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Different Patterns of DNA Alterations Detected by Restriction Landmark Genomic Scanning in Heterogeneous Prostate Carcinomas

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Alterations in the genomic DNAs of six heterogeneous prostate carcinomas, as well as that of individual and histologically distinct foci within the tumors, were examined using restriction landmark genomic scanning, a method employing two-dimensional gel analysis of a large number of DNA fragments generated by digestion with highly specific endonucleases. Upon autoradiographic imaging, these fragments appear as spots of varying intensity and location specific for each sample. In our study, comparison of cancer DNAs against normal prostate DNA controls yielded alterations in at least 35 spots. Despite differences in the histological grading of tumors, 3 spots common to all tumor samples showed consistent amplification of intensity and 8 other common spots demonstrated consistent reduction of intensity when compared with control. In addition, spot alterations occurred between histologically identical foci isolated from within single tumors. We suggest that these spot changes detected in DNA profiles generated by restriction landmark genomic scanning reflect aberrations in as yet unidentified oncogenes and tumor suppressor genes and indicate that prostate cancer is not only histologically heterogeneous and multifocal but also genetically multicentric. (Am J Pathol 1997, 150:305–314)

Prostate cancer is the third most common malignant disease in developed countries, surpassed only by lung and colorectal cancers.[1] Despite intensive studies, its etiology remains unclear. The number of clinical cancers worldwide differs markedly among various populations,[2] suggesting the involvement of possible inherent genetic predispositions and/or environmental factors in initiation and in progression.

The heterogeneous nature of both latent and clinical prostate cancer is well known and it is not unlikely that small foci within the tumor proper progress and develop more aggressive phenotypes. Based on the evaluation of such features as histological grade, tumor volume, pathological stage, and multiplicity of primary tumor sites within a gland, prostate cancer has been recognized as a multifocal disease,[3] but the question remains as to whether or not these multifocal lesions represent multicentric origins. An understanding of the molecular mechanisms underlying transformation and progression is crucial for predicting the course of disease and instituting the correct therapeutic regimen for each patient.

Progress in molecular biology, as the result of both technical and conceptual advances, has provided much information regarding carcinogenesis. Recently, we demonstrated that *ras* activations and *p53* mutations occasionally occur in focal areas within prostate carcinoma.[4] These findings suggest these events may be associated with the histogenesis of specific cell populations within a lesion. Similarly, small tumor nodules and prostatic intraepithelial neoplasias have shown varying incidences of allelic loss of sequence on chromosomes 8p, 10q, and 16q independent of dominant or primary tumors.[5] However, no cancer gene(s) has been identified in the initiation or malignant progression of prostate cancer, and we remain unable to predict its biological behavior.

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This lack of any known or highly suspect target gene or gene sequence makes molecular methodologies relying on tailored probes hit-or-miss propositions for investigating prostatic tumors; an analytic method that allows scanning for alterations in genomic DNA without the use of specific oligonucleotide probes is required. Comparative genomic hybridization serves as a screening test for regions of copy number changes in genomes. With this approach, frequent chromosomal gains and losses were detected in prostate carcinomas.[6] Another recently developed technique, restriction landmark genomic scanning (RLGS), is capable of breaking down genomic DNA into over 2000 fragments in a two-dimensional gel analysis by using specific restriction endonucleases rather than scripted probe sequences.[7–9] The fragments are radioactively end-labeled and separated by high-resolution two-dimensional gel electrophoresis. After autoradiography, highly reproducible site/spot profiles or patterns are generated, and the intensity of each spot is then compared and quantitated automatically against equivalent ones in control profiles. Spot intensity is related to gene copy number and methylation status; thus, loss of heterozygosity or deletion can be inferred from reductions in intensity, whereas amplification can be detected as an increase in signal.[7,9] In this study, we systematically compared the status of genomic DNA isolated from morphologically distinct foci within larger tumorous prostate glands by RLGS to investigate the similarities and variations in genetic alterations.

Materials and Methods

Tumor Samples and Histology

The six prostate carcinomas evaluated in this study were obtained from radical prostatectomies performed on patients who had had no previous chemotherapy or hormone treatments. The patients ranged in age from 58 to 70 years with a mean of 65 years. A slice of whole prostate was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm and remaining tissues were frozen at −70°C for later DNA extraction. Mounted sections were stained with hematoxylin and eosin (H&E) for histopathological diagnoses and to serve as a guide for the resection of specific foci and extraction of DNA.

