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Analysis of Genetic Changes Underlying Local Recurrence of Prostate Carcinoma during Androgen Deprivation Therapy

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The molecular mechanisms and genetic changes that lead to the progression of prostate cancer during endocrine therapy are poorly characterized. Here, paired specimens from both untreated primary tumors and from local recurrences were collected from 10 prostate cancer patients treated by conventional androgen deprivation therapy. The genetic progression of the tumors was studied by using interphase fluorescence in situ hybridization and chromosome-specific probes. Six primary tumors (60%) and all ten recurrent tumors were aneuploid by fluorescence in situ hybridization. The recurrent tumors also showed a high degree of chromosome copy number variability from one cell to another. Increased copy number of chromosome X was particularly common in the recurrent tumors. In addition, specific high level amplification of the androgen receptor (AR) gene (Xq12) was detected in three highly aneuploid recurrent tumors. Our findings suggest that hormone-refractory prostate cancers are genetically very complex and show intratumor genetic heterogeneity. Increased copy number of chromosome X and the amplification of the androgen receptor (AR) gene may confer proliferative advantage during androgen deprivation and thus contribute to the development of recurrence. (Am J Pathol 1995, 147:1608–1614)

Cancer development and progression is a complex multistep process involving a large number of different genes. A specific cascade of genetic events has been implicated in the development of colorectal cancer,[1,2] but in prostate cancer details of the genetic factors underlying tumorigenesis and malignant progression have remained unclear.[3] Finding of loss of heterozygosity at chromosomal regions 8p, 10q, 13q, 16q, and 18q suggests that inactivations of putative tumor suppressor genes at these regions are important for prostate tumorigenesis, whereas less evidence is available on the role of the dominant oncogenes in prostate cancer progression.[4–11]

More information is particularly required on the genetic events that underlie progression of prostate cancer during hormonal therapy. Androgen deprivation therapy is often used to treat patients with advanced prostate cancer. Approximately 70 to 80% of prostate cancer patients respond favorably and achieve at least partial remission, but during the subsequent months and years treatment failure and tumor recurrence often take place.[12] The study of genetic changes underlying prostate cancer recurrence is important to understand how the hormone-refractory tumors differ from the hormone-dependent ones. The finding of specific genetic differences between these tumors could give insights to the molecular mechanisms of treatment failure and might help to develop new types of therapies.[13,14] Our recent studies by comparative genomic hybridization have suggested that increased copy number of chromosomes 7, 8, and X may be associated with recurrent prostate cancer.[15] Furthermore, we reported that specific amplification of the Xq12 region involving

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Table 1. *Genetically Abnormal Cell Populations Found in the FISH Analysis of 10 Paired Primary and Recurrent Prostate Cancers*

Patient	Tumor	TNM	Grade	Probes						
				1q12	D7Z1	D8Z2	D12Z3	D18Z1	DXZ1	AR
1	Primary	T4NXM1	II	–	3	–	–	–	–	–
	Recurrent			3	3	3,4	3	3,4	2	–
2	Primary	T3NXM1	II	–	3	–	–	–	–	–
	Recurrent			3	3	1,3	3	4	–	–
3	Primary	T1NXM0	II	–	–	–	–	–	–	–
	Recurrent			3	3	–	–	–	–	–
4	Primary	T4NXM0	II	3	3	3	3	3	–	–
	Recurrent			3	3	3	3	–	2	–
5	Primary	T3NXM1	II	–	–	–	–	–	–	–
	Recurrent			4	3,4	–	–	4	–	–
6	Primary	T1NXM0	I	3	3	3	3	3	2	–
	Recurrent			3	3	3	3	3	2	–
7	Primary	T4NXM0	II	–	–	–	–	–	–	–
	Recurrent			3	1	3,4	3	–	2	–
8	Primary	T4NXM0	III	–	3,4	4,6	–	4	2	–
	Recurrent			6	4	4,6	3	4	2	+
9	Primary	T1NXM0	II	–	–	–	–	3	–	–
	Recurrent			4,6	3,4,5	4,6,8	4	4,5,6	2	+
10	Primary	T4NXM0	III	3	3	–	3	3	–	–
	Recurrent			3,4	3,4,5	3,4	3	3,4	2	+

