Gene Amplifications Associated with the Development of Hormone-Resistant Prostate Cancer

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ABSTRACT

Purpose: **Hormone resistance remains a significant clinical problem in prostate cancer with few therapeutic options. Research into mechanisms of hormone resistance is essential.**

Experimental Design: **We analyzed 38 paired (prehormone/posthormone resistance) prostate cancer samples using the Vysis GenoSensor. Archival microdissected tumor DNA was extracted, amplified, labeled, and hybridized to Amplionc I DNA microarrays containing 57 oncogenes.**

Results: **Genetic instability increased during progression from hormone-sensitive to hormone-resistant cancer ($P = 0.008$). Amplification frequencies of 15 genes (*TERC*, *MYBL3*, *HRAS*, *PI3KCA*, *JUNB*, *LAMC2*, *RAF1*, *MYC*, *GARP*, *SAS*, *FGFR1*, *PGY1*, *MYCL1*, *MYB*, *FGR*) increased by >10% during hormone escape. Receptor tyrosine kinases were amplified in 73% of cases; this was unrelated to development of hormone resistance. However, downstream receptor tyrosine kinase signaling pathways showed increased amplification rates in resistant tumors for the mitogen-activated protein kinase (*FGR/Src-2*, *HRAS*, and *RAF1*; $P = 0.005$) and phosphatidylinositol 3′-kinase pathways (*FGR/Src-2*, *PI3K*, and *Akt*; $P = 0.046$). Transcription factors regulated by these pathways were also more frequently amplified after escape (*MYC* family: 21% before *versus* 63% after, $P = 0.027$; *MYB* family: 26% before *versus* 53% after, $P = 0.18$).**

Conclusions: **Development of clinical hormone escape is linked to phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. These pathways may function independently of the androgen receptor or via androgen receptor activation by phosphorylation, providing novel therapeutic targets.**

INTRODUCTION

Treatment of advanced prostate cancer has relied on androgen deprivation therapy for the past 50 years. Response rates are initially high (70–80%), however, almost all patients experience treatment failure (hormone escape), with a median time to relapse of 18 months (1). Their cancer then progresses, frequently with metastasis and increased risk of morbidity and death (2). Unlike breast cancer, alternative approaches (chemotherapy and radiotherapy) do not provide significant disease control after the development of hormone escaped. The high rate of prostate cancer mortality is, therefore, linked strongly to both development of hormone escape and the lack of alternate therapies.

The lack of progress in developing novel therapies to treat hormone escape is linked closely to a lack of understanding of the molecular mechanisms underlying the development of this disease. Understanding why this occurs will also enable us to use therapies that are currently available for treatment of other solid tumor types.

To date, the majority of research on hormone escape has focused on the AR[2]; however, mutations and amplifications of the *AR* are not found frequently enough to alone explain the development of hormone escape (3–7). Furthermore, we have recently demonstrated a significant amplification of the *AR* before antiandrogen therapy in a patient who had a full clinical response to antiandrogens, including a reduction in PSA (8). Hormone escape may, therefore, develop because of alternative methods of regulating AR protein activation rather than modifications to the receptor itself.

Phosphorylation of the AR seems to play a role in both ligand-dependent and ligand-independent AR activation (9–13). Therefore, although it seems that activation of the AR is central to the development of hormone escape, it may be because of activation of the AR in an androgen-independent manner by alternative pathways.

Cell line studies have demonstrated that various pathways [PI3K/Akt (14, 15), Ras/MAPK (16, 17), protein kinase A (18), and protein kinase C (19)] may have a role in the development of hormone escape; however, the significance of these pathways in the clinical setting has not been investigated. This study has used two matched tumors from each patient with prostate cancer, one tumor before and one tumor after the development of hormone escape. Analysis of tumor DNA using CGH arrays will allow us to identify gene abnormalities in tumors after the

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[2] The abbreviations used are: AR, androgen receptor; PSA, prostate-specific antigen; PI3K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; CGH, comparative genomic hybridization; DOP, degenerate oligonucleotide primed; FISH, fluorescence *in situ* hybridization; RTK, Receptor tyrosine kinase; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; TURP, transurethral resection of the prostate.

Table 1 Patient information

			Information at diagnosis			Information at relapse			
Patient no.	Age (yr)	Primary surgery	Gleason score	PSA	Relapse surgery	Gleason score	PSA	Time to relapse (days)	Treatment
1	71	TURP	5	39	TURP	6	8	1553	Finesteride
2	72	TURP	6	64	TURP	9	39	1097	Orch, zoladex
3	74	TURP	4	1	TURP	10	29	893	Orch, zoladex
4	54	TURP	7	10	TURP	8	59	2087	Orch, flutamide
5	71	TURP	9	152	TURP	9	116	693	Orch, zoladex, casodex
6	66	TURP	10	306	TURP	10	44	850	Orch, flutamide
7	62	Tru-cut biopsy	9	207	TURP	8	31	1046	Zoladex, casodex
8	83	Tru-cut biopsy	10	42	TURP	8	7	765	Zoladex, flutamide
9	67	Tru-cut biopsy	8	55	TURP	9	7	3031	Zoladex
10	71	Tru-cut biopsy	8	112	TURP	8	16	714	Zoladex, casodex
11	76	Tru-cut biopsy	8	1300	TURP	8	796	686	Zoladex
12	68	TURP	9	10	TURP	9	3	966	Zoladex
13	76	TURP	7	136	TURP	9	140	1410	Zoladex
14	69	Tru-cut biopsy	6	7	TURP	10	15	616	Zoladex, casodex
15	64	TURP	8	384	TURP	7	36	356	Zoladex
16	70	TURP	6	6	TURP	9	17	60	Zoladex
17	73	TURP	9	208	TURP	7	52	1224	Orch
18	63	TURP	7	24	TURP	9	33	1593	Orch
19	81	TURP	7	2	TURP	8	3	1821	Orch

development of hormone escape that are not present in the matched tumor that responded to androgen deprivation therapy. Although this CGH array method does not generally differentiate between amplification and gain in copy number, the results obtained for individual genes within a chromosomal region can aid differentiation between amplification and gain in copy number.

MATERIALS AND METHODS

Patients. Nineteen patients (38 matched tumor pairs) were retrospectively selected for analysis; all tumors had patient identification removed, including block number and hospital number, and were coded to make the database anonymous. Ethical approval was obtained from the Multicenter Research Ethics Committee for Scotland and the appropriate local research and ethical committees for use of matched hormone-sensitive and hormone-resistant tumors in our study. All patients received conventional androgen deprivation therapy (orchidectomy, antiandrogens, or androgen ablation therapy). Patients were selected for analysis if they initially responded to treatment (response was defined by PSA levels falling by at least 50%) but subsequently relapsed. PSA values and full clinical follow-up was available for each patient (Table 1). Patients were classed as having hormone-escaped cancer when sustained rising PSA levels were noted and were selected for study if a posthormone relapse sample was available. The initial tumor sample was either a TURP or a transrectal ultrasound-guided biopsy, however, the relapsed tumor sample was always a TURP, which was performed to treat clinical symptoms, most commonly bladder outflow obstruction.

DNA. Archival (formalin fixed, paraffin embedded) prostate tumors and normal colorectal tissue (normal control) were microdissected from 5-μm sections, as described by Going and Lamb (20). DOP-PCR was performed according to the method of Speicher *et al.* (21) to amplify all DNA [DNA extracted from prostate and colorectal tissue, DNA (positive control; Vysis Inc., Downers Grove, IL), and normal reference male DNA]. The concentration of PCR products was determined by spectrophotometry.

Nick Translation. DOP-PCR product (1.5 μg) from either reference (male DNA) or test (male DNA extracted from colon tissue, COSH DNA, or tumor DNA) samples were nick translated using the Vysis Nick translation kit in a final volume of 50 μl. Reference DNA were labeled with Alexa 594 dUTP (red), and test DNA with Alexa 488 dUTP (green). Samples were incubated at 37°C for 30 min, followed by enzyme denaturation at 80°C for 10 min. Samples were chilled, and 2 μl of precipitation reagent, 5 μl of 3 M sodium acetate, and 55 μl of isopropanol were added to precipitate DNA. Samples were placed at −20°C for at least 1 h, and DNA was pelted at 15,000 × *g* for 15 min at room temperature. The supernatant was removed, and pellets were washed in 300 μl of 70% ethanol, spun, and resuspended in 7.5 μl of nuclease-free water (200 ng/μl). After labeling, 0.5 μg of each product was run on a 2% agarose gel to confirm the success of the reaction.