Based on H&E evaluation, three to four focal areas from each of the six frozen tumors were selected for DNA extraction, using the Gleason system of histological classification of prostatic carcinoma[10] and well, moderately, or poorly differentiated adenocarcinomas with clinical stages A through D. Foci were additionally classed as invasive or nodular, based on their growth patterns in relation to surrounding stroma. The tumor demonstrating invasive growth included invasive areas or edges of the tumor. The foci were selected to be large enough and representative to prevent contamination of normal surrounding tissues for RLGS analysis. Adjacent normal glandular tissues were used as control samples.

DNA Extraction and Preparation

Using H&E sections as visual guides, average volumes of 3 to 5 mm^3 of tissue from each of the 20 separate selected foci were dissected from the opposite frozen slice to the H&E section and suspended in 4 ml of proteinase K buffer (500 mmol/L EDTA, 10 mmol/L Tris/HCl, 0.5% Sarcosyl) containing 1 mg/ml RNAse (Sigma type II, Sigma Chemical Co., St. Louis, MO) incubated at 37°C for 20 minutes. Proteinase K was then added to a final concentration of 1 mg/ml, and the mixture was incubated again at 65°C for 1 hour. High-molecular-weight genomic DNAs were isolated after phenol/chloroform extraction and ethanol precipitation.[11]

RLGS Analyses of Samples

The procedure for RLGS followed the standard protocol described previously.[7,12] DNAs extracted from prostate carcinoma and the normal tissue were treated simultaneously for optimal comparison. Genomic DNA (10 μg), isolated from each control sample as well from the 20 prostate foci sample, was treated with 10 U of DNA polymerase I in the presence of 0.33 μmol/L dGTPαS, 0.33 μmol/L dCTPαS, 33 μmol/L ddATP, and 33 μmol/L ddTTP. DNAs were then digested with 100 U of restriction enzyme *Not*I (Takara Co., Shiga, Japan). The cleaved ends were filled in with 20 U of Sequenase Version 2.0 (United States Biochemical, Cleveland, OH) in the presence of 0.33 μmol/L [α-^{32}P]dGTP (3000 Ci/mmol) and 0.33 μmol/L [α-^{32}P]dCTP (6000 Ci/mmol) (DuPont, Wilmington, DE). A second digestion was then performed using *Eco*RV I (New England Biolabs, Beverly, MA), and the resulting fragmented DNA was loaded onto an 0.8% Seakem GTG agarose gel (FMC Biological, Rockland, ME) and electrophoresed at 8 V/cm for 12 hours. A third digestion was performed in the gel on the separated DNA with 1500 U of *Hin*fI (New England Biolabs); that portion of the agarose gel containing the fractionated DNA was excised and fused to a separate 5% polyacryl-

