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MICROSATELLITE MUTATION ($CAG_{24\to18}$) IN THE ANDROGEN RECEPTOR GENE IN HUMAN PROSTATE CANCER

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SUMMARY - The androgen receptor (AR) gene contains a polymorphic CAG microsatellite that codes for a variable length of glutamine repeats in the AR protein. Microsatellite DNA sequences may be potential sites of genetic instability. Using the polymerase chain reaction (PCR), we screened 40 human prostate cancer specimens for expansions or deletions of this microsatellite. In one patient, nontumor DNA yielded a single PCR product, as expected for the AR, but the tumor DNA yielded two discrete products, one identical to normal, and a second smaller one. Direct sequencing revealed that the nontumor tissue contained 24 CAGs, whereas the tumor contained one fragment with 24 CAGs (wild-type) and a second fragment with 18 CAGs (mutant), representing a somatic contraction of the AR CAG repeat ($CAG_{24}\to CAG_{18}$) in the tumor. Interestingly, this patient manifested a paradoxical agonistic response to hormonal therapy with the antiandrogen flutamide.

Prostate cancer is the most common malignant tumor of American men, and the second most common cause of cancer death in this group (1). Androgen is required for prostate differentiation and growth (2) and is believed to play a critical role in prostate carcinogenesis (3). Androgens act via the androgen receptor (AR), a member of the steroid receptor superfamily of ligand-dependent transcription factors (4, 5). AR gene mutations that cause androgen insensitivity syndrome inactivate AR function (6), but AR gene mutations that occur in prostate cancer do not inactivate AR (7-9). The LNCaP human prostate cancer cell line contains mutated AR that can be transcriptionally activated not just by androgen but also, paradoxically, by the antiandrogen hydroxyflutamide (7). We recently reported the presence of

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a somatic point mutation in the AR gene in human prostate cancer; the mutation was in codon 730 (val→met), which is in a region of the hormone binding domain that is highly conserved among members of the steroid receptor superfamily (8). Preliminary data indicate that this mutation does not ablate androgen binding or androgen-dependent transcriptional activity (9).

We sought to determine whether mutations might be located in other parts of the AR coding sequence. We chose to focus on structural motifs that are predisposed to instability. The AR gene contains two regions within the first exon of the coding sequence that fulfill this criterion. The most 5' of these is a polymorphic trinucleotide microsatellite (CAG), the length of which varies in the population [average size, 21 $\pm$ 2 CAGs (10); range, 11-31 CAGs (11)] and thus codes for a variable length of glutamine repeats in the AR protein. The 3' motif (GGN) codes for a glycine repeat that also is polymorphic (12).

The CAG repeat in the AR is particularly interesting because expansion (to 40-52 CAGs) occurs in patients with the X-linked neuromuscular disorder spinal and bulbar muscular atrophy (SBMA) (10). In addition, 4 other neurological diseases have been linked to microsatellite expansions in or near genes that are thought to be involved in disease development (13-15). To study whether the CAG microsatellite of the AR gene is subject to deletion or expansion in prostate cancer, we screened 40 prostate cancers by PCR amplification of the region containing the repeat and subsequent electrophoretic analysis of PCR fragment length. Nonmalignant control tissue was analyzed simultaneously.

MATERIALS AND METHODS

DNA Preparation. Genomic DNA was isolated from 34 frozen prostate adenocarcinomas obtained from patients undergoing radical prostatectomy for organ-confined (clinical stage B) prostate cancer, and from control nontumor DNA (nonmalignant prostate tissue, seminal vesicles, or peripheral lymphocytes) of the same patients (16,17). DNA was also isolated from 6 paraffin-embedded prostate cancers and adjacent nonmalignant prostate tissue. Four of these tumors were designated stage D-3 prostate carcinoma because they were removed *post mortem* from patients who died with progressive, metastatic cancer after androgen withdrawal. Two patients (including #P-6) had clinically localized disease.

