ABSTRACT

Genetic changes leading to the development of prostate cancer and factors that underlie the clinical progression of the disease are poorly characterized. Here, we used comparative genomic hybridization (CGH) to screen for DNA sequence copy number changes along all chromosomes in 31 primary and 9 recurrent uncultured prostate carcinomas. The aim of the study was to identify those chromosome regions that contain genes important for the development of prostate cancer and to identify genetic markers of tumor progression. CGH analysis indicated that 74% of primary prostate carcinomas showed DNA sequence copy number changes. Losses were 5 times more common than gains and most often involved 8p (32%), 13q (32%), 6q (22%), 16q (19%), 18q (19%), and 9p (16%). Allelic loss studies with 5 polymorphic microsatellite markers for 4 different chromosomes were done from 13 samples and showed a 76% concordance with CGH results. In local recurrences that developed during endocrine therapy, there were significantly more losses (P < 0.001) and losses (P < 0.05) of DNA sequences than in primary tumors, with gains of 8q (found in 89% of recurrences versus 6% of primary tumors), X (56% versus 0%), and 7 (56% versus 32%), as well as loss of 8p (78% versus 32%), being particularly often involved. In conclusion, our CGH results indicate that losses of several chromosomal regions are common genetic changes in primary tumors, suggesting that deleterial inactivation of putative tumor suppressor genes in these chromosomal sites is likely to underlie development of prostate cancer. Furthermore, the pattern of genetic changes seen in recurrent tumors with the frequent gains of 7, 8q, and X suggests that the progression of prostate cancer and development of hormone-independent growth may have a distinct genetic basis. These chromosomal aberrations may have diagnostic utility as markers of prostate cancer progression.

INTRODUCTION

Genetic changes underlying the development and progression of prostate cancer are poorly known. Classical cytogenetic studies are very difficult to carry out in prostate cancer because of the preferential growth of nonmalignant cells. In many cases only a normal karyotype has been found (1). The aberrations that have been reported most often include deletions of 7q, 10q, and 8 as well as gains of chromosome 7. Occasionally, double minute chromosomes have been reported (1). LOH studies, which are thought to highlight chromosomal sites harboring mutated tumor suppressor genes, have implicated 8p, 10q, 13q, 16q, and 18q (2–7). However, LOH studies are typically limited to extensive analyses of a single chromosome or analysis of all chromosomes with only 1–3 probes/chromosome arm, thus leaving the vast majority of the genome unexamined. The specific roles of any of the known TSGs and oncogenes in prostate cancer development are also poorly known. For example, ras oncogene, which is commonly affected in human malignancies, is involved relatively infrequently in prostate cancer (8, 9). Furthermore, factors that determine the prognosis of patients with prostate cancer are poorly known, and genetic markers of tumor progression are urgently required (10).

CGH is a newly developed molecular cytogenetic method that makes it possible to survey the entire genome for gains and losses of DNA sequences (11–17). CGH is based on the simultaneous hybridization of differentially labeled tumor and normal DNA to normal metaphase chromosomes. In such a hybridization, differences between the binding of the labeled DNA sequences, as evidenced by fluorescence intensity ratio measurements along all chromosomes, indicate those regions of the genome that were either over- or under-represented in the tumor genome. The utility of CGH is based on the concept that regions with increased copy number reveal chromosome sites that may contain dominant oncogenes, whereas regions with decreased copy number may be putative TSG loci (11). Thus, in a single hybridization, CGH allows screening of all chromosomal sites that are likely to contain genes with an important role in tumor development.

We took advantage of the potential of CGH to screen for losses and gains of DNA sequences in 31 primary uncultured prostate carcinomas. The aim was to identify those chromosomal regions that are often involved in copy number aberrations and may thus contain genes implicated in the development of prostate cancer. In the second part of the study, genetic aberrations in the primary tumors were compared with those detected in 9 recurrent prostate tumors. The aim of this part of the study was to identify genetic changes that underlie clinical tumor progression.