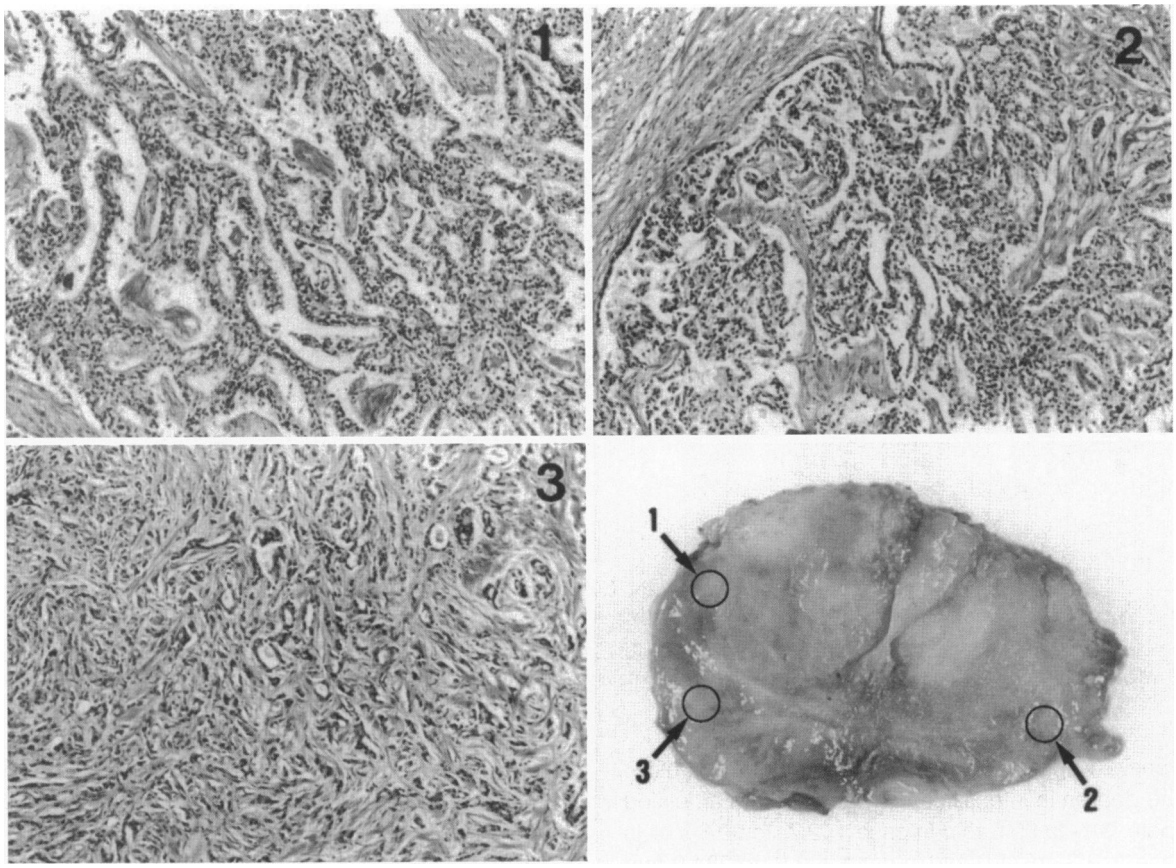

Figure 1. *The different areas within a tumor (case 3) from radical prostatectomy were selected on the basis of the histological grades and the growth patterns.* **1:** *A moderately differentiated adenocarcinoma of Gleason pattern 3 with invasive growth.* **2:** *A moderately differentiated adenocarcinoma of Gleason pattern 3 with nodular growth.* **3:** *A poorly differentiated adenocarcinoma of Gleason pattern 5 with invasive growth.*

amide gel, and a second electrophoresis was performed, in a direction perpendicular to the first, at 8 V/cm for 7 hours. Gels were dried and exposed to x-ray films (Kodak X-Omat, Eastman Kodak Co., Rochester, NY) with intensifying screens for 3 to 14 days. The resulting autoradiographs were initially inspected by eye and then compared with the previous data,[8,9] and selected spots were subjected to automated analysis by PDQUEST (PDI, Huntington Station, NY), a software program that matches and calibrates the intensities of specified spots by standardization with contiguous copy spots.[13]

Results

Tumor Histology and Growth Patterns

The tumors evaluated in this study were selected based both on their heterogeneous natures (Figure 1) and the lack of any previous chemotherapeutic or hormonal treatments to preclude any treatment-induced aberrations in lesion DNA. The criteria for clinical staging were the same as in a previous report.[4] As shown in Table 1, the 20 tumor foci evaluated were distributed throughout the spectrum of the Gleason classification system. Intratumor foci demonstrating the same histological grade were combined in characterizing whole-tumor DNA alterations.

The growth patterns of various focal samples did not always correlate with the Gleason definition, even given the relatively small number of samples. For example, tumors with Gleason grades of 3, 4, or 5 usually demonstrate some degree of invasive growth; however, nodular patterns were found in some grade 3 and 4 foci (Table 1, cases 2-1 and 3-2).

RLGS Profiles and Clinical/Histological Grades

The RLGS profiles of foci within one prostate tumor are shown in Figure 2. By comparing each pair of RLGS profiles, we detected reproducible increases and decreases in the intensity of carcinoma samples. However, the majority of the prostate carcinomas and normal spots were the same. In one sample

Table 1. *Clinical Stages, Histological Characteristics, and Number of Altered Spots in Different Areas of Heterogeneous Prostate Carcinomas*