Six chromosome-specific centromeric probes (1q12, D7Z1, D8Z2, D12Z3, D18Z1, and DXZ1) as well as a gene-specific androgen receptor probe were used.

the androgen receptor (AR) gene is important for tumor progression during endocrine therapy.[16]

In this study, we sought to evaluate in more detail the genetic evolution and progression mechanisms that lead to tumor recurrence during androgen deprivation. Genetic changes in paired specimens from the primary tumors and local recurrences of 10 prostate cancer patients were studied by fluorescence *in situ* hybridization (FISH) and chromosome-specific repeat-sequence probes. This strategy made it possible to distinguish progression-related genetic changes from patient-to-patient differences in the genetic composition of the tumors. Furthermore, whereas comparative genomic hybridization results reflect the average pattern of genetic changes in a tumor, FISH makes it possible to evaluate genetic changes in individual uncultured tumor cells, thereby revealing the degree of intratumor genetic heterogeneity.

Materials and Methods

Ten prostate cancer patients who experienced a local tumor recurrence after treatment with conventional androgen deprivation were included in this study. All patients had initially responded favorably to androgen deprivation and the response had on average lasted 37 months (range, 9 to 87 months). Paraffin-embedded specimens were available from both the primary and the recurrent tumor in all cases. TNM-stage distribution[17] at diagnosis was T1NXM0 (three cases), T3NXM1 (two), T4NXM0 (four), and T4NXM1 (one). The distribution of histological grades was as follows[18]: one grade I, seven grade II, and two grade III cases. There were three cases each of Gleason scores 5 and 7 and two cases each of Gleason scores 6 and 8. Primary tumor specimens were either transurethral resection specimens (four cases, 2, 3, 4, and 10 in Table 1) or Tru-Cut needle biopsies (six cases) taken before administration of any hormonal therapy. Recurrent prostate carcinomas were all transurethral resection specimens taken from patients who had been treated by conventional androgen deprivation consisting of either orchiectomy (eight cases), luteinizing hormone releasing hormone agonist (one), or orchiectomy and parenteral estrogen (one). Recurrence was defined based on a substantial increase in the level of serum prostatic specific antigen and the presence of symptoms and clinical signs of urethral obstruction indicating local progression of the disease. Three of the patients also had concurrent bone metastases.

Probes specific for the pericentromeric repeat regions of chromosomes 1 (locus 1q12/probe pUC177), 7 (D7Z1/p7αtet), 8 (D8Z2/pJM128), 12 (D12Z3/pA12H8), 18 (D18Z1/p18R), and X (DXZ1/BamX7) were selected for this study. In addition, a P1 probe to the human AR gene was used.[16] Selection of the probes was based on our previous studies of recurrent prostate cancers by comparative

genomic hybridization[15] as well as previous studies of primary prostate cancers by FISH.[19] The probes were labeled by nick translation (BioNick kit; GIBCO BRL, Gaithersburg, MD) with either fluorescein isothiocyanate- or Texas Red-labeled dUTP (DuPont, Wilmington, DE). Two differentially labeled probes for two different chromosomes were used in each hybridization.

The most representative paraffin-embedded tumor blocks, containing more than 70 to 80% malignant cells, were selected by histopathological examination of hematoxylin-and-eosin-stained tumor sections. Nuclei for FISH analysis of paraffin-embedded tumors were isolated as described elsewhere.[19,20] Briefly, the sections were deparaffinized with xylene, rehydrated in an ethanol series, and placed in 1 ml of Carlsberg solution (0.1% Sigma protease XXIV, 0.1 mol/L Tris, 0.07 mol/L NaCl, pH 7.2) for 1 hour at 37°C. The nuclear suspension was pipetted on Vectabond-treated (Vector Laboratories, Burlingame, CA) slides and air dried.