Hybridization. Test DNA (2.5 μl, 0.5 μg) and 2.5 μl of reference DNA were combined with 22 μl of hybridization buffer (total volume, 27 μl). Samples were heated to 80°C for 10 min and allowed to cool to 37°C. Samples were then prehybridized for 2–6 h before placing on Genosensor Amplionc I microarrays. Microarrays were then incubated for 18 h at 37°C. Table 2 shows a list of the genes and location of genes on the array.

Posthybridization Washes. Arrays were washed in wash solution (50% formamide and 2× SSC) three times at 40°C for 10 min. This was followed by four washes for 5 min in 1× SSC at room temperature. After air drying, 4′,6-diamidino-2-phenylindole IV was applied to counterstain the DNA. After 15 min, arrays were imaged using the Genosensor and the Genosensor Reader Software.

Table 2 Gene array targets

Gene	Location	Average	SD	Gene	Location	Average	SD
FGR	1p36.2-p36.1	0.95	0.03	*WNT1*	12q12-q13	0.75	0.14
MYCL1	1p34.3	1.15	0.29	*GLI*	12q13.2-q13.3	0.80	0.10
NRAS	1p13.2	1.00	0.23	*SAS/CDK4*	12q13.3	0.96	0.17
LAMC2	1q25-q31	0.89	0.12	*MDM2*	12q14.3-q15	0.81	0.22
MYCN	2p24.1	0.80	0.10	*Akt1*	14q32.3	0.75	0.23
REL	2p13-p12	0.76	0.13	*IGFR1*	15q25-q26	0.87	0.26
RAF1	3p25	1.14	0.06	*FES*	15q26.1	0.69	0.12
TERC	3q26.3	1.12	0.03	*MRP1*	16p13.1	1.02	0.08
P13K	3q26.3	1.14	0.05	*TOP2A*	17q21-q22	0.86	0.17
PDGFRA	4q12	0.87	0.23	*HER2*	17q21.2	0.95	0.21
MYB	6q22	1.06	0.17	*RPS6KB1*	17q23	0.96	0.09
ESR	6q25.1	1.18	0.19	*D17S1670*	17q23	0.98	0.16
EGFR	7p12.3-p12.1	0.89	0.11	*YES1*	18p11.3	1.02	0.09
PGY1	7q21.1	0.99	0.15	*BCL2 3′*	18q21.3	1.06	0.12
MET	7q31	0.88	0.06	*BCL2 5′*	18q21.3	1.03	0.14
CTSB	8p22	0.99	0.31	*INSR*	19p13.2	1.10	0.06
FGFR1	8p11.2-p11.1	0.94	0.21	*JUNB*	19p13.2	0.56	0.07
MOS	8q11	0.97	0.09	*CCNE1*	19q13.1	0.92	0.16
MYC	8q24.12-q24.13	0.89	0.17	*AIB1*	20q12	1.05	0.21
ABL1	9q34.1	0.88	0.20	*STK15*	20q13	0.98	0.08
FGFR2	10q26	1.18	0.07	*CSE1L*	20q13	1.00	0.15
HRAS	11p15.5	1.03	0.20	*MYBL2*	20q13.1	0.87	0.16
CCND1	11q13	0.98	0.13	*PTPN1*	20q13.1-q13.2	0.82	0.09
FGF4/FGF3	11q13	0.80	0.20	*ZNF217*	20q13.2	0.93	0.19
EMS1	11q13	1.02	0.24	*CBFA2*	21q22.3	1.01	0.20
GARP	11q13.5-q14	1.08	0.21	*BCR*	22q11.21	0.93	0.15
PAK1	11q13.5-q14	1.01	0.10	*PDGFB*	22q12.3-q13.1	0.74	0.17
MLL	11q23	0.91	0.16	*AR 5′*	Xq11-q12	0.86	0.15
CCND2	12p13	0.90	0.25	*AR 3′*	Xq11-q12	1.22	0.21
KRAS2	12p12.1	0.90	0.15				

Normal DNA Controls. Reference DNA arrays *versus* COSH DNA (DNA with known amplifications and losses) arrays were initially performed to confirm that DOP-PCR was not biasing the results, therefore validating the technique. Thereafter, one reference DNA array *versus* COSH DNA array was performed per run as a positive control. Reference DNA arrays *versus* normal male DNA (extracted from colorectal archival tissue) arrays were performed on 10 occasions to establish the normal range when using archival DNA amplified by DOP-PCR. The mean hybridization ratio ± 2 SD (red:green fluorescence ratio) was calculated using the Genosensor software.

Statistics. The total number of amplifications before and after hormone escape were analyzed using Wilcoxon's signed ranks test. The number of amplifications per gene or signaling pathway were analyzed using McNemar's test.

RESULTS

The mean test:normal ratio for the CGH array was calculated for 10 arrays (1770 spots) using DOP-PCR-amplified DNA extracted from archival colorectal tissue. The mean hybridization ratio and SD for each individual gene across the 10 arrays is shown in Table 2. The mean hybridization ratio for all genes across the 10 arrays was 0.944 ± 0.217 (mean ± SD). The range of normal values for hybridization, as defined by these control experiments, was 0.727–1.161. We defined the 95% confidence interval of the mean hybridization signal (0.944 ± 2 SD) as the normal range for hybridization ratios. Thus, the normal range for hybridization ratios was 0.510–1.378; values <0.510 identify loss ("deletion"), and values >1.378 identify gain ("amplification").

Amplifications Associated with Prostate Cancer. In total, 287 of 2142 (13.4%) spots from the 38 tumor samples showed ratios above 1.378 and were, therefore, scored as amplified. Fewer (18 of 2142, 0.8%) spots showed clear loss (ratios <0.5). Amplifications ranged from 2 to 14 genes/tumor with a median of 7 (interquartile range, 6–10). Deletions ranged from zero to four genes/tumor with a median of 0 (interquartile range, 0–1). Overall, tumors showed alterations (amplifications and deletions) in 2–17 genes with a median of 7 (interquartile range, 6–11). No alterations were observed in 13 genes; an additional 29 genes were altered in $<15\%$ of cases, and 16 genes were altered in $>15\%$ of cases. The 16 most commonly altered genes are shown in Fig. 1, this graph shows the copy number for each gene in each individual tumor and the average copy number ± SD for the colon controls (Fig. 1). The *AR* copy number was most frequently out of the normal range; because *AR* is the only gene on the array that is on the X chromosome, AR results in this study measure *AR* copy number, not amplification. The *AR* copy number was confirmed by FISH; 65% of tumors had an abnormal copy number by the array method compared with 61% by FISH, and there was a 92% correlation between the methods (Table 3). Eighteen percent of the tumors were found to be amplified for the *AR* using FISH (Table 3).

