[CANCER RESEARCH 59, 803–806, February 15, 1999]

Advances in Brief

Survey of Gene Amplifications during Prostate Cancer Progression by High-Throughput Fluorescence *in Situ* Hybridization on Tissue Microarrays[1]

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Abstract

Prostate cancer development and progression is driven by the accumulation of genetic changes, the nature of which remains incompletely understood. To facilitate high-throughput analysis of molecular events taking place in primary, recurrent, and metastatic prostate cancer, we constructed a tissue microarray containing small 0.6-mm cylindrical samples acquired from 371 formalin-fixed blocks, including benign prostatic hyperplasia ($n = 32$) and primary tumors ($n = 223$), as well as both locally recurrent tumors ($n = 54$) and metastases ($n = 62$) from patients with hormone-refractory disease. Fluorescence *in situ* hybridization (FISH) was applied to the analysis of consecutive tissue microarray sections with probes for five different genes. High-level (≥3X) amplifications were very rare (<2%) in primary prostate cancers. However, in metastases from patients with hormone-refractory disease, amplification of the androgen receptor gene was seen in 22%, MYC in 11%, and Cyclin-D1 in 5% of the cases. In specimens from locally recurrent tumors, the corresponding percentages were 23, 4, and 8%. ERBB2 and NMYC amplifications were never detected at any stage of prostate cancer progression. In conclusion, FISH to tissue microarray sections enables high-throughput analysis of genetic alterations contributing to cancer development and progression. Our results implicate a role for amplification of androgen receptor in hormonal therapy failure and that of MYC in the metastatic progression of human prostate cancer.

Introduction

Prostate cancer is the most frequent cancer among men in industrialized countries and the second leading cause of cancer-related death (1). Given the substantial variability in the clinical behavior of prostate cancer, it would be important to better understand the biological basis of tumor development and progression, to develop markers for assessing prognosis or prediction of therapy outcome, as well as to identify targets for the development of novel therapies (2). The number of potential cancer-related genes and genetic alterations is increasing rapidly. The evaluation of the clinical utility of each of these genes would require multiple consecutive experiments with hundreds of tumors. This would be both time-consuming as well as impractical for more than a handful of genes.

We recently developed a novel tissue microarray ("tissue chip") technology (3) for rapid molecular profiling of large numbers of cancers in a single experiment. Tissue microarrays are constructed by bringing minute cylindrical tissue samples (diameter, 0.6 mm) from hundreds of different tumors into a single paraffin block. Five-μm sections from these tissue microarray blocks can then be applied in the analysis of copy number or expression of multiple genes by DNA and RNA *in situ* hybridization or by immunohistochemistry. Here, we constructed a tissue microarray containing samples from different stages of human prostate cancer progression to survey genetic alterations that may contribute to hormone refractory and metastatic disease. We decided to investigate the role of gene amplifications, because these alterations have been implicated in the progression of many tumor types. Most previous studies have found few if any gene amplifications in prostate cancer, but the majority of these have been based on relatively small materials or evaluated only a single gene (4–9). The comparison of data from different studies is also difficult, because a large variety of different techniques have been used, including Southern blot, slot blot, quantitative PCR, and FISH.[3] For example, substantially discordant results have been published on the role of *ERBB2* oncogene with the reported amplification frequencies ranging from 0 to 44% (4, 10–12). The two gene amplifications that have been studied in more detail include the *AR* and *MYC* oncogene amplifications reported in hormone-refractory or metastatic tumors, respectively (7–9). In this study, we constructed a tissue microarray containing 339 tumor specimens from different stages of prostate cancer progression and assayed five different gene amplifications (*AR*, *MYC*, *ERBB2*, *CCND1*, and *NMYC*) by FISH to consecutive formalin-fixed tissue microarray sections. The aim was to obtain a comprehensive survey of gene amplifications in different stages of prostate cancer progression, including specimens from distant metastases.