Methods for FISH analysis have been previously described in detail.[19,21] Before FISH, the slides were pretreated by heating in a 50% glycerol/0.1X standard saline citrate (SSC; 1X SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate) solution at 90°C for 3 minutes to decondense the chromatin and to improve hybridization efficiency. The slides were denatured in 70% formamide/2X SSC (pH 7) at 74°C for 5 minutes, dehydrated in an ethanol series, and treated with 8 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) in a 20 mmol/L Tris mmol/l $CaCl_2$ buffer at 37°C for 7.5 minutes followed by dehydration. A 10-μl volume of hybridization mixture was denatured at 70°C for 5 minutes. Hybridization was performed under a coverslip for 24 hours at 37°C. After hybridization, the slides were washed three times in 50% formamide/2X SSC at 45°C and once in 4X SSC at room temperature. The slides were counterstained with 4,6-diamidino-2-phenylindole in an antifade solution.

A Nikon SA epifluorescence microscope (Nikon Corp., Tokyo, Japan) was used for scoring signal copy numbers from a minimum of 100 nuclei per hybridization. We used scoring guidelines published previously by Hopman and co-workers.[22] Tumors were considered trisomic or tetrasomic (or disomic for chromosome X) if more than 15% of nuclei showed three or four signals (or two signals with probe for X) and monosomic (or nullisomic for X) if more than 20% of nuclei showed only one signal (or no signal with probe for X). In addition, cell subpopulations with five or more signals were registered if present in >2% of cells. These cutoffs were based on our previous studies of benign prostatic hyperplasia specimens and represent the mean + 4 SDs of the signal counts seen in these control hybridizations.[19] AR amplification was defined as previously explained[16] based on the presence of individual tumor cells with tight clusters of AR signals, with more than five AR signals per cell or with more than twofold higher number of signals with the AR probe than with DXZ1.[16]

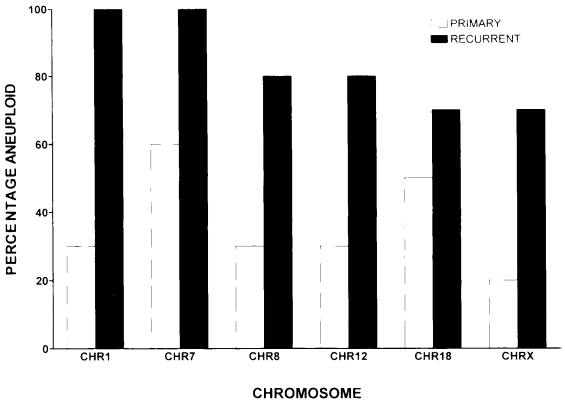

Figure 1. *Percentage of aneuploidy found with each of the chromosome-specific probes (1q12, D7Z1, D8Z2, D12Z3, D18Z1, and DXZ1) in 10 primary and recurrent prostate cancers.*

Results

Table 1 summarizes the results obtained from the hybridization of six different chromosome-specific probes to paired specimens of primary and recurrent prostate cancers from ten patients. One or more non-disomic cell populations were found in six (60%) primary prostate cancers and in all recurrent tumors. Four tumors became aneuploid at the time of recurrence and in three others the degree of aneuploidy (the number of non-disomic cell populations with all probes) increased. On average, 2.2 (range, 0 to 6) of the six chromosome probes revealed aneuploidy in the primary tumors and 5.0 (range, 2 to 6) in the recurrent tumors. Figure 1 shows a comparison of aneuploidy percentages between primary and recurrent tumors with each of the six probes. There was a general tendency towards tetraploidization, but many clearly chromosome-specific changes were also found.