Case	Clinical stage	Histology (Gleason)	Growth pattern	Altered spots			
				Amplified	Dwindling	Total	Mean ± SD
1-1	B2	MD (3)	I	27	20	47	50.0 ± 2.94
-2		MD (3)	I	23	29	52	
-3		PD (4)	I	23	30	53	
-4		PD (4)	I	27	21	48	
2-1	C	MD (4)	N	20	17	37	37.7 ± 3.06
-2		PD (4)	I	19	22	41	
-3		PD (5)	I	17	18	35	
3-1	D2	MD (3)	I	13	29	42	50.2 ± 9.61
-2		MD (3)	N	18	31	49	
-3		PD (5)	I	28	33	61	
4-1	B2	MD (3)	I	22	23	45	44.5 ± 7.42
-2		MD (3)	I	11	15	36	
-3		PD (4)	I	21	33	54	
-4		PD (5)	I	30	13	43	
5-1	B2	PD (4)	I	18	26	44	41.3 ± 4.62
-2		PD (4)	I	17	27	44	
-3		PD (5)	I	19	17	36	
6-1	B2	WD (1)	N	18	27	45	48.3 ± 6.66
-2		WD (2)	N	26	30	56	
-3		WD (2)	N	16	28	44	
				20.6 ± 5.08	24.4 ± 6.21		45.6 ± 7.07

WD, well differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; I, invasive growth; N, nodular growth.

alone, 61 spots showed changes in signal intensity. The degree in intensification was varied in amplified spots: 3.1- to 99.0-fold in case 1, 2.7- to 90.3-fold in case 2, 2.3- to 71.0-fold in case 3, 3.6- to 284.9-fold in case 4, 3.7- to 77.9-fold in case 5, and 3.2- to 74.3-fold in case 6. The intensity of dwindling spots was from 0.0 to 0.5 as measured by densitometer. The average number of spot alterations for the 20 foci evaluated was 45.6 ± 7.07. Table 1 summarizes the numbers of altered fragments by case and focus designation. As can be seen, the focus demonstrating the highest number of total alterations (case 3-3) occurred within one of the histologically most malignant tumors, yet this high number of alterations appeared to be an isolated occurrence, as the other two foci within the same tumor had approximately the average number of alterations.

Table 2 summarizes the number of amplified and reduced spots by focal histological grade. There were no significant correlations between grade and quantity, but the numbers of spots showing signal amplification demonstrated a tendency to increase whereas the numbers showing signal reduction tended to decrease with higher Gleason scores.

Detection of Consistent Spot Amplification/ Reduction in Tumor Samples

Many spots were found to be commonly amplified or reduced in various samples of tumor foci, indicating that certain subsets of DNA alterations were persistent at differing frequencies within the tumors. More significantly, 11 separate spots were found common to all six prostate carcinomas; 3 (spots A, B, and C) of these were always amplified to some degree over control samples, whereas the remaining 8 spots (a to h) consistently showed reduction of signal relative to control (Figure 3). We compared the altered spots with the chromosome-assigned RLGS profile of genomic DNA from GM0130B cells[14] (Table 3). The range of signal amplification was 2.3- to 22.4-fold over normal. Signal reductions ranged in value from 0.5 (50% reduction relative to control) to 0.0 (considered undetectable).

We detected several spots that seem to be altered with transition in histological grade (Figure 4). When the Gleason classification changed from grade 1 to 2, we noted two spots that always showed signal intensification and four that showed reduction of signal. One other spot demonstrated consistent reduction on score shifts from 2 to 3. Amplification was noted in still another fragment when the grade increased from 4 to 5. Additional spots showed alterations 50 to 80% of the time with grade changes.

Discussion

A definitive marker gene (or genes) has not been identified in prostate cancers, despite intensive investigation.[15,16] DNA transfection assays were commonly