Materials and Methods

Prostate Cancer Tissue Microarray. Formalin-fixed and paraffin-embedded tumor and control specimens were from the archives of the Institutes for Pathology, University of Basel and the Tampere University Hospital. All tumors and controls were reviewed by one pathologist (L. B.). The least differentiated tumor area was selected to be sampled for the tissue microarray. The specimens that were interpretable for at least one FISH probe included: (*a*) transurethral resections from 32 patients with BPH to be used as controls; (*b*) 223 primary tumors, including 64 cancers incidentally detected in transurethral resections for BPH; (*c*) 145 clinically localized cancers from radical prostatectomies, and 14 transurethral resections from patients with primary, locally extensive disease (13); (*d*) 54 local recurrences after hormonal therapy failure including 31 transurethral resections from living patients and 23 specimens obtained from autopsies; and (*e*) 62 metastases collected at the autopsies from 47 patients who had undergone androgen deprivation by orchiectomy and had subsequently died of end-stage metastatic prostate cancer. Metastatic tissue was sampled from pelvic lymph nodes ($n = 8$), lung ($n = 21$), liver ($n = 16$), pleura ($n = 5$), adrenal gland ($n = 5$), kidney ($n = 2$), mediastinal lymph nodes ($n = 1$), peritoneum ($n = 1$), stomach ($n = 1$), and ureter ($n = 1$). In 23 autopsies, material was available from both the primary and from the metastatic site. More than one sample per tumor specimen was arrayed in 44 of the

Received 11/10/98; accepted 1/6/98.

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[1] L. B. is supported by the Swiss National Science Foundation (81BS-052807), G. S. is supported by Clinical Cancer Foundation, Basel, Switzerland, and P. K. by the Academy of Finland and the TAUH-EVO Foundation.

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[3] The abbreviations used are: FISH, fluorescence *in situ* hybridization; AR, androgen receptor; CCND1, Cyclin-D1; BPH, benign prostatic hyperplasia.

339 cases. A tumor was considered amplified if at least one sample had amplification.

The original array also included 48 pathologically representative samples that consistently failed in the analysis of sections with all FISH probes. The number of samples evaluable with the different probes was variable because: (*a*) the hybridization efficiency of the probes was slightly different (see "Results"); (*b*) some samples on the array were occasionally lost during the sectioning or FISH procedure; and (*c*) some tumors were only representative on the surface of the block, and the morphology changed as more sections were cut.

Construction and Sectioning of Tissue Microarrays. The prostate tissue microarray was constructed as described previously (3). Briefly, a tissue arraying instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle with an inner diameter of 0.6 mm, held in an X-Y precision guide. The cylindrical sample was retrieved from the selected region in the donor and extruded directly into the recipient block with defined array coordinates. A solid steel wire, closely fit in the tube, was used to transfer the tissue cores into the recipient block. After the construction of the array block, multiple 5-μm sections were cut with a microtome using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ). H&E-stained sections were used for histological verification of tumor tissue on the arrayed samples.

FISH to Formalin-fixed Tissue Microarray Sections. Two-color FISH to sections of the formalin-fixed samples on the tissue microarray was performed using Spectrum Orange-labeled AR, MYC, ERBB2, and CCND1 probes with corresponding FITC-labeled centromeric probes (Vysis, Downer's Grove, IL). In addition, one-color FISH was done with Spectrum Orange-labeled NMYC probe (Vysis). The hybridization was performed according to the manufacturer's instructions. The following tissue treatment protocol was developed to allow formalin-fixed tumors on the array to be reliably analyzed by FISH. The slides of the prostate microarray were first deparaffinized, immersed in 0.2 N HCl, incubated in 1 M sodium thiocyanate solution at 80°C for 30 min, and immersed in a protease solution (0.5 mg/ml in 0.9% NaCl; Vysis) for 10 min at 37°C. The slides were then postfixed in 10% buffered formalin for 10 min, air dried, denaturated for 5 min at 73°C in 70% formamide/2× SSC (SSC is 0.3 M sodium chloride and 0.03 M sodium citrate) solution and dehydrated in 70, 80, and 100% ethanol, followed by proteinase K (4 μg/ml PBS; Life Technologies, Inc., Rockville, MD) treatment for 7 min at 37°C. The slides were then dehydrated and hybridized. The hybridization mixture contained 3 μl of each of the probes and Cot1-DNA (1 mg/ml; Life Technologies, Inc.) in a hybridization mixture. After overnight hybridization at 37°C in a humid chamber, slides were washed and counterstained with 0.2 μM DAPI. FISH signals were scored with a Zeiss fluorescence microscope (Jena, Germany) equipped with a double-band pass filter using ×40-×100 objectives. The relative number of gene signals in relation to the centromeric signals was evaluated by visual analysis of the hybridization signals. Criteria for gene amplification were: tight clusters of signals in multiple cells or at least three times more test probe signals than centromeric signals per cell in >10% of the tumor cells. Test:control signal ratios in the range between 1 and 3 were regarded as low-level gains and were not scored as evidence of specific gene amplification. Evidence for amplification of NMYC without reference probe was considered in the case of tight clusters of gene signals or >5 signals in at least 10% of the tumor cells.