Recurrent tumors showed a greater degree of intratumor heterogeneity in chromosome copy number than the primary tumors (Figure 2). Chromosome copy numbers ranging from 1 to 6 per cell, occasionally up to 8 to 10 per cell, could be detected in recurrent tumors, whereas the corresponding pri-

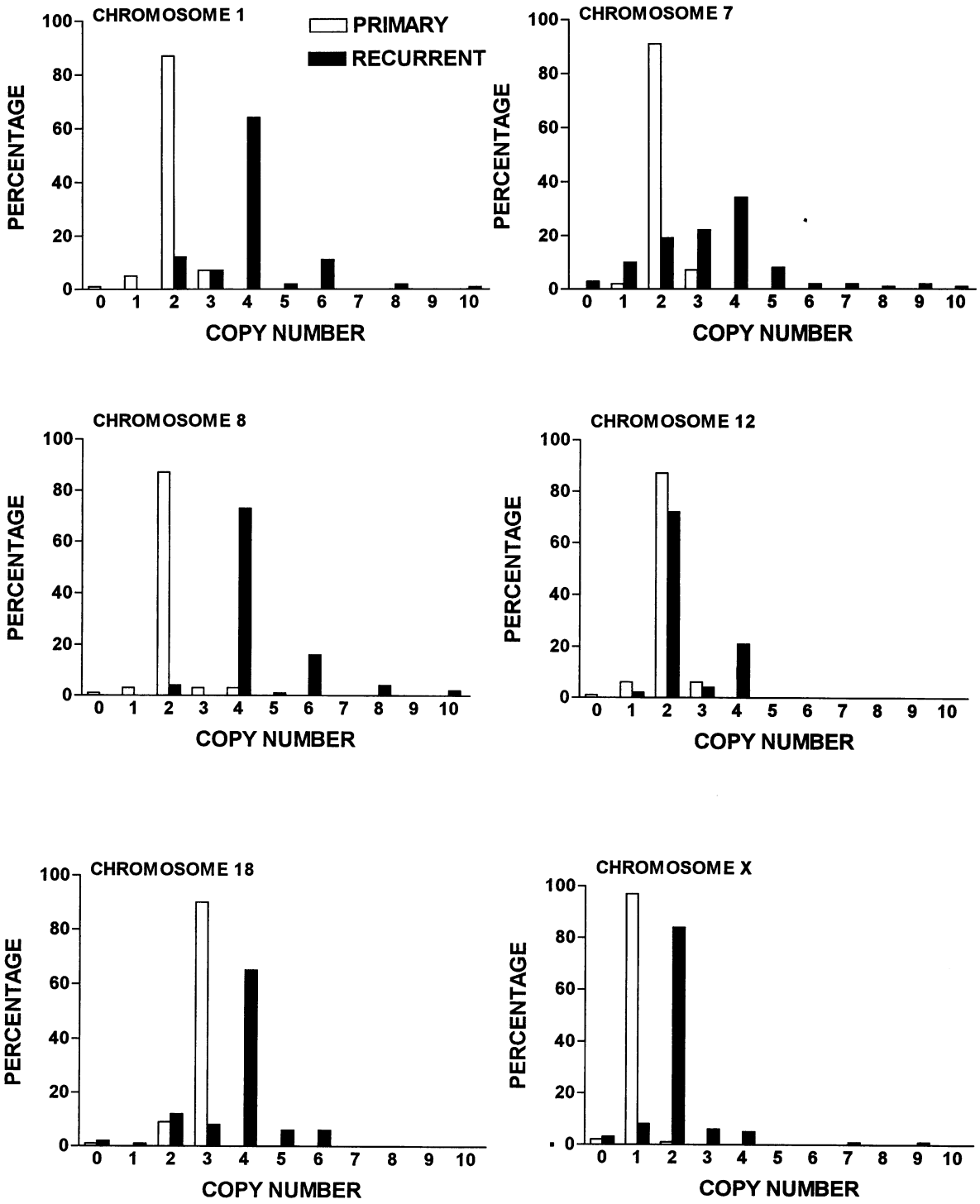

Figure 2. *Comparison of chromosome copy number distributions between a primary and recurrent prostate cancer from the same patient (case 9). The recurrent tumor shows a higher degree of aneuploidy and extensive intratumor heterogeneity.*

mary tumors showed a much more homogeneous distribution of chromosome copy number per cell.