MATERIALS AND METHODS

The material consisted of 31 primary and 9 recurrent uncultured prostate carcinomas. Six benign prostate hyperplasia samples were also evaluated. The TNM stage distribution (18) of the primary prostate carcinomas was: T1N0M0, 1; T2N0M0, 14; T2N1M0, 3; T2N2M0, 2; T2N3M0, 2; T3N0M0, 1; T3N1M0, 1; T3N2M0, 1; T3N3M0, 1; T4N1M0, 2; and unknown, 1. The histological grade distribution (19) of primary tumors was: grade I, 10; grade II, 19; and grade III, 2. That of recurrent tumors was: grade II, 4; and grade III, 5. The primary tumor specimens were prostatectomy specimens (20 cases), transurethral resection specimens (7 cases), or Tru-Cut needle biopsy specimens (4 cases), all taken prior to the administration of any hormonal therapy, whereas recurrent prostate carcinomas were all transurethral resection specimens taken from patients who received only endocrine therapy (orchietomy 6 cases, luteinizing hormone-releasing hormone agonist 2 cases, estrogen 1 case). The recurrent tumors came from patients with symptoms of urethral obstruction, indicating local progression of the disease despite ongoing therapy.

Five-μm sections were cut from freshly frozen tumor blocks embedded in Tissue-Tek (Miles, Inc., Diagnostic Division, Elkhart, IN) and stained with hematoxylin and eosin to ensure the histological representativeness of the samples. Some specimens were trimmed by cutting normal tissue away with a scalpel. High-molecular-weight tumor DNA was isolated either from homogenized tissue samples or from 200-μm frozen sections of the tumors using standard protocols. DNA also was isolated from the peripheral blood of 13 patients who received only endocrine therapy (orchiectomy 6 cases, luteinizing hormone-releasing hormone agonist 2 cases, estrogen 1 case). The recurrent tumors came from patients with symptoms of urethral obstruction, indicating local progression of the disease despite ongoing therapy.
patients (for LOH studies) and from normal male donors (reference DNA for CGH).

Comparative Genomic Hybridization

Hybridization. CGH was performed using directly fluorochrome-conjugated DNAs, as described previously (11, 20). Briefly, DNA samples from the tumors were labeled with FITC-UTP (DuPont, Boston, MA), and normal male DNA was labeled with Texas red-UTP (DuPont) using nick translation. Labeled tumor and normal DNAs (400 ng each) together with 10 μg of unlabeled Cot-1 DNA (Gibco BRL, Gaithersburg, MD) in 10 μl of hybridization mixture [50% formamide, 10% dextran sulfate, 2× SSC (1× SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7)] were denatured at 70°C for 5 min and applied on normal lymphocyte metaphase preparations. Prior to hybridization, the metaphase preparations were denatured at 72–74°C for 3 min in a formamide solution (70% formamide, 2× SSC, pH 7) and dehydrated in a series of 70, 85, and 100% ethanol; this was followed by proteinase K (0.1 mg/ml in 20 mM Tris-HCl, 2 mM CaCl2, pH 7.5) treatment at room temperature and dehydration once again as described above. The hybridization was done at 37°C for 48 h. After hybridization, the slides were washed three times in 50% formamide/2× SSC (pH 7), twice in 2× SSC, and once in 0.1× SSC at 45°C followed by 2× SSC and 0.1 M Na2HPO4-0.1 % NP40 (pH 8), and distilled water at room temperature for 10 min each. After air drying, the slides were counterstained with 4',6-diamidino-2-phenylindole, 0.1 μg/ml, in an antifade solution.

Digital Image Analysis. Three single-color images (matching 4',6-diamidino-2-phenylindole, FITC, and Texas red fluorescence) were collected from each metaphase spread using a Nikon epifluorescence microscope (Nikon Corp., Tokyo, Japan) and a Xillix charge-coupled-device camera (Xillix Technologies Corp., Vancouver, BC, Canada) interfaced to a Sun LX workstation (Sun Microsystems Computer Corp., Mountain View, CA). Four to six three-color digital images were collected from each hybridization. Relative DNA sequence copy number changes were detected by analyzing the hybridization intensities of tumor and normal DNAs along the length of all chromosomes in the metaphase spreads, as described earlier (20). The absolute fluorescence intensities were normalized so that the average green:red ratio of all chromosome objects in each metaphase was 1.0. The final results were plotted as a series of green:red ratio profiles and corresponding SDs for each human chromosome from pter to qter.

Interpretation of CGH results followed previously described protocols (20). Hybridizations of FITC-labeled normal female DNA with Texas red-labeled normal male DNA were used as negative controls. The mean green:red ratio and the corresponding SD for all autosomes remained between 0.9 and 1.1 in these control hybridizations. Chromosomal regions where the mean ratio and the corresponding SD were less than 0.85 were therefore considered lost, and regions where the mean and the corresponding SD were greater than 1.15 gained in the tumor genome. The entire Y chromosome was excluded from analysis. Hybridizations of DNA from the MCF-7 breast cancer cell line against normal female DNA were used as additional positive controls in each hybridization batch.