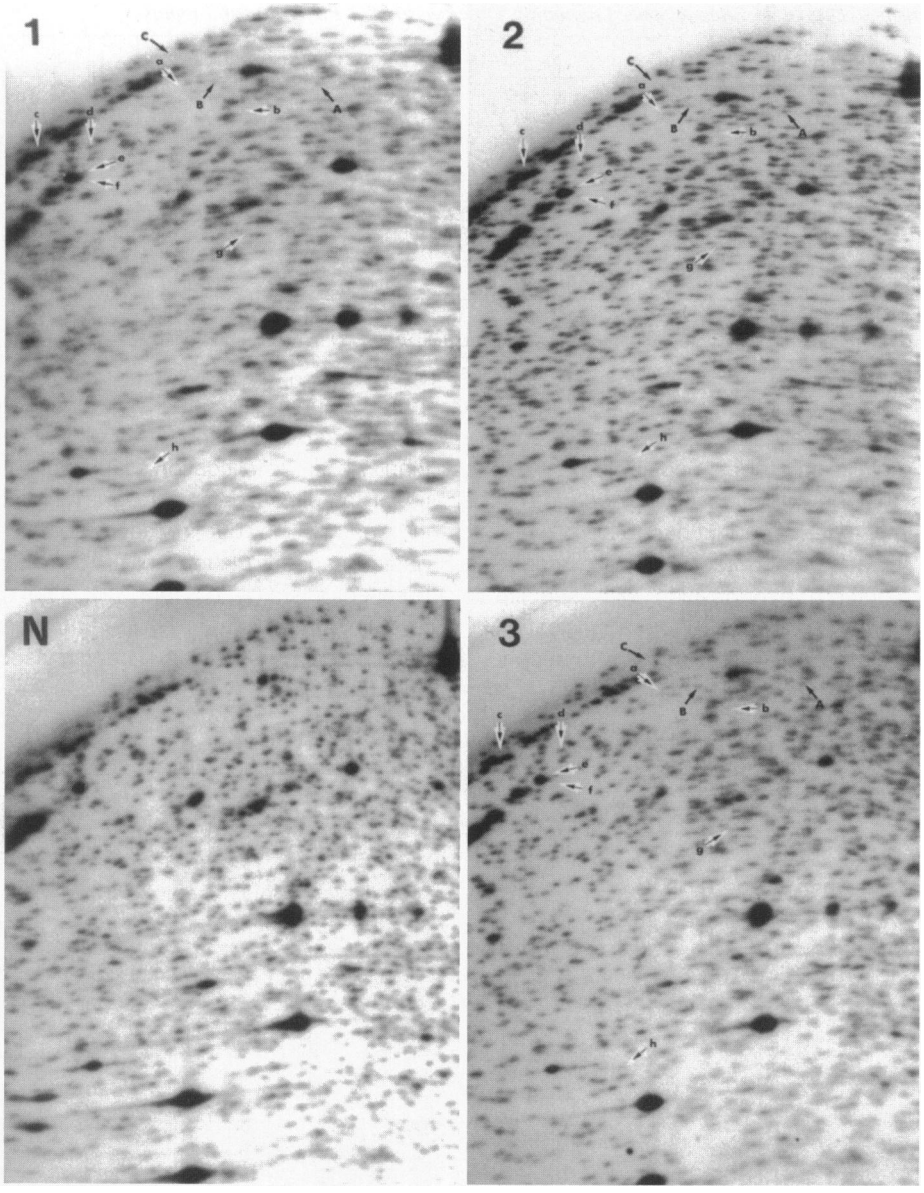

Figure 2. *Whole RLGS profiles of DNA from different foci within a prostate carcinoma (case 3) and from surrounding normal prostate. Approximately 2000 spots were identified and compared in one gel. The data of amplified and dwindling spots were summarized in Table 1.*

used in the early 1980s to identify active oncogenes, but the transfection method could not pinpoint a genetic event correlated with the initiation or progression of the disease. We have previously noted an increased incidence of *ras* activation in prostate tumors of Japanese patients compared with that found in patients from Western countries; however, the highest frequency ascertained in Japanese tumors was still only 27%.[2,4,17–20] Activation or overexpression of other oncogenes, including c-*myc*, *sis*, and *neu,* have been investigated as well, but although this appears to be a common occurrence *in vitro*, such activation appears to be sporadic *in vivo*.[21–23]

Table 2. *Number of Altered Spots According to Gleason's Grading of Prostate Carcinoma*

Gleason's grade	Number of foci	Amplified spots	Dwindling spots
1	1	18.0 ± 0.0	27.0 ± 0.0
2	2	21.0 ± 7.07	29.0 ± 1.41
3	6	19.0 ± 6.16	24.5 ± 6.25
4	7	20.7 ± 3.40	25.1 ± 5.52
5	4	23.5 ± 6.45	20.3 ± 8.77

The apparent lack of consistent genetic events associated with prostate cancer may be a function of the analytical methods used. As was previously

Table 3. *Intensified Degree of Altered Spots Commonly Observed in Six Prostate Carcinomas*

	Amplified spots			Dwindling spots							
Case	A (3)	B (ND)	C (ND)	a (10)	b (6)	c (16)	d (15)	e (20)	f (15)	g (10)	h (ND)
1	17.3	18.6	6.8	0	0	0	0	0.3	0	0.5	0
2	9.6	5.9	6.6	0.2	0	0.4	0.5	0.3	0.5	0	0.3
3	2.4	15.4	17.6	0	0	0.1	0	0.2	0.2	0.5	0.2
4	22.4	3.8	7.3	0	0.5	0.4	0	0.3	0.5	0.5	0
5	18.5	2.3	14.8	0.2	0	0	0	0.3	0.2	0	0
6	16.6	3.0	4.6	0.4	0	0	0.4	0.3	0.3	0.5	0

