70% of men have autopsy evidence of carcinoma.[13]

Many lines of evidence suggest that prostate cancer begins as microscopic foci of prostatic intraepithelial neoplasia (PIN) early in middle age. Normal, PIN, and neoplastic prostate cells are subjected to a relentless barrage of genome-damaging stress that may be modulated by inherited genotype, diet, male sex steroids, and environmental factors. For example, inactivation of the carcinogen-detoxification enzyme GSTP1, glutathione S-transferase pi, may be an initiating genetic lesion for prostate cancer.[14] Cancer and PIN cells fail to express GSTπ, as a result of epigenetic silencing due to promoter methlyation.[14] Therefore, in the aging prostate, molecular alterations may be present and identifiable even in histologically benign cells. Thus, PIN lesions may provide a resource for tumor suppressor gene identification, and characterizing their development and progression represents a tractable model for translatable animal research.[15]

Prostate Cancer and Race

Race-associated prostate cancer appears to reflect an interplay between host determinants and geographical/

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environmental factors. For example, epidemiological studies have consistently shown that the incidence of prostate cancer is highest in the Scandinavian countries while Asian countries have the lowest rates. However, prostate cancer incidence and mortality rates in immigrants and their offspring soon approach those of their adopted homeland. In one well-documented study, significantly higher incidence and mortality rates, approaching those of the native U.S. population, were identified in Spanish-surnamed white and Japanese men compared with their native counterparts in their respective homelands following their immigration to Los Angeles County.[16]

Key genetic determinants underlying these racial differences are now being uncovered. For example, African-American men have the highest risk for prostate cancer development, present with more advanced disease than whites, and have higher mortality rates, even when diagnosed at the same clinical stage as whites.[4] A potential molecular explanation is the presence in African populations of significantly shorter CAG and GGC trinucleotide alleles of the androgen receptor gene than non-African populations.[17] These short CAG and GGC repeats have been associated with increased risk, and higher grade and advanced stage of prostate cancer at diagnosis.[18] Large-scale studies are currently underway to identify other genes accounting for these important differences in clinical behavior and outcome in African-Americans.[19]

Hereditary Prostate Cancer

A positive family history has also been shown to be a major predictor of prostate cancer risk based on a variety of methodologies, including retrospective or cohort study designs, case-control, and twin studies [reviewed in reference [5]]. These studies have yielded several conclusions: prostate cancer risk is increased among the first and second-degree relatives of index patients; the risk for developing prostate cancer among the first and second-degree relatives is proportionate to the number of affected individuals in the families and to earlier age at diagnosis of index cases; and, the risk associated with having an affected brother is greater than with having a father with prostate cancer. In general, men who have either a brother or father affected with prostate cancer are twice as likely to develop prostate cancer as men without affected relatives.[20] Risk is increased 5- and 11-fold, respectively, in men having two or three affected first-degree relatives.[20]

Twin studies[21–23] and complex segregation analyses[24–26] have been used to model the relative contributions of genetic and environmental factors in familial prostate cancer. Each has implicated a genetic contribution for familial aggregation of prostate cancer. Carter et al suggests that familial aggregation can be best explained by autosomal-dominant inheritance of a rare, high-risk allele leading to an early onset of prostate cancer.[24] The estimated cumulative risk of prostate cancer by age 85 years in his study was 88% for carriers, *versus* 5% for non-carriers. This inherited form of prostate cancer was estimated to account for a significant proportion of early-onset disease, and, overall, to be responsible for approximately 9% of all cases.[24] Gronberg et al also suggested that familial aggregation of prostate cancer is best explained by a high-risk allele inherited in a dominant fashion, although this model would require a gene with a higher population frequency and a moderate lifetime penetrance (63%).[25] Schaid et al reported that the best-fitting model was also that of a rare autosomal-dominant susceptibility gene, although no single-gene model of inheritance clearly explained the familial clustering observed in men undergoing radical prostatectomy for clinically localized disease at the Mayo Clinic.[26] The best fit was observed in probands diagnosed at <60 years of age.