Results

FISH Analysis of Formalin-fixed Tissue Microarray Sections. After optimizing pretreatment of tissue microarray sections, the quality of FISH results from formalin-fixed tumors was in most instances comparable with that obtained with ethanol-fixed frozen tissues used in our previous studies (Fig. 1). The hybridization efficiency of the probes could be estimated from the analysis of BPH tissue samples that were present on the same tissue microarray. High-quality hybridization signals with both centromeric, and gene-specific probes were obtained in 96% of the BPH samples for chromosome X/*AR* gene, 84% for chromosome 8/MYC, 81% for chromosome 17/ERBB2, and 83% for chromosome 11/CCND1. In the evaluable BPH samples, the average percentage of epithelial cells with two signals for autosomal probes was ~75%, with ~20% showing one signal and ~5% no signals. The percentage of cells with one or zero signals is probably mostly attributable to the truncation of nuclei with sectioning (14). In the punched samples from biopsy cancer specimens, AR, MYC, ERBB2, and CCND1 FISH data could be obtained from 92, 78, 82, and 86% of the cases, respectively. The success rate of FISH was lower in punches from autopsy tumors (44–58%). Amplifications were only scored to be present when the copy number of the test probe exceeded that of the chromosome-specific centromere reference probe by ≥3-fold in 10% or more of the tumor cells. This criterion was chosen, because low-level amplification is likely to be less relevant, and because locus-specific probes often display slightly higher copy numbers than centromeric probes, due to signal splitting or the presence of G_2-M-phase cells.

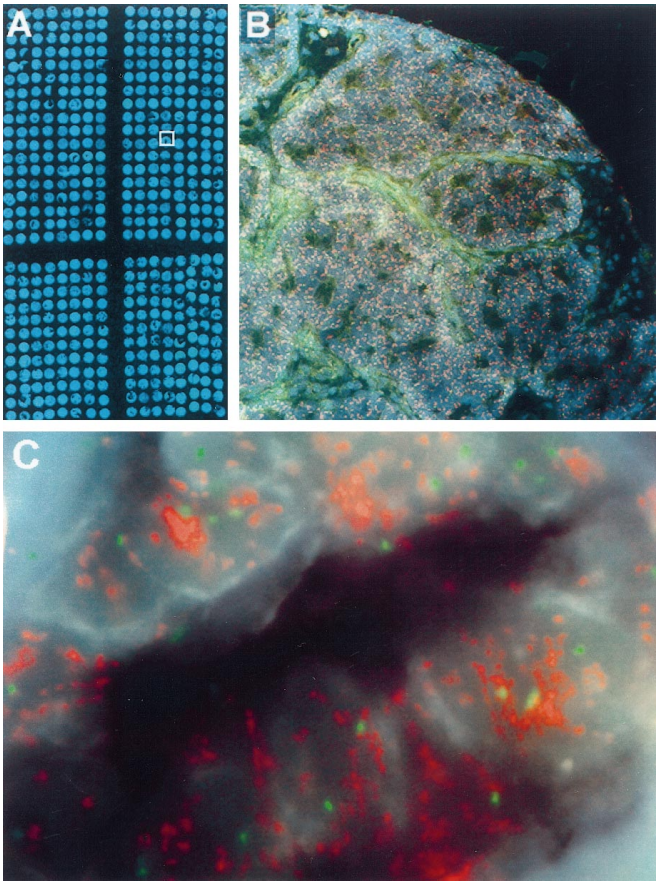

Fig. 1. Detection of AR amplifications in prostate cancer by FISH on sections of a prostate tissue microarray from formalin-fixed tissue specimens. *A*, overview of a tissue microarray section containing hundreds of different tumor samples (Ø 0.6 mm, each). ×3. *B* and *C*, AR amplification with many clustered *AR* gene signals (*red*) and a few centromere X reference signals (*green*). *B*, ×200; *C*, ×1000.