When oncogenes associated with signal transduction pathways were investigated, it was noted that 22% of tumors were amplified for members of the type I RTK family (*EGFR*, 11%;

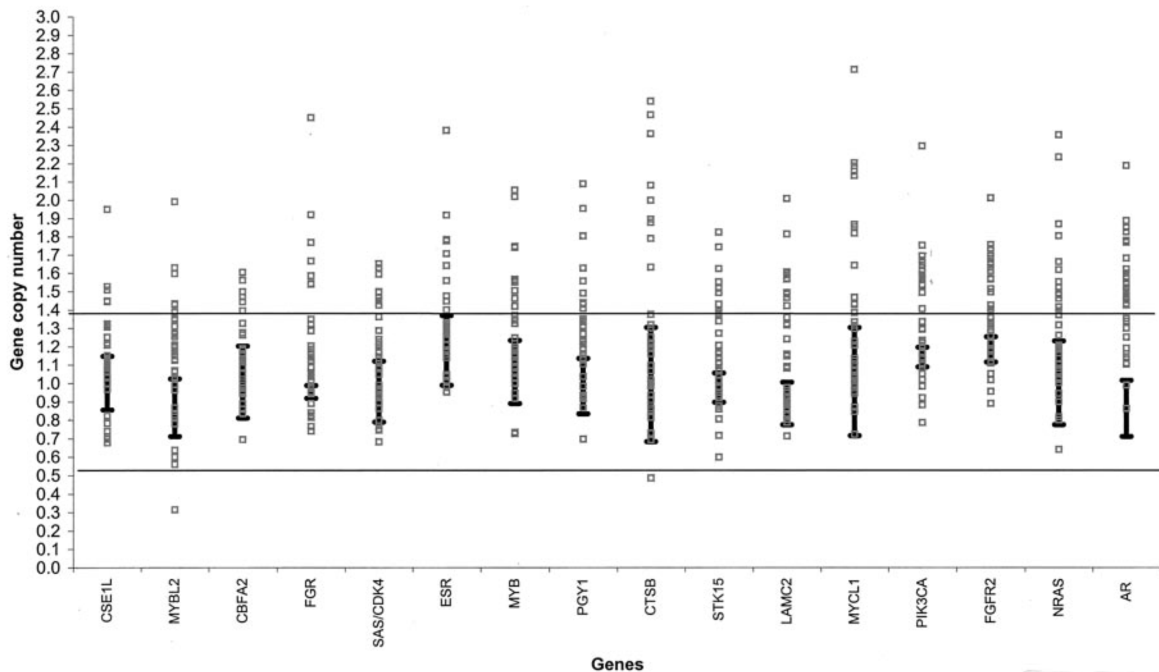

Fig. 1 The copy number of each gene for each tumor is indicated by a square, and the average copy number for each gene calculated from the normal colon controls is indicated by a *circle*. The *error bars* on the *circles* denote the SD, and the *horizontal lines* on the graph at 0.378 and −0.51 are the cutoffs used in this study to determine the normal range.

HER2, 11%). Nineteen percent of cases were amplified for members of the type II RTK family of receptors (*INSR*, 6%; *IGF1* receptor, 13%), 11% were amplified for type III RTKs/ligands (*PDGFRA*, 8%; *PDGFB*/*c-sis*, 3%), and 47% for type IV RTKs (*FGFR1*, 9%; *FGFR2*, 47%; Fig. 2). In total, 73% of prostate cancer cases showed amplification of classical growth factor cell surface RTKs or their ligands. Only 18% of cases were amplified for more than one type of RTK. There was no evidence for nonrandom association of amplification.

In addition to amplification of cell surface receptors and their ligands, frequent amplification of members of the MAPK cascade were observed (*FGR/Src-2*, 18%; *NRAS*, 48%; *HRAS*, 11%; *KRAS*, 14%; *RAF1*, 11%). When all cases were taken into consideration, 74% (28 of 38) of tumors showed amplification for key elements of the MAPK cascade. It was also observed that 44% (16 of 36) of cases were amplified for *PI3K*. When the above data were combined, 87% (33 of 38) of prostate tumors showed amplification of *RTK* genes or genes downstream of these (*i.e.*, *FGR/RAS/RAF1*/*PI3K*; Fig. 3). Only five cases (13.2%) showed no detectable amplification of these pathways.

When transcription factors regulated by the RTK signal transduction pathways were investigated, 40% (15 of 38) of tumors were amplified for members of the MYB family (*MYB*, 29%; *MYBL2*, 16%) and 47% of tumors were amplified for the MYC family (*MYCL1*, 35%; *MYCN*, 14%; *MYC*, 8%; Fig. 2).

Amplifications Associated with Hormone Escape. Significantly fewer genes were amplified in hormone-sensitive tumors when compared with hormone-escaped tumors (11.7% *versus* 15.2%; $P = 0.008$). Seventeen genes were amplified in ≥15% of hormone-sensitive tumors, and 24 genes were amplified in ≥15% of hormone-escaped tumors (Table 4). *AR* (63% prehormone and 67% posthormone; only gene on X chromosome), *FGFR2* (50% prehormone and 44% posthormone; only gene on chromosome 10), *PI3K* (39% prehormone and 50% posthormone), and *NRAS* (47% prehormone and 47% posthormone) all demonstrated copy number gains in >35% of both hormone-sensitive and hormone-escaped tumors (Table 4).

Forty-six percent (27 of 59) of genes investigated were amplified and increased copies relative to adjacent genes on the same chromosome more frequently in hormone-escaped tumors compared with hormone-sensitive tumors. An increase of >10% in the frequency of amplification or gain during transition from hormone-sensitive to hormone-escaped prostate cancer was observed for 15 genes (Fig. 4). *FGR* (*Src-2*; control *MYCL1*) was amplified in significantly more hormone-escaped tumors (37%, 7 of 19) compared with hormone-sensitive tumors (0%, 0 of 19; $P = 0.016$).

When RTK oncogenes were compared in hormone-sensitive and hormone-escaped prostate cancer, there was no significant difference in the amplification rate when compared as families (type I RTK: 5 of 19 *versus* 3 of 19, $P = 0.69$; type II RTK: 3 of 19 *versus* 4 of 19, $P = 0.41$; type III RTK: 2 of 19 *versus* 2 of 19, $P = 1$; type IV RTK: 9 of 19 *versus* 9 of 19, $P = 0.1$) or when combined (15 of 19 *versus* 13 of 19, $P = 0.71$). An increase was, however, observed for *FGFR1* (control MOS; 0 of 18 *versus* 3 of 17, $P = 0.25$).

A significant increase was seen in the frequency of amplifications for members of the MAPK cascade [*FGR* (*Src-2*), *HRAS*, and *RAF1*] with the development of hormone escape [10% (2 of 19) *versus* 58% (11 of 19), $P = 0.005$; Table 5]. In

Table 3 Correlation between *FISH* gene array results

Case no.	Tumor no.	Abnormal copy number by array	Abnormal copy number by FISH	Amplified by FISH
1	1	√	√	
	2	No result	√	
2	3	√	√	
	4	√	√	
3	5	√	√	√
	6	√	√	√
4	7			
	8	√	√	
5	9			
	10	No result		
6	11			
	12	√	√	
7	13			
	14			
8	15	√		
	16			
9	17	√	√	
	18	No result		
10	19			
	20			
11	21	√		
	22	√	√	
12	23			
	24			
13	25	√	√	
	26	√	√	
14	27	√	√	
	28	No result	√	√
15	29			
	30		√	√
16	31	√	√	
	32	√	√	
17	33	√	√	
	34	√	√	
18	35	√	√	
	36	√	√	
19	37	√	√	
	38	√	√	

47% (9 of 19) of cases, either *FGR* (Src-2) or *HRAS* was amplified during hormone escape, and in only 1 case (5%) were they both amplified; an additional 2 cases (11%) showed amplification of *RAF1* at hormone escape (Table 5).

It was observed that 39% of hormone-sensitive tumors and 50% of hormone-escaped tumors were amplified for *PI3K* (Table 5). No tumors were observed to be amplified for *Akt1*. Coamplification of *PI3K* and *FGR (Src-2)* was observed in 0% (0 of 19) of hormone-sensitive tumors and 26% (5 of 19) of hormone-escaped tumors ($P = 0.046$; Table 4).

Transcription factors (*MYC* and *MYB*) regulated by the MAPK or PI3K cascade were amplified more frequently in hormone-escaped tumors (*MYC* and *MYCL1*: 21% prehormone *versus* 63% posthormone, $P = 0.02$; *MYB* and *MYBL2*: 26% prehormone *versus* 53% posthormone, $P = 0.18$; Table 5). Amplifications for *FGR* (*Src-2*)/*HRAS*/*RAF1*/*MYC*/*MYCL1* were observed in 32% (6 of 19) of hormone-sensitive cases and 74% (14 of 19) of hormone-escaped cases ($P = 0.02$). Amplifications for *FGR* (*Src*-2) and *PI3K*/*MYBL2*/*MYB* were observed in 26% (5 of 19) of hormone-sensitive cases and 53% (10 of 19) of hormone-escaped cases ($P = 0.18$). Amplification rates increase for either the MAPK or PI3K pathways in 79% (15 of 19) of cases with the development of hormone escape, and 58% (11 of 19) of these were gains in a novel pathway.