These studies clearly indicate a strong inherited component in prostate cancer risk, and perhaps even in influencing the progression of the disease. Men with a familial risk of prostate cancer represent an enriched pool for the identification of mutations and polymorphisms in tumor suppressor genes. This is based on the paradigm established for other cancer predisposition gene discoveries, wherein families with a known hereditary cancer predisposition are first studied to identify a gene by use of linkage analysis and positional gene cloning.[27] Cancer-related genes identified by this approach can then be applied to prospective studies examining their role in the development of sporadic cancer. This approach has successfully localized a number of PCa-susceptibility loci.[5] From among these loci, three candidate genes have emerged, HPC2/ELAC2,[28] RNASEL,[29] and MSR1;[30] their overall importance has, however, been confined to mostly a small subset of familial cases. Subsequent independent confirmatory studies have now provided support for the role of RNASEL in prostate cancer susceptibility.[31–33]

Prostate Cancer Genetics: The Biology and Tumor Suppressor Function of KLF6

Despite efforts to tie specific disease-related genes to prostate cancer risk, the number of candidate genes implicated in its pathogenesis has been limited. In part, this reflects the surprisingly small number of consistent molecular abnormalities in sporadic or familial prostate cancer, compared to other tumors where loss in tumor suppressor genes or overexpression of oncogenes has frequently been observed. Although mutations in a wide variety of tumor suppressor genes and oncogenes in prostate cancer have been reported,[8,34] no single gene has been identified as a major "gatekeeper." Even the best-known tumor suppressor genes seem to play a role only in late-stage disease. For example, p53 mutations are uncommon in localized disease but present in approximately 25% of bone metastases.[35] Similarly, mutations or homozygous deletions of PTEN occur just as infrequently in localized tumors[36,37] and biallelic PTEN inactivation is found in only approximately 30% of metastatic prostate cancers.[38] Mutations in k-Ras are also relatively uncommon and occur in less than 5% of tu-

mors[39] while allelic loss, without mutation, of the Rb gene can be seen in up to 80% of advanced tumors.[40,41]

We recently reported that KLF6 is a tumor suppressor gene inactivated in a significant percentage of sporadic prostate cancers.[10] KLF6 is a ubiquitously expressed zinc finger transcription factor that is part of a growing family of Krüppel-like factors (KLF). These KLF proteins share a nearly identical carboxy terminal DNA binding domain, but have widely divergent amino terminal activation domains, patterns of expression, and transcriptional targets.[42,43] The KLF family is broadly involved in differentiation and development, growth-related signal transduction, cell proliferation, apoptosis, and angiogenesis.[42,43] These functions have drawn attention to their possible roles in tumorigenesis.[42,43]

Recent evidence suggests a generalized role for KLF6 in regulating cell growth and differentiation. KLF6 is a 283 amino acid (a.a.) protein containing a 201 a.a. activation domain and an 82 a.a. zinc finger DNA binding domain. It was originally cloned from rat and human liver mesenchymal stellate cells[44] as well as placenta,[45] and is expressed in all mammalian tissues. In culture models, KLF6 regulates expression of a placental glycoprotein,[45] collagen α_1(I),[44] TGFβ1, types I and II TGFβ receptors[46] and urokinase-type plasminogen activator.[46] KLF6 is an immediate-early gene up-regulated in hepatic stellate cells during acute liver injury,[44] in hepatocytes following partial hepatectomy (S. Friedman, Mt. Sinai School of Medicine, unpublished observations), during differentiation from preadipocytes toward adipocytes,[47] and in endothelial cells following vascular injury.[48] KLF6 has also been shown to regulate the expression of the keratin-4 gene, a protein associated with epithelial differentiation in stratified squamous esophageal epithelium.[49]

What is the evidence that KLF6 is a tumor suppressor gene? Using the definition of Haber and Harlow,[50] a tumor suppressor gene is a gene that sustains loss-of-function mutations in the development of cancer. Does KLF6 meet this requirement? Normally, KLF6 appears to be growth suppressive, in part by up-regulating p21, a cyclin-dependent kinase inhibitor that also accounts for the growth suppressive effects of the classical tumor suppressor p53.[10] However, up-regulation of p21 by KLF6 occurs independently of p53.[10] Thus, loss of KLF6 might lead to removal of a "brake" on cellular proliferation. Indeed, we recently demonstrated that the KLF6 gene is functionally inactivated, by "two hits" in prostate cancer[10] due to deletion and an inactivating mutation, in accordance with Knudson's[51] original definition of a tumor suppressor gene.