AR. FISH with the AR probe revealed amplification in 23.4% of the 47 evaluable hormone-refractory local recurrencies (Fig. 2). Amplification was seen equally often (22.0%) in 59 metastases of hormone-refractory tumors. The strong association between AR amplification and hormone-refractory prostate cancer is evident from the fact that only 2 of the 205 evaluable primary tumors (1%) and none of the 32 BPH controls showed any AR amplification. The two exceptions included a patient with locally advanced and metastatic prostate cancer and another patient with clinically localized disease. Whereas the patient records did not mention hormonal therapy, prior exposure of these patients to such therapy cannot definitively be ruled out. Paired tumors from the primary site of the cancer and from a distant metastasis of 17 patients were successfully analyzed for AR amplification. In 11 of these patients, no AR amplification could be seen at either site. Of the six remaining patients, three patients showed amplification in the local tumor mass, as well as in the distant

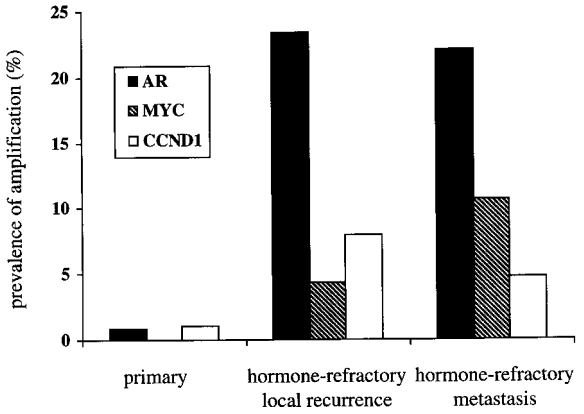

Fig. 2. Prevalence of oncogene amplifications during the progression of prostate cancer by FISH on prostate tissue microarray (n = 371 specimens). There were no amplifications of ERBB2 and NMYC.

metastases. In two cases, amplification was only found in the sample from the primary site, whereas in another case, only the distant metastasis showed amplification.

MYC. High-level MYC amplifications were found in 5 of 47 evaluable metastatic deposits (10.6%), in 2 of the 47 local recurrencies (4.3%, both from cancers that also had metastases), but in none of the 168 evaluable primary cancers or 31 BPH controls (Fig. 2). There was a significant association between AR and MYC amplifications. MYC was amplified in 12.5% of 24 evaluable tumors with AR amplifications but only in 1.8% of 219 tumors without AR amplifications (P = 0.003, contingency table analysis). AR was independently amplified in 21 tumors, whereas only 4 tumors had MYC amplification, but no AR amplification.

CCND1, ERBB2, and NMYC. CCND1 amplifications were found in 2 (1.2%) of the 172 evaluable primary tumors, in 3 (7.9%) of 38 local recurrencies, and in 2 (4.7%) of the 43 metastases. CCND1 amplification appeared independent from AR or MYC amplification with only two of seven and one of seven CCND1-amplified tumors showing also AR or MYC amplification, respectively. There were no ERBB2 amplifications among any of the 262 evaluable tumors or 31 BPH controls. Finally, a subset of the tumors was analyzed with the NMYC probe in a single-color FISH analysis. Of the 164 tumors available, none showed evidence of amplification.