DISCUSSION

The development of hormone resistance in prostate cancer is among the most significant causes of morbidity and mortality from this disease. The object of this study was to identify molecular aberrations that occur at the development of hormone-escaped prostate cancer using matched tumors before and after hormone escape. Using a targeted CGH microarray approach, we were able to identify individual genes that are commonly amplified in prostate cancer and those likely to be involved in the development of hormone escape. A series of gene amplifications associated with the development of hormone escape were identified linking cell surface receptors, through signal transduction pathways, to nuclear transcription factors.

The most frequent regions of amplification detected were within the 1p amplicon, frequently associated with prostate cancer in conventional CGH studies (22, 23), with amplification of *FGR* (1p36; Refs. 22 and 23), *MYCL1* (1p34.3; Ref. 22), and *NRAS* (1p13.2) occurring in over 60% of cases. In addition, frequent amplification of *LAMC2* (1q25; Refs. 24 and 25) was observed. Within the 1p amplicon, however, there appeared to be little evidence of linkage between gene alterations, but this region is commonly reported as gained by CGH (22, 23). Other hot spots for amplification were similar to those reported in the literature as detected by CGH in prostate cancer, including *PI3K* (3q26.3; Refs. 23, 25, and 26), *PGY1* (7q21.1; Refs. 23 and 27), *STK15* (20q13; Ref. 23), and *AR* (Xq12; Refs. 22–24 and 28). In the case of *PI3K*, *PGY1*, and *STK15*, genes found in similar loci (*TERC*, 3q26.3), *MET* (7q21), and *CSE1L* (20q13), respectively, were infrequently coamplified, suggesting these genes are specific targets within these regions. In each case, however, expression analysis will be required for final confirmation that these are indeed the target genes within these frequently amplified regions. The presence of multiple, but apparently independent, alterations within the 1p amplicon reflects similar data we, and others, have observed in genomic mappings studies. *AR* was the most frequently modified gene (Table 3), with the copy number being increased in 65% of cases, which is within the range reported in the literature for *AR* copy number (35–80%; Refs. 5, 7, and 8). *AR* copy number rates correlate (92%) with the results we found using FISH for *AR* and X chromosome copy number in the same patient cohort. This figure is higher than the *AR* amplification rate reported in the literature (20–30%; Refs. 29 and 30), because it measures *AR* copy number, not amplification; however, using FISH, the level of amplification in this cohort was 18%, which is similar to that reported in the literature (5–8). We have noted that, although there exists a strong correlation between gene copy number detected by FISH and that detected on microarrays, a doubling of gene copy detected by FISH does not equate to a doubling of the hybridization ratio seen on microarrays. This can be demonstrated clearly by the observation that ratios above 1.378 on the microarray are indicative of abnormal gene copy numbers whereas

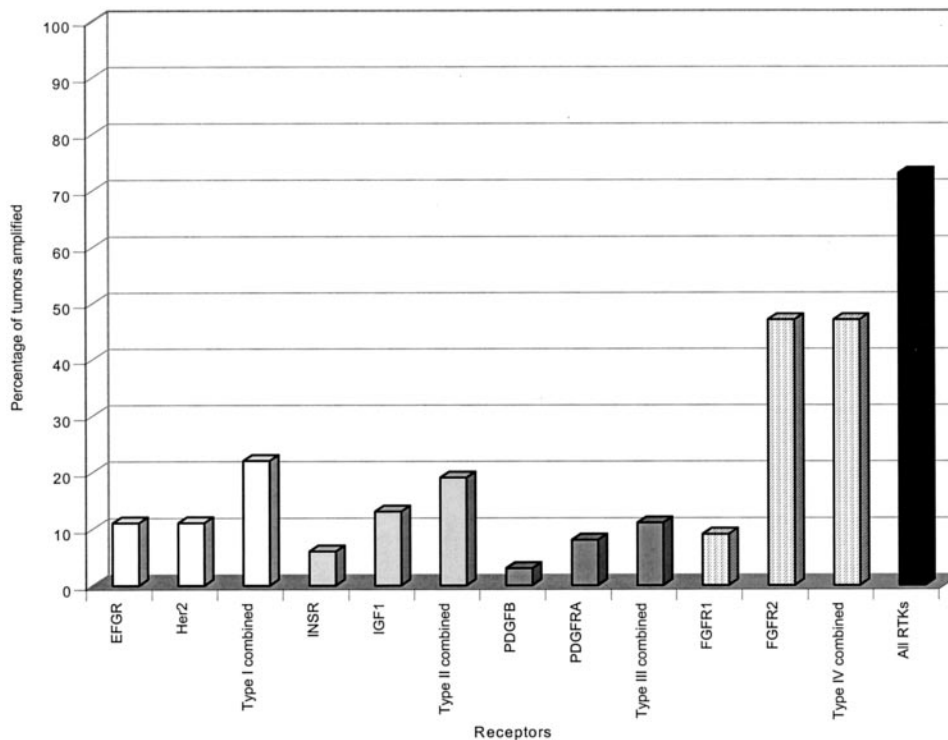

Fig. 2 The total percentage of tumors amplified for cell surface RTK are shown in Fig. 1. Each family [type I (*EGFR* and *HER2*), type II (*INSR* and *IGF1*), type III (*PDGFB* and *PDGFRA*), and type IV (*FGFR1* and *FGFR2*)] is shown for each individual family member and the family in total.

a significantly higher figure (>1.5–2.0) is required to identify abnormal gene copy numbers by FISH (29, 30).

The molecular mechanisms underlying the development of hormone resistance in prostate cancer is still poorly understood. Within this study, we have the unique opportunity to compare molecular alterations within tumors collected before and after hormone relapse, where each patient's pretreatment tumor acts as a linked control. We have used this approach previously to confirm that AR amplification cannot always explain hormone escape and in some cases may be present in hormone-sensitive tumors (8). Data from the current study further emphasizes this by confirming our finding that *AR* gene copy does not increase with hormone escape. We, therefore, looked for other genetic patterns associated with hormone resistance. Our results identify growth factor pathways, which may play a role in the development of hormone resistance.

We show that type I–IV RTKs are amplified in ~73% of prostate cancers (Fig. 2). Frequency of amplifications were low for type I–III RTKs (type I, 22%; type II, 19%; type III, 11%), however, 47% of cases were amplified for type IV RTKs (*FGFR1* and *FGFR2*). There was no significant difference between amplification frequencies in the transition from hormone-sensitive to hormone-insensitive prostate cancer, but a trend was observed with the *FGFR1* amplification rate increasing from 0% to 17.65%. Many studies have investigated the role of type I RTKs in hormone-escaped prostate cancer. Here, we report that 11% of tumors are amplified for *EGFR*. This value is marginally higher than that reported in the literature (3–9%; Ref. 29), however, it is still within the general range. This figure could be higher because higher-grade tumors are used or because of duplication of the short arm of chromosome 7, because the other genes present on the chip are found on the long arm of chromosome 7. Duplication of the short arm would not be noted by amplification of other genes on the chip, therefore, there is no internal control of this gene. Although *EGFR* amplification might result in an increase in *EGFR* expression, which has been reported previously to predict prostate cancer progression, it has been reported that the mutated form of *EGFR*, which is constitutively active (EGFRvIII), is frequently overexpressed in prostate cancer (30, 31). This study also reported that 11% of tumors were amplified for *HER2*, also within the range (0–44%) reported in the literature [*e.g.*, Mark *et al.* (32) reported 9.3%, and Ross *et al.* (33) reported 41%]. Overexpression of p185$^{\mathrm{HER2}}$ is associated with poor prognosis (31, 32, 34), and we have demonstrated by FISH that *HER2* amplification rates increase with grade in prostate tumors.[3]

The FGFs and their receptors are involved in the development of the fetal prostate gland and have been reported to be involved in prostate cancer (35). FGFR2 is expressed in normal prostate epithelium and, with its ligand KGF/FGF-7 (androgen-inducible paracrine factor), is involved in regulating the development and growth of the normal prostate (36). Although *FGFR2* mutations or polymorphisms are not commonly found in prostate cancer, increased mRNA expression has been detected (36) that could be a consequence of *FGFR2* gains seen in this study (no control gene is on the array to allow differentiation between amplification or gain of *FGFR2*; Fig. 1). We noted that during the transition to hormone resistance the rate of

[3] J. Edwards, personal communication.