Loss of Heterozygosity

Oligonucleotides. Primers for amplification of the microsatellite loci D6S283 (6q16.3–q21), D8S265 (8p23.1), D8S282 (8q22), D13S153 (13q14– q22), and D16S413 (16q) were synthesized according to the sequences given in the Genethon catalogue. The 5’ ends of the upstream (AC strand) D6S283, D13S153, and D16S422 primers and the downstream (GT strand) D8S265 and D8S282 primers were fluorescence labeled during the synthesis using the FluoroPrime reagent (Pharmacia Biotech AB, Uppsala, Sweden).

PCR. Twenty ng of DNA isolated from patients’ leukocytes or tumor samples were amplified separately with each set of primers. The PCR mixtures contained 50 pmol of both primers, the four deoxynucleotide triphosphates at 0.2 mM concentration, and 1.25 units of DynaZyme DNA polymerase (Finnzymes Oy, Espoo, Finland) in 50 μl of buffer supplied with the enzyme. The PCR was initiated by heating the samples at 95°C for 3 min, followed by addition of enzyme at 80°C. Twenty-five PCR cycles of 1 min at 95°C, 1 min at 58°C (markers D6S283, D8S282, and D16S422) or at 54°C (markers D8S265 and D13S153), and 1 min at 72°C were carried out in a programmable heat block (PTC 100; MJ Research Inc., Watertown, MA).

Analysis and Interpretation of the LOH Studies. Aliquots (3 μl) of the fluorescent PCR products were denatured in 50% formamide containing blue dextran and analyzed on 6% denaturing polyacrylamide gels using an automatic DNA sequencer (ALF; Pharmacia Biotech AB). The relative quantities of the PCR products were determined with the aid of ALF DNA Fragment Manager program V1.1 (Pharmacia Biotech AB). When the ratio between the two alleles amplified from a tumor sample differed significantly (2–5-fold) from that obtained in the leukocyte sample, it was interpreted as a sign of LOH.

Statistical Analysis

The statistical significance between the primary and recurrent tumors in the total number of genetic aberrations and the frequencies of selected changes was calculated with the nonparametric Kruskal-Wallis test and 2-tailed Fisher’s exact test, respectively. The k statistic was used to analyze the level of agreement between CGH and LOH results.

RESULTS

Overview of Genetic Changes. None of the benign prostate hyperplasias showed any gains or losses of DNA sequences by CGH. Six (19%) of the primary prostate cancers showed relative DNA sequence gains, and 23 (74%) showed losses at 1 or more chromosomal sites (Fig. 1). Eight tumors (26%) had no copy number alterations. On average, there were 2.9 (range, 0–12) aberrations per primary tumor: 0.5 gain (range, 0–4) and 2.4 deletions (range, 0–9). Fig. 2 shows an example of green:red fluorescence ratio profiles of a primary prostate carcinoma analyzed by CGH.

All recurrent prostate cancers showed both relative gains and losses of DNA sequences (Fig. 3). The total numbers of aberrations per tumor (mean, 7.8; range, 4–15), as well as gains (mean, 2.2; range, 1–4) and losses (mean, 5.6; range, 3–12) were significantly higher in the recurrences than in primary tumors (Fig. 4A). Significance values for these differences were P < 0.01 for all aberrations, P < 0.001 for gains, and P < 0.05 for losses.

Losses and Gains. Chromosome arms that were lost most frequently in primary prostate cancers were 8p (32% of the cases), 13q (32%), 6q (22%), 16q (19%), 18q (19%), and 9p (16%). The minimal overlapping regions of loss in each chromosome were 8p12–pter, 13q21–31, 6q–q21, 16cen–q23, 18q22–qter, and 9p23–pter. Gain of the entire long arm of chromosome 8 was found in two (6%) cases.

Chromosome arms that were lost most frequently in recurrent prostate cancers were 8p (78%), 13q (56%), 16q (56%), 6q (44%), and 5q (44%), and the minimal regions 8p21–pter, 13cen–q21, 16q22–qter, 6q13–q21, and 5q14–q23. Gain of 8q was seen in 8 of 9 (89%) recurrent prostate cancers and usually affected the entire arm. Gains of chromosome 7 (minimal common region 7p13) and X (Xp11–q13 and Xq23–qter) were both seen in 56% of cases. Fig. 4, B and C, illustrates the main differences in the frequencies of genetic changes in primary and recurrent prostate cancer.