Discussion

Development and progression of prostate cancer to lethal, hormone-refractory, and metastatic disease is believed to be driven by multiple genetic alterations, the nature and sequence of which have remained poorly understood. For this study, a tumor tissue microarray was constructed that allowed us to investigate the pattern of amplifications of multiple genes in samples representing the entire spectrum of prostate cancer progression, including distant metastases. The advantage of the tissue microarray strategy is that it facilitates standardized analysis of multiple genes in the same tumors, even in the same specific tumor sites using the same technology, same kind of probes, and similar interpretation criteria. In just five FISH experiments, we were able to screen a material of 371 specimens with five gene-specific probes, resulting in a total of >1400 evaluable FISH results. The ability to achieve reliable detection of gene amplifications from formalin-fixed tissues substantially extends the range of possible applications for the tissue microarray technology (3) by enabling studies of tumor progression using archived surgical or even autopsy specimens. In this study, we focused on high-level gene amplifications that are relatively straightforward to detect and score visually. However, we expect that in the future, automated digital image scanning and spot counting of entire tissue microarray slides will become possible and will increase reproducibility and facilitate detection of other alterations, such as deletions or low-level gene copy number gains.

A possible limitation of the tissue microarray technology is that the minute tissue samples acquired from the original tissues may not always be representative of the entire tumor, in light of the intratumor heterogeneity characteristic to most cancers. The frequency of involvement of the different genes in our study is therefore likely to be an underestimate, although the comparison of the present results with the previous literature suggests that this problem is not as substantial as one would expect (see below). The effect of this sampling bias is also primarily reflected in the analysis of absolute frequencies of genetic alterations in a given tumor type. The comparisons between similarly acquired specimens from different stages of tumor progression placed on the same tissue microarray should be less problematic. Also, comparisons of the frequencies of involvement of specific genes with those of other genes evaluated from consecutive tissue microarray experiments should suffer little if at all of sampling biases. Moreover, it is very likely that "punching" from multiple sites from each original tumor can significantly reduce the sampling problem. Nevertheless, at the moment, one should consider the tumor tissue microarray technology as a rapid, high-throughput survey method to pinpoint the biologically most prevalent or clinically most promising genes and molecular markers for detailed studies with conventional tissue specimens.

Gene amplifications have been reported to be more infrequent in prostate cancer than in many other carcinomas. According to our results, this is indeed the case for primary prostate cancers, where high-level amplifications of all of the tested loci were rare (<2%). However, in samples from hormone-refractory local recurrencies or metastatic deposits, the amplification frequencies were substantially more common for three of the five genes evaluated. This is in agreement with the hypothesis that accumulation of multiple genetic changes, perhaps as a result of genetic instability, is associated with prostate cancer progression (15–17).

In our previous studies, up to 30% of patients failing hormonal treatment were found to have AR amplification (8, 9). In these studies, only tissues from the locally recurrent tumors were available. The present tumor tissue microarray analyses of end-stage metastatic patients indicated that AR amplification is equally common in the distant metastatic deposits. Studies of the molecular genetic changes in the metastatic specimens are important, because the distant metastatic sites are primarily responsible for the clinical outcome, and represent the primary targets of systemic therapies (18). In one-half of the patients with end-stage hormone-refractory disease associated with AR amplification, amplified cells were present in all sites sampled, both in the locally recurrent tumors, as well as in distant metastases. In the remaining patients, AR amplification was only present in either the local site or in the metastases. Because most of the patients have metastatic disease already before androgen deprivation therapy is initiated, it is likely that the different sites of cancer in the same patient may sometimes respond to hormonal treatment in a unique manner. Our results suggest that tumor progression to hormone refractory cancer develops via different molecular mechanisms. This heterogeneity may also explain why any therapy against metastatic, hormone-refractory prostate cancer often tends to be ineffective.

MYC amplifications were most often found in the distant metastatic deposits sampled at autopsy. This 11% prevalence is somewhat lower than the previously reported 21% frequency of MYC involvement in prostate cancer metastases to pelvic lymph nodes (7). It is possible that the sampling from only one distinct region of each tumor may have led to an underestimation of the true prevalence of gene amplification in our study. However, in the study by Jenkins *et al.* (7), also a less stringent

criterion (>2X) for amplification was used than in our study (≥3X). Because low-level increases of copy numbers of the long arm of chromosome 8 are so common (15, 16, 19), it is possible that the more stringent cutoff is more appropriate to identify cases, where specific amplifications of the *MYC* oncogene region take place. One should note, however, that the finding of MYC amplification by FISH does not prove that MYC is the target gene of the amplification at 8q24.