PCR Amplification. PCR amplification of the AR CAG repeat was carried out using primers designed to flank the repeat region of interest (10). The sense (5'-TCCAGAATCTGTT-CCAGAGCGTGC-3') and antisense (5'-GCTGTGAAGGTTGCTGTTCCTCAT-3') primers correspond to nucleotides 146-169 and 404-427, respectively, of the AR sequence (18). Labeled PCR products, produced by adding [α^{32}P]-dATP or [α^{33}P]-dATP (Amersham, Arlington Heights, IL) to the PCR reaction mixture, were denatured by heating (100°C, 3 min), and then analyzed by electrophoresis in a 0.4 mm x 30 cm x 40 cm sequencing gel (6% polyacrylamide/8 M urea) at 70 W constant power, at 45°C for 75 min.

DNA Sequencing. To allow resolution of the 2 PCR products amplified from the tumor DNA of patient #P-6, unlabeled PCR products from control and tumor DNA were electrophoresed in a 16 x 16 x 0.8 cm 3.5% native polyacrylamide gel at 150 V for 90 min. The gels were stained with ethidium bromide and individual bands were excised while viewing

on a preparative ultraviolet light box (Fotoprep I, Fotodyne, Inc., New Berlin, WI). DNA was recovered from each gel slice by electroelution (Centrielutor, Amicon, Beverly, MA). Approximately 30 ng of electroeluted double-stranded PCR product was combined with 3 pmol of internal primer (5'-AGAATCTGTTCCAGAGCGTGC-3') for sequencing (10).

Case History of Patient #P-6. The patient was a 53 y.o. caucasian male with Gleason grade 4, score 8 prostate adenocarcinoma, who underwent radical prostatectomy followed by radiation therapy because the surgical margins contained tumor. Three years later his serum prostate specific antigen (PSA) level rose to 190 ng/ml (normal range, 1-4 ng/ml); a bone scan revealed evidence of metastatic disease. After treatment with a luteinizing hormone releasing hormone agonist and the antiandrogen flutamide, his PSA fell to castrate levels (< 0.2 ng/ml); 18 months later his PSA rose to 35 ng/ml, despite a serum testosterone level still in the castrate range, apparently signaling tumor progression to androgen independence. Unexpectedly, however, cessation of flutamide therapy led to a fall in serum PSA to 7.5 ng/ml within 1 month, suggesting that flutamide was acting paradoxically as an agonist. His PSA remains low (13-16 ng/ml) 12 months after flutamide withdrawal.

RESULTS

To screen for evidence of expansion or deletion of the CAG microsatellite in the AR gene, a fragment of the coding sequence that contains the CAG repeat was amplified by PCR. Because the length of the CAG repeat varies within the population (i.e., is polymorphic) (10,11), PCR product length should vary among patients, so detection of a somatic change in repeat length in a tumor requires concurrent evaluation of nonmalignant tissue from the same patient.

When the labeled PCR products from paired tumor and nontumor DNA samples were analyzed by electrophoresis in denaturing polyacrylamide sequencing gels (Fig. 1), fragment size was found to vary among the 40 patients screened, as expected. Because the AR gene is on the X-chromosome (4), there is only a single allele per cell in the male; this accounts for the presence of a single major PCR product from each specimen (Fig. 1). In 39 of 40 patients the PCR products of each tumor/nontumor pair did not appear to differ from each other (Fig. 1).

However, in one patient (#P-6), nontumor DNA yielded a single PCR product, but the tumor DNA yielded two discrete products, one identical to normal, and a second smaller one (Fig. 2), indicating the presence of cells that contain an AR gene with a deletion. This same pattern was observed in multiple independent PCR reactions from 2 different preparations of tumor and nontumor DNA of this patient. In addition, amplification of an independent polymorphic locus, LPL (19), from tumor and nontumor DNA of patient #P-6 yielded identical PCR products (data not shown), indicating that the tumor and nontumor DNA came from the same individual. Therefore, we conclude that the smaller PCR product amplified from tumor DNA (Fig. 2, lane T, lower band) was not a PCR artifact or the result of contamination of P-6 tumor DNA with DNA from another patient specimen during paraffin embedding, DNA isolation, or PCR amplification.