Chromosome 7 was most often aberrant in both primary tumors (60%) and in recurrent tumors (100%). Increased copy number of chromosomes 1, 8, and 12 were found in 30 to 40% of primary tumors and in more than 70% of recurrent tumors. Aberrations of chromosome X were found in only two (20%) primary tumors but in seven (70%) recurrent tumors (Figure 1). In three of these seven recurrent cases

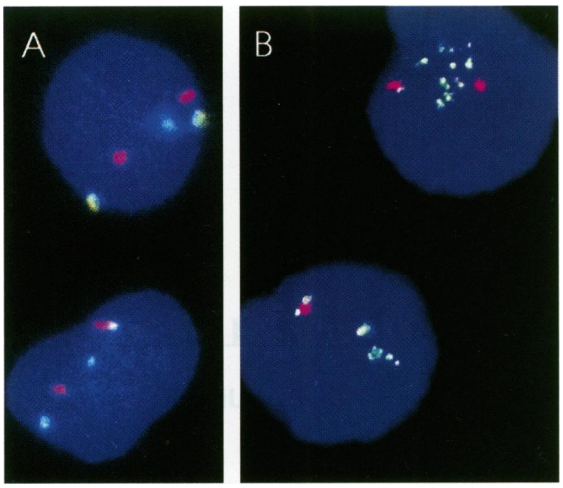

Figure 3. *Photomicrographs of two-color FISH analysis of two recurrent, hormone-refractory prostate cancers.* **A:** *Two copies of chromosome X (red) and three copies of chromosome 18 (green) are seen.* **B:** *Androgen receptor gene (green) copy number is highly increased whereas only one to two copies of chromosome X (red) are seen.*

with an extra copy of chromosome X, high level amplification of the AR gene was also detected (Figure 3). These three tumors were highly aneuploid with all probes used and also showed a high degree of genetic variability in chromosome copy number counts from one tumor cell to another.

Discussion

Many studies have already been published on genetic changes in primary prostate cancer by FISH,[19,23–24] but recurrent tumors have been included in only two studies, both describing a few individual tumors.[15,16] Here, interphase nuclei were studied by FISH from 10 untreated primary prostate carcinomas and from local recurrences appearing in the same patients after 9 to 37 months of androgen deprivation therapy. This study design is advantageous in that it makes it possible to distinguish changes related to tumor progression from patient-to-patient differences in the genetic composition of the tumor cells. Furthermore, as all patients had initially favorably responded to androgen deprivation, the genetic changes in the recurrent tumors are likely to reflect molecular mechanisms that have contributed to treatment failure and the development of androgen-refractory cell growth.

The overall frequency of aneuploidy was 60% in the primary tumors and 100% in the corresponding recurrent tumors. In addition, a higher degree of intratumor variation in chromosome copy number from one tumor cell to another was also typical of recurrent tumors. The increased frequency of aneuploidy as well as the intratumor variability suggest that the recurrent tumors are genetically advanced and probably unstable. Such instability is likely to be advantageous as it increases the probability that a critical genetic event arises that facilitates tumor cell growth during hormonal therapy.[25]

The most frequent chromosome copy number aberration in both primary and recurrent tumors was the gain of chromosome 7. This is in agreement with previous studies of primary prostate cancers by FISH[23,26] that have suggested that increased copy number of this chromosome is a marker of aggressive disease course. Gains of chromosome 8 were found in 30% of primary and 80% of recurrent tumors. Chromosome 8 aberrations have not been previously studied in paired specimens by FISH, but in primary tumors chromosome 8 changes appear to be common not only by FISH[19,23] but also by classical cytogenetics,[27,28] allelotyping,[6,9,29,30] and comparative genomic hybridization.[15,31]