The degree of intensification is represented as fold enhancement in each spot, compared with the corresponding spot in normal prostate. Chromosome numbers are indicated in parentheses. ND, not detected on chromosome-assigned RLGS profile.

stated, earlier reports depended on evaluating relatively short sequences of cancer DNAs using techniques requiring tailored probes. Sensitivity was also a problem in that amplification of a sequence or overexpression of a gene product had to be above a certain level for accurate detection. For example, conventional Southern hybridization with specific probes can detect more than a 30-fold amplification of a single locus per analysis.[7] Even with recent improvements, such as in-gel renaturation, and under stringent conditions, it is difficult to detect less than 7-fold amplification.[24] Low-grade, but functional, amplifications have been reported for *hst*-1 in hepatocellular carcinomas (5-fold),[25] for c-*myc* in gastric cancers (4- to 8-fold),[26] and for c-*erb*B-2 in breast cancers (2- to 5-fold).[27] It is not unreasonable to suggest that many DNA perturbations inherent in prostate cancers may have been missed with routine

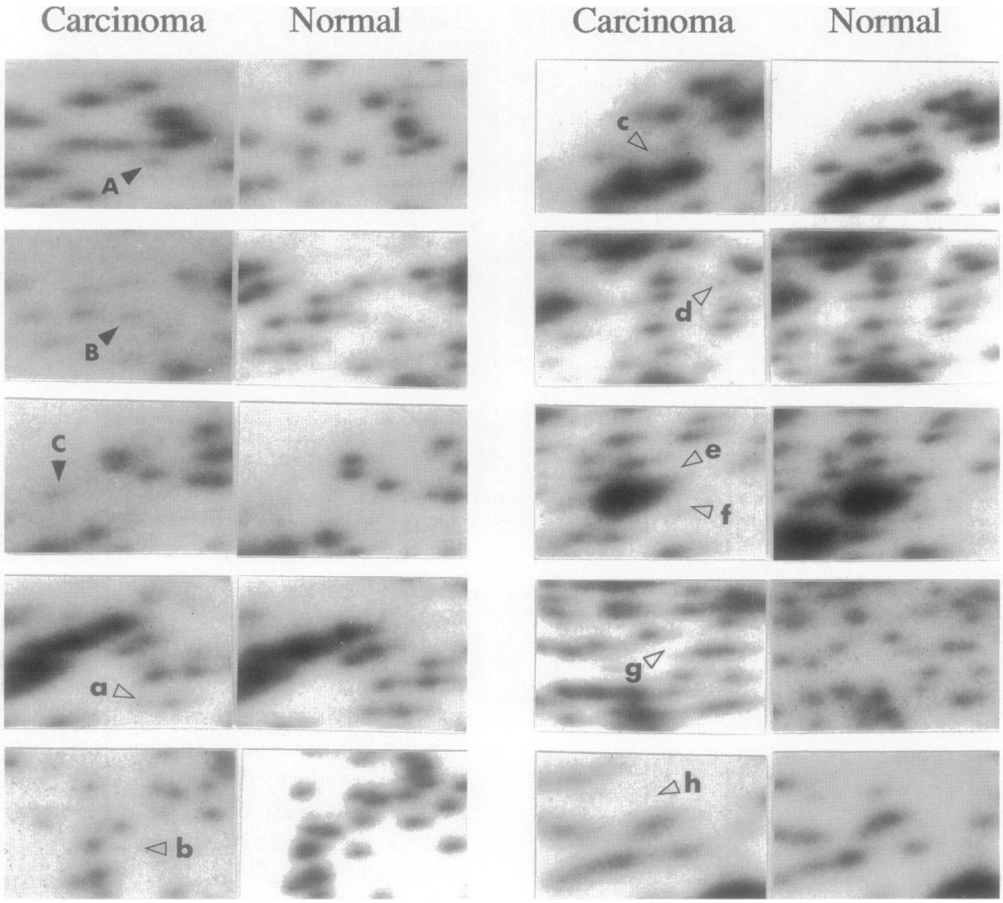

Figure 3. *The magnified regions of RLGS profiles containing the commonly amplified spots* **A**, **B**, *and* **C** *and the spots* **a** *to* **h** *with signal decreases are shown in pairs with the correlated spots in normal tissue. When compared with the normal tissue, sporadic spots besides spots* **A** *to* **C** *and* **a** *to* **h** *were occasionally observed, which were summarized in Table 1.*