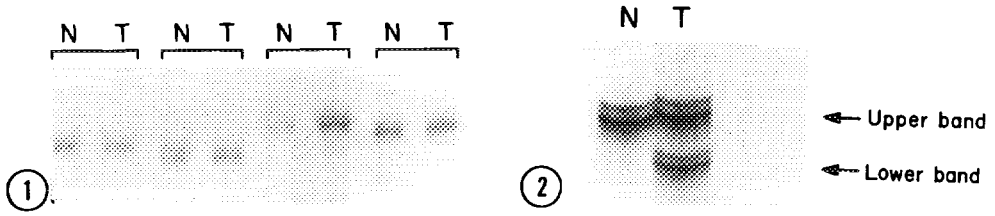

Figure 1. Comparison of the AR gene CAG repeat length in prostate cancer. In the 4 patients illustrated the PCR products of each tumor(T)/nontumor(N) pair did not differ from each other.

Figure 2. Prostate cancer (patient #P-6) with a somatic mutation in the CAG repeat of the AR gene. Nontumor DNA yielded a single major PCR product (*upper band*), but tumor DNA yielded 2 major products (*upper band* and *lower band*), indicating the presence of cells with a wild-type allele (*upper band*) and cells with a mutant allele (*lower band*) that contains a deletion. Accompanying minor bands are a common feature in denaturing gels of PCR products of repeat sequences (27).

To establish the exact nature of the somatic mutation represented by the smaller PCR product in tumor #P-6, we directly sequenced the PCR products from both tumor and nontumor DNA after electrophoretic separation of the products and electroelution of the individual bands. Direct sequencing of the larger PCR fragment of both the tumor and nonmalignant DNA (upper band in Fig. 2, lanes N and T) demonstrated the presence of 24 CAGs (Fig. 3), which represents the wild-type repeat length for this patient. The smaller PCR product in the tumor (lower band in Fig. 2, lane T) contained only 18 CAGs, indicating that the tumor contained an 18 bp deletion (Fig. 3). The remainder of the DNA sequence of the upper and lower bands and of tumor and nontumor DNA was identical to published wild-type sequence (data not shown).

DISCUSSION

We have identified a patient whose prostate cancer contains a somatic mutation in the length of a trinucleotide microsatellite ($CAG_{24\rightarrow18}$) in the first exon of the coding sequence of the AR gene. PCR amplification of this region of the gene yielded only one product from nontumor DNA, but two major PCR products from tumor-derived DNA. One PCR product from the tumor contained the same number of CAGs as nontumor tissue, whereas the other PCR product contained a CAG repeat that was 6 codons shorter than control, representing an in-frame contraction of 18 bp. Interestingly, this patient had a paradoxical response to flutamide tharapy.

The high relative abundance of the mutant allele in tumor P-6 suggests that the mutation occurred in cells with a growth advantage. If the mutation had occurred in cells without a growth advantage, it would not have been detected because its presence would have been masked by the proliferation of cells with a growth advantage. However, whether the mutation itself conferred a growth advantage on the cells in which it occurred is not known.