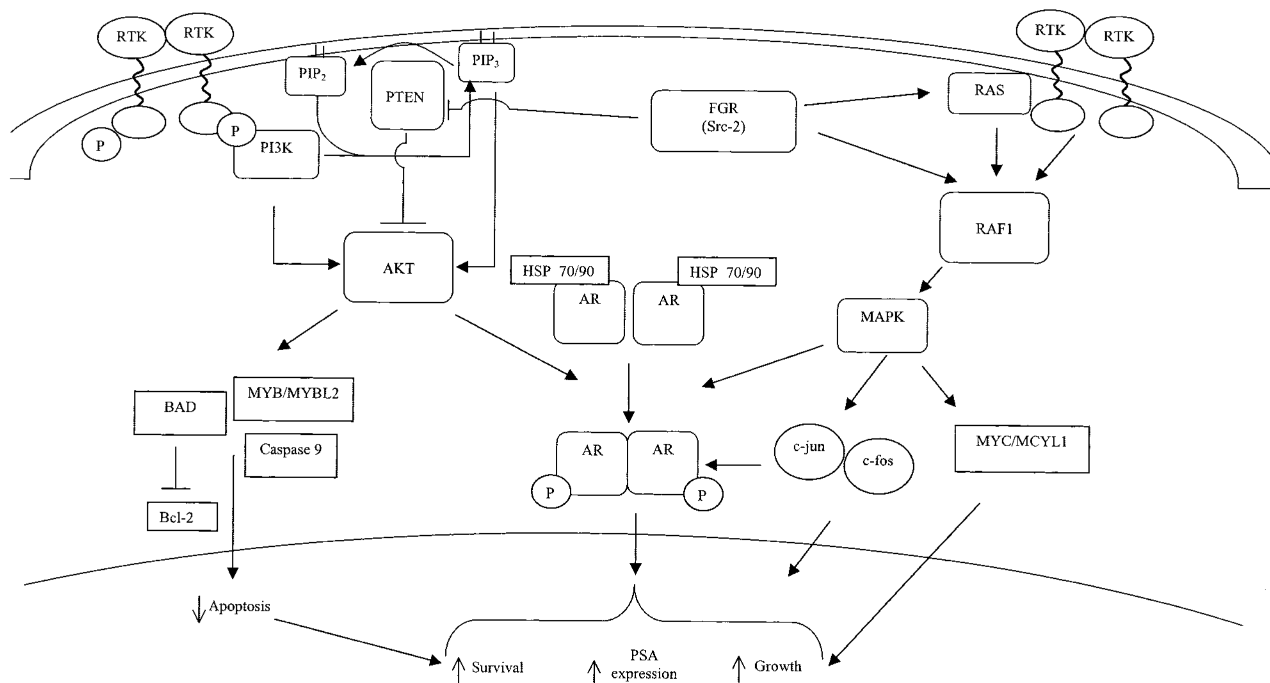

Fig. 3 Interactions between the Src-2/RAS/RAF/MAPK and Src-2/PI3K/Akt pathways and related transcription factors in the hormone-resistant prostate cancer cell. Activation of both these pathways may result in phosphorylation and, hence, activation of the AR in the absence of androgens.

FGFR1 amplification of increases (Table 4), suggesting that this receptor may be important in the development of hormone escape. In the Dunning rat model, FGFR1 expression can induce hormone escape concurrent with loss of FGFR2 (37). Although this study observes amplification of *FGFR1* with the development of hormone escape, this was not significant; the role of this receptor, therefore, seems to be minor in this cohort. Because there is no significant change in the cell surface RTK amplification rate with the development of hormone escape, we have to conclude from these data that RTK amplification is not involved. We are, however, currently studying protein expression and activation status of RTKs to determine whether, in prostate cancer, protein expression is not amplification driven.

RTKs are responsible for the regulation of downstream signaling pathways, including the RAS/RAF/MAPK and PI3K/Akt pathways. These pathways control cell proliferation, differentiation, and apoptosis (Refs. 15 and 38; Fig. 3) and have been implicated previously in the development of hormone escape in prostate cancer cell lines (14, 38).

Alterations to *HRAS*, *NRAS*, and *KRAS*, the three human *RAS* genes (39, 40), are associated with many advanced human cancers (*e.g.*, lung, thyroid, breast, bladdern and ovarian tumors; Ref. 41). They are amplified frequently in this study, and for *HRAS* an increase in amplification rates is associated with hormone resistance (Table 4). These oncogenes function via the activation of MAPK, although many of the upstream components of the MAPK cascade are activated via a series of phosphorylation events, activated MAPK relays signals to the nucleus via other kinases, and nuclear transcription factors including *fos*, *myc*, and *jun* (41). In addition, a significant increase in the frequency of *Src-2* (*FGR*) amplifications was seen at hormone escape (Table 4; $P = 0.016$). In only one case were *HRAS* and *Src-2* both amplified; therefore, in 47% of cases, either *Src-2* or *HRAS* was gained during the process of hormone escape. Furthermore, *HRAS* or *Src-2* were amplified infrequently (5%) before hormone escape (Table 5). *RAS* amplifications are often a consequence of *RAS* mutations that result in activated Ras protein (42) and may, therefore, be considered an indicator of activated Ras. Furthermore, mutated *Ras* has been associated previously with chemoresistance in breast cancer. Srcs are activated by binding to SH2 domains of RTKs (43) but also interact with Ras in activating the Raf/MAPK pathway (44–47), which has been associated with hormone escape in prostate cancer cell lines. MAPK has been shown to stimulate prostate cancer cell growth in the absence of androgens via phosphorylation (serine 515) and activation of the AR (48). Therefore, amplification and, presumably, activation of one or the other partner in this interaction could act to stimulate signaling via RAF1 and the MAPK cascade, activating AR-responsive genes and facilitating hormone resistance. In almost 60% of cancers, we have shown evidence for modification of the *Src-2/HRAS/RAF1* cascade during hormone escape. This study has, therefore, identified a key molecular mechanism responsible for the development of hormone escape, because the MAPK pathway is activated by a range of growth factors and growth factor receptors and results in the activation of many nuclear transcription factors.

RTKs also activate the PI3K/Akt pathway (Refs. 48 and 49; Fig. 3), predominantly to regulate apoptosis. This pathway has been reported to be involved with many human cancers

Table 4 Gene amplifications detected by array CGH

Gene	Hormone-sensitive tumors			Hormone-escaped tumors			*P*
	Amplifications	Tumors	Percentage	Amplifications	Tumors	Percentage	
MET	3	16	18.75	0	19	0.00	0.250
PTPN1	2	18	11.11	0	18	0.00	0.500
KRAS2	4	19	21.05	1	18	5.56	0.375
HER2	3	19	15.79	1	18	5.56	1.000
MYCN	3	18	16.67	2	18	11.11	1.000
D17S1670	1	19	5.26	2	18	11.11	1.000
CBFA2	3	18	16.67	3	19	15.79	1.000
IGFR1	2	19	10.53	3	19	15.79	1.000
TERC	1	18	5.56	3	19	15.79	0.500
HRAS	1	19	5.26	3	19	15.79	0.500
MYC	0	18	0.00	3	19	15.79	0.250
CSE1L	2	15	13.33	3	18	16.67	0.500
JUNB	1	18	5.56	3	18	16.67	0.500
RAF1	1	19	5.26	3	18	16.67	0.500
FGFR1	0	18	0.00	3	17	17.65	0.250
MYBL2	2	19	10.53	4	19	21.05	0.625
GARP	1	17	5.88	4	18	22.22	0.250
CTSB	5	17	29.41	6	19	31.58	1.000
ESR	4	18	22.22	6	19	31.58	0.625
STK15	5	18	27.78	6	18	33.33	1.000
SAS/CDK4	3	18	16.67	6	18	33.33	0.250
FGR(Src-2)	0	19	0.00	7	19	36.84	0.016
LAMC2	5	18	27.78	7	18	38.89	0.500
PGY1	3	15	20.00	7	18	38.89	0.375
MYB	3	19	15.79	8	19	42.11	0.063
FGFR2	9	18	50.00	8	18	44.44	1.000
NRAS	9	19	47.37	9	19	47.37	1.000
MYCL1	4	18	22.22	9	19	47.37	0.125
PI3K	7	18	38.89	9	18	50.00	0.375
AR	12	19	63.16	10	15	67.23	1.000