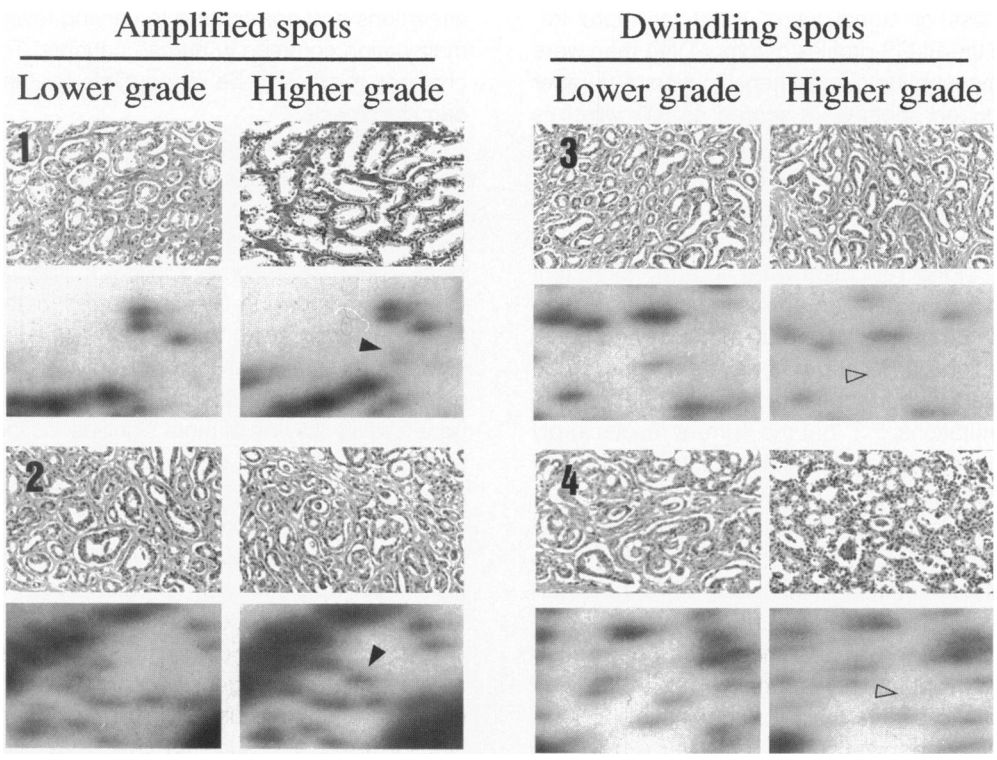

Figure 4. *Specific spot changes in histological grades of prostate carcinoma. Amplified spots are noted when Gleason grade increases from 1 to 2* (**1**) *and 2 to 3* (**2**). *The spot intensity decreases when Gleason grade increases from 2 to 3* (**3**) *and 3 to 4* (**4**).

analytical methods. In contrast, RLGS allows scanning of thousands of loci at one time and is sensitive enough to catch as low as a 2-fold amplification as well as permit estimation of copy number. As noted earlier, the RLGS profiles for different individuals are the same as long as the same set of restriction enzymes are used.[9] The intensification was reproducible to varying degrees for the prostate carcinomas tested. The three spots we found to be consistently amplified in all tumor samples showed less than 25-fold increases in signal intensity and thus might have been undetected with conventional methods. One amplified spot A was located in chromosome 3, whereas the other two spots were not detected on the chromosome-assigned RLGS profile. As the RLGS map was assigned from GM0130B cells originating from a spontaneously immortalized lymphoblast cell line, the latter two spots may be tissue specific.