Taking primary and recurrent tumors together, 10 cases had a gain at 8q. In one tumor, gain was limited to 8q24, while nine were gains of the entire long arm. Seven of these tumors also had loss of 8p.

Comparison between CGH and LOH Results. Detection of losses by CGH was compared with data from LOH studies using five polymorphic microsatellite markers (D6S283, D8S265, D8S282, D13S153, and D16S413) for four different chromosome arms. In total, 37 comparisons were done in 5 loci. A 76% concordance was found between CGH and allelic loss results (Table 1). The k coefficient was 0.507. In 13% of cases, LOH was found without loss by CGH; in another 11% of cases, CGH showed loss of DNA sequences, but no LOH was detected.
Fig. 1. Summary of all gains and losses of DNA sequences observed in 31 primary prostate carcinomas by CGH. Gains are shown on the left side of the chromosome ideograms and losses on the right. Chromosome Y was excluded from analysis.

Fig. 2. Mean green:red ratio profiles for all chromosomes (except Y) from pter to qter obtained from CGH analysis of a primary prostate cancer. ———, the baseline value (1.0) representing the mean green:red ratio for the entire sample; ·····, ratios 0.5 and 1.5. Changes in the green:red ratio profile indicate losses at 6cen–q22, 8p, 11q14–q23, 16q, and 22q; and gains at 5p, 7p, 7q32–qter, and 19p. Bars, SD.
DISCUSSION

This study represents a genome-wide survey of DNA sequence copy number changes in prostate cancer using CGH. We found that 74% of the primary carcinomas showed gains and/or losses of DNA sequences, which is a significantly higher number than seen by cytogenetic studies. This reflects the power of CGH in revealing aberrations across the genome in uncultured cells. In contrast, none of the benign prostatic hyperplasias showed any genetic alterations by CGH. In primary tumors, losses predominated over gains with a ratio of 5:1. The complete absence of high-level amplification and the low overall frequency of gains in primary prostate cancer are striking as the benign prostatic hyperplasias showed any genetic alterations by cytogenetic studies. This reflects the power of CGH in revealing sequences, which is a significantly higher number than seen by 74% of the primary carcinomas showed gains and/or losses of DNA cancer (13). This suggests that inactivation of putative recessive TSGs compared to the extensive amplifications seen, for example, in breast cancer (13). This suggests that inactivation of putative recessive TSGs in several chromosomal sites is especially important in prostate cancer development.

The most commonly lost chromosomal regions in primary prostate cancer were 8p, 13q, 6q, 18q, and 9p. Several studies have shown loss of heterozygosities at 8p, 13q, 16q, and 18q in prostate cancer (2–7), but 6q and 9p losses have not been reported previously in prostate cancer. In one previous study, possible LOH at 6q and 9p was studied but not found. However, only a single probe per chromosome arm was used (7). According to CGH, the critical region at 6q was 6cen–q21, indicating that this region may harbor a TSG important in the development of prostate cancer. Previously, LOH of the same region was reported in melanoma and in ovarian and breast cancers (21–23). Furthermore, transfection of a normal human chromosome 6 suppresses the tumorigenicity of both breast and melanoma cancer cell lines (24, 25). Taken together, these results support the presence of a TSG in 6q that may be involved in several tumor types, including prostate cancer. The putative TSG at 9p also remains unknown. Recently, a new TSG, $MTS_1$, was identified at 9p21 (26). Whether this gene is also involved in prostate cancer remains to be determined. According to CGH, the minimal deleted region was 9p23–pter, which suggests that the target TSG for 9p loss in prostate cancer may reside distal to $MTS_1$.

The overall concordance between CGH and LOH results was 76%. In five cases (13%), LOH was detected but there was no loss by CGH. CGH is sensitive only to physical losses of DNA sequences and not to losses of specific alleles. Mitotic recombinations and losses followed by duplication of the remaining allele cannot be seen by CGH but may be common mechanisms of LOH (27). Furthermore, CGH can detect only physical losses affecting regions larger than 10 mega-base pairs. In four cases (11%), a loss was clearly detected by CGH, but no LOH was found. The reason for this inconsistency remains unknown, but it is possible that in these cases the microsatellite markers, which have been only genetically mapped, were localized outside the region of loss.