[*e.g.*, breast and ovarian (50)]. Here, we report that *PI3K* amplification rates increase from 39% to 50% with the development of hormone resistance; however, Akt was not amplified in any of the tumors we analyzed. The PI3K/Akt pathway has been implicated in hormone-resistant prostate cancer (14, 48, 49) because activated Akt modifies and stimulates AR in the absence of androgens via phosphorylation of serine 213 (14). Akt activation status is controlled by the PTEN phosphatase, with inactivating mutations of PTEN leading to activation of Akt (48–53). PTEN, a protein phosphatase that is one of the most widely mutated tumor suppressor genes in prostate cancer, is mutated in 29–42% of prostate cancers and in up to 60% of prostate cancer cell lines (50). Src-2 directly inhibits the action of PTEN, leading to activation of Akt via PI3K (54). Therefore, Srcs in addition to modifying the RAS/MAPK pathway, may act synergistically with *PI3K* amplifications to modify Akt (43) and thereby modify apoptotic control in prostate cancers. Activation of Akt may cause Bcl-2 and Bcl-XL proteins to function as inhibitors of apoptosis. Akt phosphorylates a proapoptotic member of the Bcl-2 family that, when dephosphorylated, displaces Bax from Bcl-2 and Bcl-XL complexes, ultimately resulting in cell death (51). Overexpression of Bcl-2 has been implicated previously in the conversion of androgen-dependent to androgen-independent disease (50), and expression of Bcl-2 in LNCaP cells protects cells from apoptosis caused by androgen withdrawal and enables cells to grow in the absence of androgens (51). Therefore, there is strong evidence that increased activation of the PI3K/Akt pathway could induce hormone resistance via an increase in Bcl-2 expression. The development of hormone resistance in this patient group was associated with gain of coamplification of *Src-2* and *PI3K* (26%; Table 5). Therefore, we hypothesize that PTEN inactivation, because of *Src-2* amplification, combined with amplification of *PI3K* results in increased Akt activity and activation of the AR and/or Bcl-2, leading to the development of hormone escape.

The Src-2/HRAS and the Src-2/PI3K pathway effects are mediated by different classes of transcription factors (MYC, *jun*, and *fos* and MYB, Bad, and caspase 9, respectively; Refs. 55 and 56). Amplification rates of *MYC* (*MYC*, *MYCL1*) and *MYB* (*MYB*, *MYBL2*) transcription increased significantly during development of hormone escape. Amplification of *MYC* has been reported previously in clinical samples to increase with the development of hormone escape (23, 26, 57) and in presence of distant metastases (7). Protein expression of c-*myc* also increases after androgen deprivation (58) and binds to a *myc* consensus site on the *AR* coding region (59). Binding of c-*myc* to the *AR* coding region results in an increase in AR mRNA expression and AR protein levels (58). Akt is the principal transducer of the MYB family of transcription factors, which are highly regulated throughout the cell cycle and are required for cell cycle progression (21, 56). Overexpression of the MYB family has been demonstrated to protect cells from programmed cell death and also to regulate cell adhesion (21, 56). Combining amplification of these transcription factors with their respective

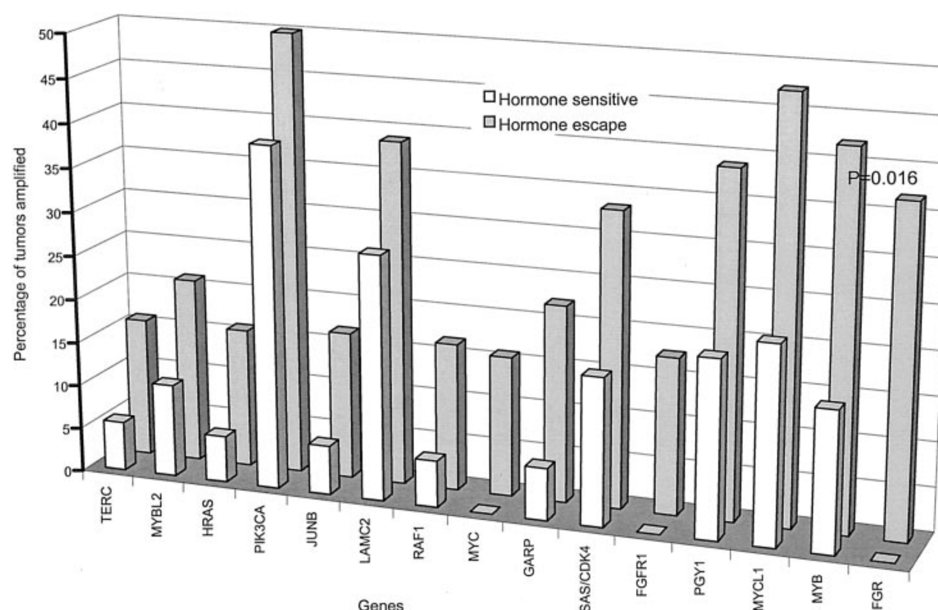

Fig. 4 Percentage of tumors amplified for each individual gene in both the hormone-sensitive (*white*) and hormone-escaped (*gray*) tumors. Only genes with an amplification rate increased by ≥10% in the transition from hormone sensitive to hormone escape are shown. The *FGR* (*Src-2*) amplification rate significantly increased with the development of hormone escape ($P = 0.016$, McNemar's test).

Table 5 Signal transduction pathway genes

	Hormone sensitive	Hormone escaped	*P*
MAPK pathway			
FGR(Src-2)(control *MYCL2*)	0% (0/19)	37% (7/19)	0.016
HRAS (control *FGF3*)	5% (1/19)	16% (3/19)	0.500
RAF1 (control *TERC*)	5% (1/19)	17% (3/18)	0.500
MYC/MYCL1 (control *MOSF/FGR*)	21% (4/19)	63% (12/19)	0.027
FGR/HRAS/RAF1	10% (2/19)	60% (11/19)	0.005
FGR/HRAS/RAF1MYC/MYCL1	31% (6/19)	74% (14/19)	0.024
P13K pathway			
FGR(Src-2) (control *MYCL2*)	0% (0/19)	37% (7/19)	0.016
PI3K (control *TERC*)	39% (7/18)	50% (9/18)	0.375
FGR(Src-2) and *PI3K*	0% (0/19)	26% (5/19)	0.046
Akt (no control)	0% (0/19)	0% (0/19)	1.000
MYB/MYB12 (control *ERS/CSE1L*)	26% (5/19)	53% (10/19)	0.180
FGR(Src-2) and *PI3K/MYB/MYB12*	26% (5/19)	53% (10/19)	0.180

upstream regulators (Table 5) demonstrated that 73% of cases were amplified for *HRAS/RAF1/Src-2/MYC/MYCL1* after hormone escape *versus* 31% before hormone escape. Overall, 53% of cases were amplified for *Src-2* and *PI3K* and/or *MYB/MYBL2* after hormone relapse *versus* 26% before hormone relapse. In 58% of cases, this represented an amplification in a novel pathway arising during the development of hormone escape. We, therefore, conclude from the data presented here that amplification of key genes within the RAS/MAPK and PI3K pathways are involved in the development of hormone escape in the majority of prostate cancers. Both of these pathways are increasingly implicated the development of prostate cancer and are also implicated with developing resistance in an increasing number of other cancers, most notably breast cancer, which is also a hormonally regulated disease (41, 50).