RLGS also appears to be of great value for looking at DNA methylation patterns, as it employs methylation-sensitive restriction enzymes such as *Not*I.[28,29] Both the increases and decreases of signal given off by DNA fragments observed on a RLGS film are related to the methylation status within the *Not*I recognition sequence. In the vertebrate genome, there are approximately 4000 *Not*I sites, with approximately 3500 of those believed to lie within cytosin-p-guanine islands[30]; roughly 1000, or 25%, of these sites will register on the film generated by one RLGS gel. These CpG islands contain many of the transcriptional elements of genes. Demethylation of these islands is associated with oncogene activation and tumor suppressor gene inactivation both *in vivo* and *in vitro*.[29,31] Signal amplification and reduction, therefore, implies that there has been demethylation and subsequent activation of a presumed oncogene and inactivation of a tumor suppressor gene, respectively. Hypomethylation in CpG islands has been linked previously with inactivation of the tumor suppressor retinoblastoma gene,[31] and hypomethylation at chromosome 17p 13.3 has frequently been found in human prostate adenocarcinomas.[32] Deletion analyses have additionally pointed to allelic loss of tumor suppressor genes in prostate cancers from chromosomes 8p (60% of informative cases evaluated), 10q (34% of informative cases), 16q (>50% of informative cases), and 18q (>40% of informative cases).[33,34] These reports indicate that inactivation of tumor suppressors is involved at some point in prostate neoplasia.[35–37] As was mentioned earlier, we identified not only three amplified spots but also eight spots showing consistent reduction of signal intensity in all of our tumor samples. In fact, spots

showing loss of signal were noted far more frequently in the RLGS profiles of tumor DNA than were spots demonstrating signal intensification. A number of the reduced spots were scored as 0.0, which is essentially undetectable and could be interpreted as deletions, whereas scores of 0.5, which indicate a 50% signal reduction, might represent loss of heterozygosity. The chromosomal location of the eight spots with signal reduction were, in part, consistent with the previous deletion analysis; however, others such as chromosome 15 and 20 might be new ones. Copy deletions in *RB* and *p53* have appeared to be less frequent occurrences in prostate cancer than are *p53* mutations,[38,39] but the current results imply that deletions may be more common than previously supposed, irrespective of heterogeneous histologies. Cloning of these fragments to identify the specific genes and locations involved are in progress.

Of particular interest in this study were the differing profiles generated by foci having the same histological grade and appearance. It can be argued that little consideration has been given to the individual genetic events taking place within the morphological subpopulations of a larger tumor. We have previously reported occasional alterations in *ras*, *p53*, and *p16* in isolated areas within prostate tumors that appear to be closely associated with invasive growth patterns,[4,40] corroborating the assertion that prostate cancer is a multifocal disease.[3,41] In a related study, samples of normal prostate gland, of high-grade prostatic intraepithelial neoplasias, and of invasive carcinoma all showed differing patterns of allelic loss on chromosome 18p 12–21, despite the fact that all samples were taken from a single patient.[42] The results of these earlier studies, however, were difficult to interpret because of intratumor heterogeneity. In addition, the varying mutation frequencies found may have been due, in part, to the analytical methods employed. It has been suggested that, in the multistep process of carcinogenesis, clonal populations arising independently within hyperplastic tissues or established tumors may undergo separate and individual genetic changes leading to growth advantages. Although intraglandular metastasis should be ruled out, the present investigation clearly demonstrates that such clonal genetic changes do occur, thus supporting the concept of multicentricity, but, as yet, it is not possible to determine what or if a specific event or set of events confers any advantages to growth. As was shown in Tables 1 and 2, higher clinical stages and histological grades do not necessarily give evidence of more genetic alterations. It is possible that many of the spot changes noted represent sporadic alterations and correspond to varying levels of DNA methylation common in human cancers. These spot changes might also be responsible for the prostate carcinogenesis.

Tumor progression, driven by genetic events subsequent to initiation and proliferation, may also be an area amenable to study and elucidation by RLGS. Although the sample size in the present study was admittedly small, there were some consistent spot alterations found with increasing Gleason scores, notably in the transition from grade 1 to 2, from 2 to 3, and from 3 to 4. Most of these alterations involved decreases in signal, rather than amplification, suggesting that loss of gene activity may be of more significance than activation when it comes to tumor progression. If there are specific and reproducible DNA aberrations correlatable to clinical and histological progression, it would be of great importance to analyze metastatic lesions and lesions from patients with sporadic *versus* hereditary forms of prostate cancer.

It must be pointed out that approximately 25% of all *Not*I sites in the genome can be detected with RLGS[8] and that the use of methylation-sensitive restriction enzymes, such as *Not*I, may, by chance, cleave a gene sequence of tumorigenic importance. Even so, the data obtained in this study are consistent with the hypothesis that carcinoma of the prostate is genetically multicentric as well as histologically heterogeneous and multifocal. Cloning of specific fragments identified by RLGS should help to clarify the spectrum of genetic alterations occurring and, potentially, the sequence of these events as they pertain to tumor development and progression. This information will be of critical value in the evolution of more effective patient treatments and management practices that address the clinical idiosyncrasies imposed by tumor heterogeneity.

Acknowledgments

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