Increased copy number of chromosome X was particularly closely associated with local recurrence. Gain of chromosome X was seen in only 20% of primary tumors but in 70% of recurrent tumors. We have recently reported that the AR gene located at Xq12 is highly amplified in approximately 30% of recurrent prostate carcinomas. In the present study based on another set of patients, three tumors showed high level amplification of the AR gene. These three tumors also showed gains of chromosome X and other chromosomes as well as extensive intratumor heterogeneity in copy number of all chromosomes. This suggests that tumors with AR amplification are genetically highly advanced, a finding that is compatible with the view that gene amplification can occur only in genetically unstable cells. Both in the present as well as in our previous study,[16] AR gene amplification was exclusively found in tumors recurring during endocrine therapy when the supply of growth-promoting androgens is low and amplification of this receptor gene is likely to provide an advantage for cell growth.[16] Although the gain of chromosome X was associated with AR amplification, its frequency was two times higher. Duplication of chromosome X may be selected for because it results in a slightly increased copy number of AR. Alternatively, there may be other important genes on chromosome X, the increased copy number of which facilitates tumor cell growth during endocrine therapy.

Finally, although it is clear that those tumor cell clones that are able to grow during androgen deprivation have a distinct genetic composition, it is not

known to what extent the recurrent tumor is actually clonally related to the primary tumor. Primary prostate cancers are often multifocal and genetically heterogeneous. In four of the patients, both the primary and recurrent specimens came from transurethral resections. In these cases, a direct clonal relationship is likely. In the rest of the patients, the primary tumor specimen was obtained from a Tru-Cut needle biopsy, which samples the peripheral zone, which may harbor tumor clones that are different from the transitional zone targeted by transuretheral resection. However, based on the results of the centromere counts, the genetic progression during androgen therapy was similar regardless of how the specimens had been obtained from the primary and recurrent tumor tissues.

In conclusion, our findings suggest that recurrent hormone-refractory prostate cancers are genetically very complex and show intratumor genetic heterogeneity. These findings suggest that genetic instability may underlie tumor progression and thereby accelerate the tumor evolution rate and adaptability to different growth conditions. Gain of chromosome X and amplification of the AR gene represent two specific genetic changes that were selected for during androgen deprivation therapy.

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References

1. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Prisinger AC, Leppert M, Nakamura Y, White D, Smits AMM, Bos JL: Genetic alterations during colorectal tumor development. N Engl J Med 1988, 319:525–532
2. Stanbridge EJ, Nowell PC: Origins of human cancer revisited. Cell 1990, 63:867–874
3. Visakorpi T, Kallioniemi O-P, Koivula T, Isola J: Review of new prognostic factors in prostate carcinoma. Eur J Urol 1993, 24:438–449
4. Carter BS, Ewing CM, Ward WS, Treiger BF, Aalders TW, Schalken JA, Epstein JI, Isaacs WB: Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Natl Acad Sci USA 1990, 87:8751–8755
5. Bergerheim USR, Kunimi K, Collins VP, Ekman P: Deletion mapping of chromosomes 8, 10 and 16 in human prostatic carcinoma. Genes Chromosomes & Cancer 1991, 3:215–220
6. Bova GS, Carter BS, Bussemakers MJG, Emi M, Fujiwara Y, Kyprianou N, Jacobs SC, Robinson JC, Epstein JI, Walsh PC, Isaacs WB: Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Cancer Res 1993, 53:3869–3873
7. Philips SMA, Morton DG, Lee DJ, Wallace DMA, Neoptelemos JP: Loss of heterozygosity of the retinoblastoma and adenomatous polyposis susceptibility gene loci and in chromosomes 10p, 10q and 16q in human prostate cancer. Br J Urol 1994, 73:390–395
8. Brewster SF, Browne S, Brown KW: Somatic allelic loss at the DCC, APC, nm23-H1 and p53 tumor suppressor gene loci in human prostatic carcinoma. J Urol 1994, 151:1073–1077
9. Kunimi K, Bergerheim USP, Larsson I-L, Ekman P, Collins VP: Allelotyping of human prostatic adenocarcinoma. Genomics 1991, 11:530–536
10. Carter BS, Epstein JI, Isaacs WB: *Ras* gene mutations in human prostate cancer. Cancer Res 1990, 50:6830–6832
11. Gumerlock PH, Poonamallee UR, Meyers FJ, de Vere White RW: Activated *ras* alleles in human carcinoma of prostate are rare. Cancer Res 1991, 51:1632–1637
12. Blackard CE, Byar DP, Jordan WP Jr, for the Veterans Administration Cooperative Urological Research Group: Orchiectomy for advanced carcinoma: a re-evaluation. Urology 1973, 1:533–560
13. Stearn ME, McGarvey T: Biology of disease: prostate cancer: therapeutics, diagnostic, and basic studies. Lab Invest 1992, 67:540–552
14. Berges RR, Furuya Y, Remington L, English HF, Jacks T, Isaacks JT: Cell proliferation, DNA repair, and p53 function are not required for programmed death of prostatic glandular cells induced by androgen ablation. Proc Natl Acad Sci USA 1993, 90:8910–8914
15. Visakorpi T, Kallioniemi AH, Syvänen A-C, Hyytinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi O-P: Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 1995, 55:342–347
16. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi O-P: *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995, 9:401–406
17. UICC: TNM. Classification of Malignant Tumours, ed 4. Berlin, Springer-Verlag, 1987
18. Mostofi, FK: Histological typing of prostate tumours. World Health Organization, Geneva, 1980
19. Visakorpi T, Hyytinen E, Kallioniemi A, Isola J, Kallioniemi O-P: Sensitive detection of chromosome copy number aberrations in prostate cancer by fluorescence *in situ* hybridization. Am J Pathol 1994, 145:624–630
20. Heiden T, Wang N, Tribukait B: An improved Hedley method, for preparation of paraffin-embedded tissues for flow cytometric analysis of ploidy and S-phase. Cytometry 1991, 12:413–421
21. Hyytinen E, Visakorpi T, Kallioniemi A, Kallioniemi O-P, Isola J: Improved technique for analysis of formalin-fixed, paraffin-embedded tumors by fluorescence *in situ* hybridization. Cytometry 1994, 16:93–99

22. Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Vooijs GP: *In situ* hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. Histochemistry 1988, 89:307–316
23. Takahashi S, Qian J, Brown JA, Alcaraz A, Bostwick DG, Lieber MM, Jenkins RB: Potential markers of prostate cancer aggressiveness detected by fluorescence *in situ* hybridization in needle biopsies. Cancer Res 1994, 54:3574–3579
24. Macoska JA, Micala MA, Sakr WA, Benson PD, Wolman SR: Extensive genetic alterations in prostate cancer revealed by dual PCR and FISH analysis. Genes Chromosomes & Cancer 1993, 8:88–87
25. Nowell PC: Mechanism of tumor progression. Cancer Res 1986, 46:2203–2207
26. Alcaraz A, Takahashi S, Brown JA, Herath JH, Bergstralh EJ, Larson-Keller JJ, Lieber MM, Jenkins RB: Aneuploidy and aneusomy of chromosome 7 detected by fluorescence *in situ* hybridization are markers of poor prognosis in prostate cancer. Cancer Res 1994, 54:3998–4002
27. Brothman AR, Peehl DM, Patel AM, McNeal JE: Frequency and pattern of karytotopic abnormalities in human prostate cancer. Cancer Res 1990, 50:3795–3803
28. Lundgren R: Cytogenetic studies of prostate cancer. Scand J Urol Nephrol Suppl 1991, 136:1–35
29. Bergerheim US, Collins VP, Ebman P, Kunimi K: Recessive genetic mechanisms in the oncogenesis of prostatic carcinoma. Scand J Urol Nephrol Suppl 1991, 138:93–96
30. Chang M, Tsuchiya K, Batchelor RH, Rabinovitch PS, Kulander BG, Haggitt RC, Burmer GC: Deletion mapping of chromosome 8p in colorectal carcinoma and dysplasia arising in ulcerative colitis, prostatic carcinoma, and malignant fibrous histocytomas. Am J Pathol 1994, 144:1–6
31. Cher ML, MacGrogan D, Bookstein R, Brown JA, Jenkins RB, Jensen R: Comparative genomic hybridization, allelic imbalance, and fluorescence *in situ* hybridization on chromosome 8 in prostate cancer. Genes Chromosomes & Cancer 1994, 11:156–163