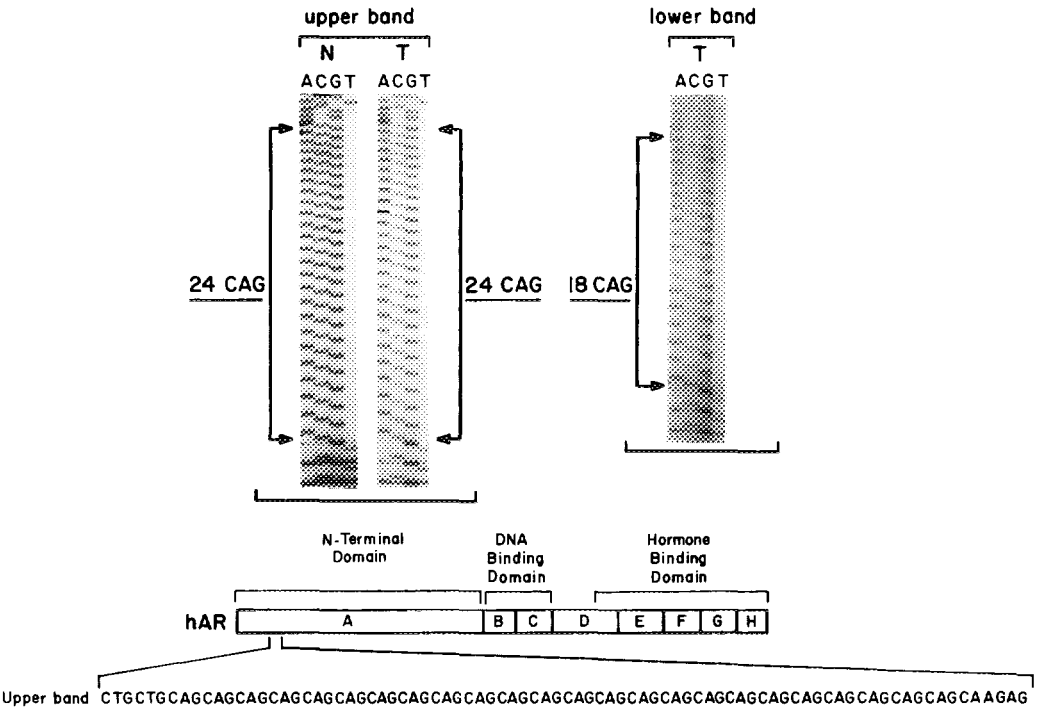

Figure 3. DNA sequence analysis of PCR products from tumor (T) and nontumor (N) DNA of patient P-6. The lanes marked *upper band* or *lower band* refer to the PCR products in Fig. 2. The upper band products from nontumor and tumor-derived DNA contain 24 CAGs; the lower band product, present only in tumor-derived DNA, contains 18 CAG repeats. A schematic representation of the AR gene, the location of the CAG repeat, and the wild-type and mutant sequences appear below the sequencing gel autoradiograms.

It is not known whether the CAG_{18} mutation alters AR function in tumor cells, whether it accounts for the paradoxical response to flutamide, or whether it reflects genomic instability. The presence of repeat sequences in several important transcription factors (20) suggests that the CAG repeat in the AR may play a role in its transcriptional activity, and therefore that changes in the length of this repeat may have subtle effects on AR function *in vivo*. Indeed, patients with SBMA have an altered CAG repeat length that does not interfere with the role of AR in masculinization but does interfere with normal AR function in specific tissues (motor neurons) at adulthood (10). In addition, a patient with androgen insensitivity syndrome was identified who had a short CAG repeat (CAG_{12}) and a point mutation in the hormone binding domain (21); functional studies revealed that an AR with 12 CAGs exacerbates the effect of the normone binding domain mutation on AR transcriptional activity (21). Whether prostate cancer P-6 contains additional mutations in the rest of the AR gene, that alone or in concert with the CAG mutation affect AR function or account for the paradoxical response to flutamide, remains to be determined. Since tumor P-6 was evaluated prior to therapy (including radiation), it is possible

that additional mutations in the AR gene may have occurred subsequently. For ethical reasons we are unable to obtain more tumor tissue from this patient, and we are thus unable to analyze the tumor AR in its current state.

The identification of a mutation in a polymorphic CAG trinucleotide repeat in the AR gene in prostate cancer is especially interesting in light of the increasing number of diseases associated with repeat mutations (10, 13-15). An important difference is that the AR mutation in prostate cancer is a somatic mutation whereas in hereditary diseases (10, 13-15) mutations are germline mutations, present in all cells. While this manuscript was in preparation, 3 other studies appeared documenting the presence of somatic microsatellite mutations in colorectal cancer (22-24). These other mutations were located at multiple polymorphic loci in the genome, and raise the intriguing question whether similar changes occur in prostate cancer, and whether the microsatellite AR mutation identified in our study might be a marker of more widespread genetic instability. However, preliminary analysis of several polymorphic loci suggests that microsatellite instability may not be common in sporadic prostate cancer (M.J.G. Bussemakers, G.S. Bova, M.P. Schoenberg, J.M. Hakimi, E.R. Barrack, and W.B. Isaacs, unpublished data).

Paradoxical response to flutamide withdrawal has been described recently (25). The salient clinical features are initial response to androgen blockade, followed by evidence of disease progression that subsequently abates after cessation of flutamide therapy. A similar clinical scenario has been reported in patients with breast cancer treated with tamoxifen (26). Whether steroid receptor mutations are the molecular basis of paradoxical responses remains to be determined, and will require studying larger numbers of patients. The existence of an AR gene mutation in a patient with prostate cancer who had a paradoxical response to flutamide withdrawal implies that an AR mediated mechanism for this syndrome may exist, and that further elucidation of the molecular defects in the AR gene in prostate cancer may aid in more effective treatment of patients with metastatic disease.

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