KLF6 maps to human chromosome 10p, a region whose deletion has been reported in approximately 55% of sporadic prostate adenocarcinomas.[52] Accordingly, in our study microsatellite markers tightly flanking the KLF6 gene were initially analyzed in paired microdissected and laser capture microdissected (LCM) specimens. In total, 16 of the 22 samples analyzed (73%) demonstrated evidence of loss of heterozygosity (LOH) across the KLF6 locus. Markers shared between these two studies, D10S533 and D10S591, were lost in 11 of 22 samples (50%). The most frequently lost markers were those which directly flank the KLF6 gene by approximately 40 and 10 kb, respectively; suggesting that a significant number of prostate tumors harbor small deletions containing the KLF6 gene locus. LOH would not be detected with distantly placed markers. In our study, the smallest region of overlap was defined and effectively narrowed the tumor suppressor locus to approximately 60 kb. All four KLF6 coding exons and intron/exon boundaries were then directly sequenced. Nineteen of 34 tumor samples (56%) from our collection were found to have tumor-specific KLF6 mutations. Unlike wild-type KLF6, these patient-derived mutations were unable to up-regulate p21 expression or decrease cell proliferation.[10]

The study by Chen et al[9] in this issue provides important new information by focusing on analysis of KLF6 in a subset of high-grade tumors and xenografts/cell lines. In these samples, LOH was present in 28% of high-grade tumors and 19% of cultured cells. Fifteen percent of primary tumor samples were identified by single-strand conformational polymorphism (SSCP) and then confirmed by DNA sequencing, as having DNA sequence mutations. Moreover, and for the first time in prostate-derived tissues, significantly decreased KLF6 gene expression was shown in 4 of 20 (20%) prostate cancer xenografts/cell lines. Thus, three potential gene-inactivating events were identified by these authors: allelic loss, mutation, and gene silencing. In accord with both an epigenetic mutation/silencing mechanism and its role as a tumor suppressor gene, a recent report identified KLF6 as one of 52 methylation-silenced genes in esophageal squamous cell carcinoma.[53]

While confirmatory, are the differences in degrees of KLF6 inactivation in prostate cancer between our study,[10] and that of Chen at al[9] significant? Most likely, these results highlight important differences between sample selection, numbers of samples, tissue isolation (microdissection *versus* LCM), and the analytic techniques used (DNA sequencing *versus* SSCP analysis; radioactive *versus* quantitative fluorescent LOH analysis; distance of microsatellite markers from locus of interest). Approaches to validate the purity of tumor tissue, and methods of analyzing mutations and deletions have not been standardized in the field of cancer genetics; their wide variability in published studies represents a significant confounding variable that makes the comparison of data between studies very difficult. Thus, the field would be greatly advantaged by developing standardized, universally accepted protocols for characterizing gene mutations, assessing allelic loss in cancer tissues, and adopting an intragenic, high-density single nucleotide polymorphism (SNP)-based assay for defining LOH.

Unlike hematological malignancies which are easily accessible and typically homogeneous, the detection of mutations in solid tumors is inherently difficult, owing to specimen selection, stromal contamination, sensitivity of detection methods, and the genetic heterogeneity of mutations within the same tumor.[54] In fact, molecular studies have begun to reveal a high degree of genetic heterogeneity within primary tumors,[55,56] while mathematical models provide insight into the staggering numbers of genetic events that account for the origin and maintenance of this