Our study provides evidence for *CCND1* gene amplification in human prostate cancer *in vivo*. The amplification frequency was low (~1%) in primary prostate cancer, which may explain why it has not been reported previously. In contrast, 4.7–7.9% of the hormone-refractory and metastatic samples had CCND1 amplification. Further studies are required to evaluate the significance of this amplification for prostate cancer progression. Interestingly, CCND1 amplification often appeared to take place independently of AR and MYC amplifications.

One group has previously suggested that *ERBB2* amplification is a frequent genetic alteration and has prognostic importance in prostate cancer (11, 12). However, other investigators have failed to detect ERBB2 amplifications in prostate cancer (4, 10). Similarly, we did not detect any ERBB2 amplifications at any stage of cancer progression, including end-stage autopsy tumors. It is likely that the high prevalence of ERBB2 amplifications reported in the study of Ross *et al.* (11, 12) was due to a less stringent definition of gene amplification and the lack of a chromosome-specific reference probe to exclude the influence of aneuploidy. On the basis of our comparative analysis of AR, MYC, CCND1, and NMYC amplifications in the identical tumor samples, using the same FISH methodology and interpretation criteria, we do not expect either ERBB2 or NMYC amplifications to play any significant roles in the *in vivo* progression of human prostate cancer.

Many symptomatic prostate cancers become both hormone-refractory and metastatic, and it is very difficult to distinguish between these two clinical features or the molecular mechanisms that contribute specifically to either one of these processes. Taking our present results together with previous information (7–9), one can formulate a hypothesis that AR amplification is more closely associated with the development of hormone-refractory cell growth, whereas MYC amplification is associated with metastatic progression. Our results suggest that the most common gene amplification in prostate cancers is that of the *AR* gene, which is usually amplified independently of both MYC and CCND1. AR has been shown to be amplified in locally recurrent tumors from patients who do not have evidence of distant metastases (8), whereas MYC amplifications have been associated with metastatic progression (7). Indeed, in our present study, MYC amplifications were more common in the distant metastases (11%) than in the locally recurrent tissues (4%; both two patients with end-stage metastatic cancers), whereas AR amplifications were equally common at both anatomical sites (22 and 23%, respectively). This suggests that AR is conferring an advantage for hormone-refractory growth and not metastatic dissemination, whereas the reverse may be true for MYC. MYC-amplified tumors may also often contain AR amplification. One could speculate that the selection force responsible for the development of AR amplification makes it necessary for the cells to overcome the checkpoints that prohibit gene amplification in normal cells. This would lead to amplification of other genes such as MYC.

In conclusion, these results illustrate that the tissue microarray technology is a powerful tool for the molecular profiling of large numbers of tumors representing the entire disease spectrum of human prostate cancer progression *in vivo*. This high-throughput, tissue microarray-based screening by FISH identified distinct patterns and interrelationships between the different gene amplifications, leading to hypotheses that can now be tested in future studies of large specimens, or by more extensive sampling from each tumor site: (*a*) the present results suggest that *AR* gene is the most frequent target, and often the first target, selected for amplification during prostate cancer progression; (*b*) in contrast to AR, amplifications of the *MYC* oncogene may be primarily associated with metastatic dissemination; and (*c*) prostate cancers occasionally also amplify the *CCND1* gene, whereas ERBB2 and NMYC amplifications are unlikely to play a significant role at any stage of the progression of prostate cancer. Additional studies with the "tissue chip" approach may be helpful to generate a more comprehensive model of the genetic and molecular steps associated with prostate cancer progression, as well as to help the translation of such biological findings to clinical applications.

Acknowledgments

We thank Hedvika Novotna, Martina Mirlacher, Martina Storz, and Heidi Oggier, University of Basel, for excellent technical support; Daryl Leja for illustration support; and Steve Leighton (Beecher Instruments, Silver Spring, MD) for help in tissue array instrumentation.

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Cancer Res 1999;59:803-806.