The majority of prostate cancer patients have disease that is no longer curable at the time of diagnosis. However, approximately 70% of these patients will respond to androgen ablation therapy. Although endocrine treatment is initially effective, the cancer cells later become androgen independent and the disease progresses despite the therapy (28). There are very few data on the genetic events that determine the malignant potential of prostate cancer and its response to endocrine therapy. Knowledge of the mechanisms underlying hormone-independent growth are important because at present, there are no effective therapies for hormone-resistant prostate cancers. We thought that the analysis of genetic changes in local recurrences that arise during hormonal therapy would highlight those aberrations causing aggressive clinical behavior.

The nine cases of androgen-resistant recurrent prostate carcinomas came from patients who had originally received endocrine therapy but developed clinical signs of local tumor progression. We found that the total number of genetic changes per tumor was almost 3 times higher in recurrences than in primary tumors. Whereas gains and amplifications were uncommon in primary tumors, all recurrences showed gains of at least one chromosomal region. In particular, gain of 8q, either alone or in association with 8p loss, was found 8 of 9 recurrent prostate cancers. This suggests that the long arm of chromosome 8 may harbor a gene(s) involved in the progression of prostate cancer and its evolution toward hormone independence. The $myc$ oncogene is located at 8q24 and has been shown to be overexpressed in poorly differentiated prostate cancers (29, 30). Because the entire long arm of chromosome 8 was usually present at an increased copy number, it is likely that other genes instead of or in addition to $myc$ are involved.

Gains of chromosomes 7 and X also were found in more than one-half of the recurrent prostate carcinomas but very infrequently in primary tumors. In two tumors, the entire chromosome 7 was gained, while three tumors showed partial gains with the minimal overlapping region at 7p13. Fluorescence in situ hybridization has shown that
trisomy 7 is common in clinically high-stage prostate cancer as well as in progression specimens (31). Recently, aneusomy of chromosome 7 was shown to be associated with poor prognosis in prostate cancer (32). Chromosome 7 contains many candidate genes, such as EGFR, PAI1, RAF, and MDR1, that may participate in the progression of prostate cancer. Gains of chromosome X were also variable. It is interesting that two recurrent tumors showed high-level amplification, one at Xp11–q13 and another at Xq23–qter. According to the Genome Data Base, the Xp11–q13 region contains many possible target genes such as AR, ARAFI, ELK1, IL2RG, PGK1, PGK1P1, PHKA1, TFE3, TIMP1, ZNF21, and ZNF81. Gains involving the same region have been found previously in about 35% of osteosarcomas by CGH, suggesting that this chromosomal region contains a dominantly acting oncogene involved in several tumor types. The other amplified region at X23–qter contains genes such as HPRT, LICAM, MCF2, and MPP1 as a possible target gene. Further studies with specific probes to these two regions and individual candidate genes are in progress. Losses in the recurrent tumors in general involved the same regions as in primary tumors, but their overall frequency was higher. However, the frequency of 5q losses was over 7 times higher in recurrent tumors than in primary tumors. Adenomatous polyposis coli TSG is localized to 5q21. LOH of the adenomatous polyposis coli region has been found in 20–30% of advanced prostate carcinomas (5, 6). These results indicate that an increased overall number of genetic changes and, specifically, gains and amplifications of certain chromosomes and chromosomal regions may underlie the progression of prostate cancer.

In conclusion, these CGH results highlight several chromosomal regions that may harbor important genes for prostate cancer tumorigenesis and progression. Losses found by CGH in primary tumors involving 6q (minimal overlapping region, 6cen–q21) and 9p (9p23–qter) suggest two new regions that may contain prostate cancer TSGs in addition to the previously reported TSG loci 8p, 13q, 16q, and 18q. Gains of DNA sequences at 7 (7p13), 8q (8q24–qter), and X (Xpl 1–qter) appear important for prostate cancer progression. Losses found by CGH in recurrent tumors involving 6q (minimal overlapping region, 6cen–q21) and 9p (9p23–qter) suggest two new regions that may contain prostate cancer TSGs in addition to the previously reported TSG loci 8p, 10q, and 16q.

ACKNOWLEDGMENTS

We thank Sari Pennanen and Ritva Timonen for their technical assistance.

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GENETIC CHANGES IN PROSTATIC CARCINOMA


Genetic Changes in Primary and Recurrent Prostate Cancer by Comparative Genomic Hybridization

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