We also reported increased genetic instability as a tumor progresses, from hormone-sensitive to hormone-escaped prostate cancer ($P = 0.008$), with amplification of multiple members of the PI3K pathway and the MAPK cascade being more commonly observed in hormone-escaped prostate cancer. Amplification rates of other genes, however, also increased with the development of hormone escape (CDK4 and PYG1; Fig. 4). CDK4 belongs to the ser/thr family of protein kinases and is involved in the control of the cell cycle (60). *PGY1* is the gene for human multidrug resistance protein 1 (61). This protein functions as an energy-dependent efflux pump and is responsible for decreasing drug accumulation in cells (61). Expression of MDR1 is associated with poor response to chemotherapy in breast carcinoma, therefore, it is not surprising that overex-

pression of this protein might cause resistance to antiandrogen therapies in prostate cancer (61). These may represent alternative pathways for hormone escape.

We have provided strong evidence that amplification or gain of key genes within the RAS/MAPK and PI3K pathways, including downstream transcription factors, drive the development of hormone resistance in prostate cancers. These pathways are not necessarily mutually exclusive, and multiple mechanisms may arise in different groups of hormone-escaped prostate cancer. Many drugs that target these pathways by inhibiting specific members at the RTK level or down stream are currently under development [*e.g.*, Ras/Sulindac, RAF1/Bay439006, and PI3K/wortmannin (62)]. We believe that additional research might identify key modulators of hormone escape within these pathways and will enable us to profile treatment for individual tumors based on expression of these key genes.

REFERENCES

1. Sciarra, A., Casale, P., Colella, D., Di Chiro, C., and Di Silverio, F. Hormone-refractory prostate cancer? Anti-androgen withdrawal and intermittent hormone therapy. Scand. J. Urol. Nephrol, *33:* 211–216, 1999.

2. Dorkin, T. J., and Neal, D. E. Basic science aspects of prostate cancer. Semin. Cancer Biol., *8:* 21–27, 1997.

3. Culig, Z., Hobisch, A., Bartsch, G., and Klocker, H. Expression and function of androgen receptor in carcinoma of the prostate. Microsc. Res. Tech., *51:* 447–455, 2000.

4. Chang, C., Saltzman, A., Yeh, S., Young, W., Keller, E., Lee, H. J., Wang, C., and Mizokami, A. Androgen receptor: an overview. Crit. Rev. Eukaryot. Gene Expr., *5:* 97–125, 1995.

5. Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanern, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J., and Kallioniemi, O. P. In vivo amplificaiton of the androgen receptor gene and progression of human prostate cancer. Nat. Genet., *9:* 401–406, 1995.

6. Linja, M. J., Savinainen, K. J., Saramaki, O. R., Tammela, T. L. J., Vessella, R. L., and Visakorpi, T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res., *61:* 3550–3555, 2001.

7. Miyoshi, Y., Uemura, H., Fujinami, K., Mikata, K., Harada, M., Kitamura, H., Koizumi, Y., and Kubota, Y. Fluorescence in situ Hybridization evaluation of c-myc and androgen receptor gene amplification and chromosomal anomalies in prostate cancer in Japanese patients. Prostate., *43:* 225–232, 2000.

8. Edwards, J., Krishna, N. S., Mukherjee, R., Watters, A. D., and Bartlett, J. M. S. Amplification of the androgen receptor may not explain the development of androgen-independent prostate cancer. Br. J. Urol. Int., *88:* 633–637, 2001.

9. Trapman, J., and Cleutjens, K. B. J. M. Androgen-regulated gene expression in prostate cancer. Semin. Cancer Biol., *8:* 29–36, 1997.

10. Wang, L. G., Liu, X. M., Kreis, W., and Budman, D. R. Phosphorylation/dephosphorylation of androgen receptor as a determinant of androgen agonistic or antagonistic activity. Biochem. Biophys. Res. Commun., *259:* 21–28, 1999.

11. Blok, L. J., de Ruiter, P. E., and Brinkmann, A. O. Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. Biochemistry, *37:* 3850–3857, 1998.

12. Zhou, Z. X., Kemppainen, J. A., and Wilson, E. M. Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol. Endocrinol., *9:* 605–615, 1995.

13. Blok, L. J., de Ruiter, P. E., and Brinkmann, A. O. Androgen receptor phosphorylation. Endocr. Res., *22:* 197–219, 1996.

14. Lin, H. K., Yeh, S., Kang, H. Y., and Chang, C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc. Natl. Acad. Sci. USA, *98:* 7200–7205, 2001.

15. Wen, Y., Hu, M. C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D. H., and Hung, M. C. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res., *60:* 6841–6845, 2000.

16. Ye, D., Mendelsohn, J., and Fan, Z. Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor p27Kip1 and costimulate proliferation of MDA PCa 2a and MDA PCa 2b. Clin. Cancer Res., *5:* 2171–2177, 1999.

17. Zhu, X., and Liu, J. P. Steroid-independent activation of androgen receptor in androgen-independent prostate cancer: a possible role for the MAP kinase signal transduction pathway? Mol. Cell. Endocrinol., *134:* 9–14, 1997.

18. Sciavolino, P. J., and Abate-Shen, C. Molecular biology of prostate development and prostate cancer. Ann. Med., *30:* 357–368, 1998.

19. Suzuki, H., and Ito, H. Role of androgen receptor in prostate cancer. Asian J. Androl., *1:* 81–85, 1999.

20. Going, J. J., and Lamb, R. F. Practical histological microdissectin for PCR analysis. J. Pathol., *79:* 121–124, 1996.

21. Speicher, N. R., Dumanoir, S., Schrock, E., Holtgrevebrex, H., Schoell, B., Lengauer, C., Cremer, T., and Reid, T. Molecular cytogenetic analysis of formalin fixed paraffin embedded solid tumor altered universal DNA amplification by comparative genomic hybridization. Hum. Mol. Genet., *2:* 1907–1914.

22. El Gedaily, A., Bubendorf, L., Willi, N., Fu, W., Richter, J., Moch, H., Mihatsch, M. J., Sauter, G., and Gasser, T. C. Discovery of new DNA amplification loci in prostate cancer by comparative genomic hybridization. Prostate, *46:* 184–190, 2001.

23. Kim, S. H., Kim, M. S., and Jensen, R. H. Genetic alterations in microdissected prostate cancer cells by comparative genomic hybridization. Prostate Cancer Prostatic Dis., *3:* 110–114, 2000.

24. Chu, L. W., Pettaway, C. A., and Liang, J. C. Genetic abnormalities specifically associated with varying metastatic potential of prostate cancer cell lines as detected by comparative genomic hybridization. Cancer Genet. Cytogenet., *127:* 161–167, 2001.

25. Sattler, H. P., Lensch, R., Rohde, V., Zimmer, E., Meese, E., Bonkhoff, H., Retz, M., Zwergel, T., Bex, A., Stoeckle, M., and Wullich, B. Novel amplification unit at chromosome 3q25–q27 in human prostate cancer. Prostate, *45:* 207–215, 2000.

26. Sattler, H. P., Rohde, V., Bonkhoff, H., Zwergel, T., and Wullich, B. Comparative genomic hybridization reveals DNA copy number gains to frequently occur in human prostate cancer. Prostate, *39:* 79–86, 1999.

27. Rokman, A., Koivisto, P. A., Matikainen, M. P., Kuukasjarvi, T., Poutiainen, M., Helin, H. J., Karhu, R., Kallioniemi, O. P., and Schleutker, J. Genetic changes in familial prostate cancer by comparative genomic hybridization. Prostate, *46:* 233–239, 2001.

28. Nupponen, N. N., and Visakorpi, T. Molecular cytogenetics of prostate cancer. Microsc. Res. Tech., *51:* 456–463, 2000.