heterogeneity.[57] Our own study reinforced this concept of genetic heterogeneity by demonstrating molecular differences in KLF6 between distinct tumor foci within the same tumor, following isolation by LCM.[10] In general, such a high degree of genetic heterogeneity increases the signal:noise ratio in DNA sequencing, and effectively makes individual sequence variations disappear. Newer technologies, such as massively parallel signature sequencing (MPSS)[58] and high-density chip-based LOH assays[59] offer great promise, as compared to direct DNA sequencing and microsatellite-based analyses, respectively. In addition, radical new approaches to discovering cancer-linked genes may be required, such as the systematic genome-wide screen recently used by the Cancer Genome Project to identify mutations in the *braf* gene in melanoma.[60] The adoption of the Gleason grading system, a single, relatively reproducible system in prostate cancer histopathology has been a key feature in accurately predicting the risk of extraprostatic infiltration and the probability of cure. Agreement on a common platform for tissue isolation and analysis techniques will similarly allow for improved and more meaningful interpretation of the results of molecular analyses, a prerequisite for establishing a molecular classification system for individual prostate tumors.

Mechanisms of Cell Growth Control and the Role of KLF6

Tight control of cell growth is an essential feature of normal tissue and its dysregulation underlies many human cancers. A key regulator of the cell growth is the protein p21, which is a cyclin-dependent kinase inhibitor responsible for preventing the phosphorylation of the Rb protein, thereby maintaining its association with the transcription factor E2F. While bound to pRb, E2F is unable to transcriptionally activate key genes regulating cell cycle progression. In this way p21 promotes cell cycle arrest primarily at the G_1/S transition of the cell cycle. The tumor suppressor p53 is a major transcriptional activator of p21 following DNA damage, oncogene activation, or cellular stress. Loss of active p53 through mutation is a major event in cancer pathogenesis, in part due to loss of its ability to up-regulate p21, leading to a failure to induce cell cycle arrest.

The recent findings with KLF6 support a model whereby mutations of this ubiquitous transcription factor may underlie carcinogenesis in a p53-independent manner.[10] While 50% of all tumors harbor p53 mutations, the pathogenetic mechanisms underlying the remaining 50% that have wild-type p53 are multiple, and in some cases uncertain. As already stated, p53 mutations are relatively infrequent in prostate cancer and are usually confined to metastatic samples.[8] Thus, the identification of KLF6, a p53-independent inducer of p21, may provide an alternative mechanism that accounts for p21 loss when p53 is normal. It remains to be determined whether loss of either KLF6 and p53 is sufficient to significantly reduce p21 in this cancer, and whether dual inactivation confers increased growth or metastatic potential.

Silencing of tumor suppressor genes as a result of promoter hypermethylation is also a common feature in human cancer, and represents an additional mechanism for loss of tumor suppressor gene function. In addition to the data of Chen et al,[9] two recent studies have identified down-regulation of KLF6 mRNA levels in primary lung cancer samples[61] and esophageal cancer cell lines[53] suggesting promoter hypermethylation as a mechanism of KLF6 inactivation. Combined with the findings of Chen et al,[9] these studies highlight an additional mechanism by which KLF6 can be functionally inactivated and further strengthen its involvement in human cancer.

The identification of KLF6 as a tumor suppressor gene in prostate cancer raises interesting and exciting questions, with broad implications. Recently, KLF6 gene mutations have been identified in nasopharyngeal carcinoma,[62] raising the possibility of a generalized role of KLF6 inactivation in cancer pathogenesis. Just as p53 exerts its tumor suppressor function through activation of many circuits, so too may KLF6 stimulate parallel pathways of tumor suppression beyond its inhibition of cell growth via p21 up-regulation. Indeed, the immediate-early induction of KLF6 in tissue injury is clearly distinct from p53, and points to potentially unique roles in tissue homeostasis. Evidence of promoter hypermethylation[53] in esophageal cancer enlarges the potential mechanisms of inactivation beyond just mutation or deletion. Efforts to assess the impact of somatic KLF6 inactivation on tumor behavior could yield important prognostic information, and analysis of genomic sequences in prostate cancer could offer evidence that germline changes in KLF6 sequence have clinical relevance for assessing risk. Thus, questions abound, and the potential paths of inquiry are many, in deciphering the role of KLF6 in human cancer.

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