29. Skacel, M., Ormsby, A. H., Pettay, J. D., Tsiftsakis, E. K., Liou, L. S., Klein, E. A., Levin, H. S., Zippe, C. D., and Tubbs, R. R. Aneusomy of chromosomes 7, 8 and 17 and amplification of HER2 and epidermal growth factor receptor in Gleason score 7 prostate carcinoma: a differential fluorescent in situ hybridization study of Gleason pattern 4 and 4 using tissue microarray. Hum. Pathol., *32:* 1392–1397, 2001.

30. Olapade-Olaopa, E. O., Moscatello, D. K., MacKay, E. H., Horsburgh, T., Sandhu, D. P., Terry, T. R., Wong, A. J., and Habib, F. K. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br. J. Cancer, *82:* 186–194, 2000.

31. Olapade-Olaopa, E. O. The expression of a mutant epidermal growth factor receptor in prostatic tumours. Br. J. Urol. Int., *87:* 224–226, 2001.

32. Mark, H. F. L., Feldman, D., Das, S., Key, H., Mark, S., Sun, D-L., and Samy, M. Fluorescence in itu hybridization study of Her2 oncogene amplification in prostate cancer. Exp. Mol. Pathol., *66:* 170–178, 1999.

33. Ross, J. S., Sheehan, C. E., Hayner-Buchan, A. M., Ambros, R. A., Kallakury, B. V., Kaufman, R. P., Jr., Fisher, H. A., Rifkin, M. D., and Muraca, P. J. Prognostic significance of HER-2/neu gene amplification

status by fluorescence in situ hybridization of prostate carcinoma. Cancer (Phila.), *79:* 2162–2170, 1997.

34. Kuhn, E. J., Kurnot, R. A., Sesterhenn, I. A., Chang, E. H., and Moul, J. W. Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma. J. Urol., *150:* 1427–1433, 1993.

35. Wu, D. Q., Foreman, T. L., Gregory, C. W., McJilton, M. A., Wescott, G. G., Ford, O. H., Alvey, R. F., Mohler, J. L., and Terrian, D. M. Protein kinase C epsilon has the potential to advance the recurrence of human prostate cancer. Cancer Res., *62:* 2423–2429, 2002.

36. Mehta, P., Robson, C. N., Neal, D. E., and Leung, H. Y. Fibroblast growth factor receptor-2 mutation analysis in human prostate cancer. Br. J. Urol. Int., *86:* 681–685, 2000.

37. Udayakumar, T. S., Klein, R. D., Maliner, M. S., Nagle, R. B., and Bowden, G. T. Aberrant expression of fibroblast growth factor receptor-1 in prostate epithelial cells allows induction of promatrilysin expression by fibroblast growth factors. Int. J. Cancer, *91:* 187–192, 2001.

38. Yeh, S., Lin, H. K., Kang, H. Y., Thin, T. H., Lin, M. F., and Chang, C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc. Natl. Acad. Sci. USA, *96:* 5458–5463, 1999.

39. Sheta, E. A., Trout, H., Gildea, J. J., Harding, M. A., and Theodorescu, D. Cell density mediated pericellular hypoxia leads to induction of HIF-1 α via nitric oxide and Ras/MAP kinase mediated signaling pathways. Oncogene, *20:* 7624–7634, 2001.

40. Wang, Z., Tseng, C. P., Pong, R. C., Chen, H., McConnell, J. D., Navone, N., and Hsieh, J. T. The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer—characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J. Biol. Chem., *277:* 12622–12631, 2002.

41. Zachos, G., and Sandidos, D. A. Expression of ras proto-oncogenes: regulation and implications in the development of human tumors. Crit. Rev. Oncol. Hematol., *26:* 65–75, 1997.

42. Shannon, K. M. The Ras pathway as a target for cancer therapy. Proc. Am. Assoc. Cancer Res., *43:* 489, 2002.

43. Frame, M. C. Src in cancer: deregulation and consequences for cell behavior. Biochim. Biophys. Acta, *1602:* 114–130, 2002.

44. Castoria, G., Migliaccio, A., Di Domenico, M., Barone, M. V., Bilancio, A., and Auricchio, F. Activation of the Src/Ras/Erk signalling pathway is required for steroid-induced DNA synthesis. Pflugers Arch., *439:* 52, 2000.

45. Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W. R., Beato, M., and Auricchio, F. Activation of the Src/p21(ras)/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J., *17:* 2008–2018, 1998.

46. Ishida, M., Ishida, T., Thomas, S. M., and Berk, B. C. Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on c-Src in vascular smooth muscle cells. Circ. Res., *82:* 7–12, 1998.

47. Thomas, S. M., and Brugge, J. S. Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. BI., *13:* 513–609, 1997.

48. Feldman, B. J., and Feldman, D. The development of androgen-independent prostate cancer. Nat. Rev., *1:* 34–45, 2001.

49. Lin, H. K., Adam, R. M., Santiestevan, E., and Freeman, M. R. The phosphatidylinositol 3′ kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP. Cancer Res. *59:* 2891–2897.

50. Gustin, J. A., Maehama, T., Dixon, J. E., and Donner, D. B. The PTEN tumor suppressor protein inhibits tumor necrosis factor-induced nuclear factor κB activity. J. Biol. Chem., *276:* 27740–27744, 2001.

51. Nicholson, K. M., and Anderson, N. G. The protein kinase B/Akt signaling pathway in human malignancy. Cell. Signal., *14:* 381–395, 2002.

52. Reiss, K., Wang, J. Y., Romano, G., Furnari, F. B., Cavenee, W. K., Morrione, A., Tu, X., and Baserga, R. IGF-I receptor signaling in a prostatic cancer cell line with a PTEN mutation. Oncogene, *19:* 2687–2694, 2000.

53. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA, *95:* 15587–15591, 1998.

54. Lu, Y., Hui Liu, J., Hang, J., Ang, H., Oul, D., McMurray, J. S., Ang, S., and Ung, W. A. Involvement of Src family protein tyrosine kinases and the SHP-1 tyrosine phosphatase in the phosphotyrosine dependent regulation of PTEN activity. Proc. Am. Assoc. Cancer Res., *43:* 154, 2002.

55. Jull, B. A., Plummer, H. K., and Schuller, H. M. Nicotinic receptor-mediated activation by the tobacco-specific nitrosamine NNK of a Raf-1/MAP kinase pathway, resulting in phosphorylation of c-myc in human small cell lung carcinoma cells and pulmonary neuroendocrine cells. J. Cancer Res. Clin. Oncol., *127:* 707–717, 2001.

56. Majka, M., Hershock, D., Ratajczak, J., Gontarewicz, A., Gewirtz, A. M., and Ratajczak, M. Z. Differentiating normal human megakaryoblasts express APO-Fas (CD95), TNF-RII, secrete several megakaryopoietic inhibitors and undergo apoptosis; an important role of thrombopoietin (TPO), MYB and PI3K-Akt-BAD axis in inhibiting apoptosis in normal megakaryocytic precursors. Blood, *94:* 2155, 1999.

57. Kaltz-Wittmer, C., Klenk, U., Glaessgen, A., Aust, D. E., Diebold, J., Lohrs, U., and Baretton, G. B. FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostatic carcinomas before and after androgen deprivation therapy. Lab. Invest., *80:* 1455–1464, 2000.

58. Latil, A., and Lidereau, R. Genetic aspects of prostate cancer. Virchows Arch., *432:* 389–406, 1998.

59. Grad, J. M., Dai, J. L., Wu, S., and Burnstein, K. L. Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. Mol. Endocrinol., *13:* 1896–1911, 1999.

60. Benard, J. Genetic alterations associated with metastatic dissemination and chemoresistance in neuroblastoma. Eur. J. Cancer, *31A:* 560–564, 1995.

61. Trock, B. J., Leonessa, F., and Clarke, R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J. Natl. Cancer Inst., *89:* 917–931, 1997.

62. Weinstein-Oppenheimer, C. R., Blalock, W. L., Steelman, L. S., Chang, F. M., and McCubrey, J. A. The Raf signal transduction cascade as a target for chemotherapeutic intervention in growth factor-responsive tumors. Pharmacol. Ther., *88:* 229–279, 2000.