

# 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 escape. 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<sup>2</sup>; 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

Received 1/22/03; revised 7/22/03; accepted 7/28/03.

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<sup>2</sup> 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

Patient no.	Age (yr)	Primary surgery	Information at diagnosis		Relapse surgery	Information at relapse		Time to relapse (days)	Treatment
			Gleason score	PSA		Gleason score	PSA		
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- $\mu$ m 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  $\mu$ g) 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  $\mu$ l. 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  $\mu$ l of precipitation reagent, 5  $\mu$ l of 3 M sodium acetate, and 55  $\mu$ l of isopropanol were added to precipitate DNA. Samples were placed at -20°C for at least 1 h, and DNA was pelleted at 15,000  $\times$  g for 15 min at room temperature. The supernatant was removed, and pellets were washed in 300  $\mu$ l of 70% ethanol, spun, and resuspended in 7.5  $\mu$ l of nuclease-free water (200 ng/ $\mu$ l). After labeling, 0.5  $\mu$ g of each product was run on a 2% agarose gel to confirm the success of the reaction.

**Hybridization.** Test DNA (2.5  $\mu$ l, 0.5  $\mu$ g) and 2.5  $\mu$ l of reference DNA were combined with 22  $\mu$ l of hybridization buffer (total volume, 27  $\mu$ l). 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 $\times$  SSC) three times at 40°C for 10 min. This was followed by four washes for 5 min in 1 $\times$  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
<i>FGR</i>	1p36.2-p36.1	0.95	0.03	<i>WNT1</i>	12q12-q13	0.75	0.14
<i>MYCL1</i>	1p34.3	1.15	0.29	<i>GLI</i>	12q13.2-q13.3	0.80	0.10
<i>NRAS</i>	1p13.2	1.00	0.23	<i>SAS/CDK4</i>	12q13.3	0.96	0.17
<i>LAMC2</i>	1q25-q31	0.89	0.12	<i>MDM2</i>	12q14.3-q15	0.81	0.22
<i>MYCN</i>	2p24.1	0.80	0.10	<i>Akt1</i>	14q32.3	0.75	0.23
<i>REL</i>	2p13-p12	0.76	0.13	<i>IGFR1</i>	15q25-q26	0.87	0.26
<i>RAF1</i>	3p25	1.14	0.06	<i>FES</i>	15q26.1	0.69	0.12
<i>TERC</i>	3q26.3	1.12	0.03	<i>MRP1</i>	16p13.1	1.02	0.08
<i>P13K</i>	3q26.3	1.14	0.05	<i>TOP2A</i>	17q21-q22	0.86	0.17
<i>PDGFRA</i>	4q12	0.87	0.23	<i>HER2</i>	17q21.2	0.95	0.21
<i>MYB</i>	6q22	1.06	0.17	<i>RPS6KB1</i>	17q23	0.96	0.09
<i>ESR</i>	6q25.1	1.18	0.19	<i>D17S1670</i>	17q23	0.98	0.16
<i>EGFR</i>	7p12.3-p12.1	0.89	0.11	<i>YES1</i>	18p11.3	1.02	0.09
<i>PGY1</i>	7q21.1	0.99	0.15	<i>BCL2 3'</i>	18q21.3	1.06	0.12
<i>MET</i>	7q31	0.88	0.06	<i>BCL2 5'</i>	18q21.3	1.03	0.14
<i>CTSB</i>	8p22	0.99	0.31	<i>INSR</i>	19p13.2	1.10	0.06
<i>FGFR1</i>	8p11.2-p11.1	0.94	0.21	<i>JUNB</i>	19p13.2	0.56	0.07
<i>MOS</i>	8q11	0.97	0.09	<i>CCNE1</i>	19q13.1	0.92	0.16
<i>MYC</i>	8q24.12-q24.13	0.89	0.17	<i>AIB1</i>	20q12	1.05	0.21
<i>ABL1</i>	9q34.1	0.88	0.20	<i>STK15</i>	20q13	0.98	0.08
<i>FGFR2</i>	10q26	1.18	0.07	<i>CSE1L</i>	20q13	1.00	0.15
<i>HRAS</i>	11p15.5	1.03	0.20	<i>MYBL2</i>	20q13.1	0.87	0.16
<i>CCND1</i>	11q13	0.98	0.13	<i>PTPNI</i>	20q13.1-q13.2	0.82	0.09
<i>FGF4/FGF3</i>	11q13	0.80	0.20	<i>ZNF217</i>	20q13.2	0.93	0.19
<i>EMS1</i>	11q13	1.02	0.24	<i>CBFA2</i>	21q22.3	1.01	0.20
<i>GARP</i>	11q13.5-q14	1.08	0.21	<i>BCR</i>	22q11.21	0.93	0.15
<i>PAK1</i>	11q13.5-q14	1.01	0.10	<i>PDGFB</i>	22q12.3-q13.1	0.74	0.17
<i>MLL</i>	11q23	0.91	0.16	<i>AR 5'</i>	Xq11-q12	0.86	0.15
<i>CCND2</i>	12p13	0.90	0.25	<i>AR 3'</i>	Xq11-q12	1.22	0.21
<i>KRAS2</i>	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  $\pm$  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  $0.944 \pm 0.217$  (mean  $\pm$  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 ( $0.944 \pm 2$  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  $\pm$  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%;

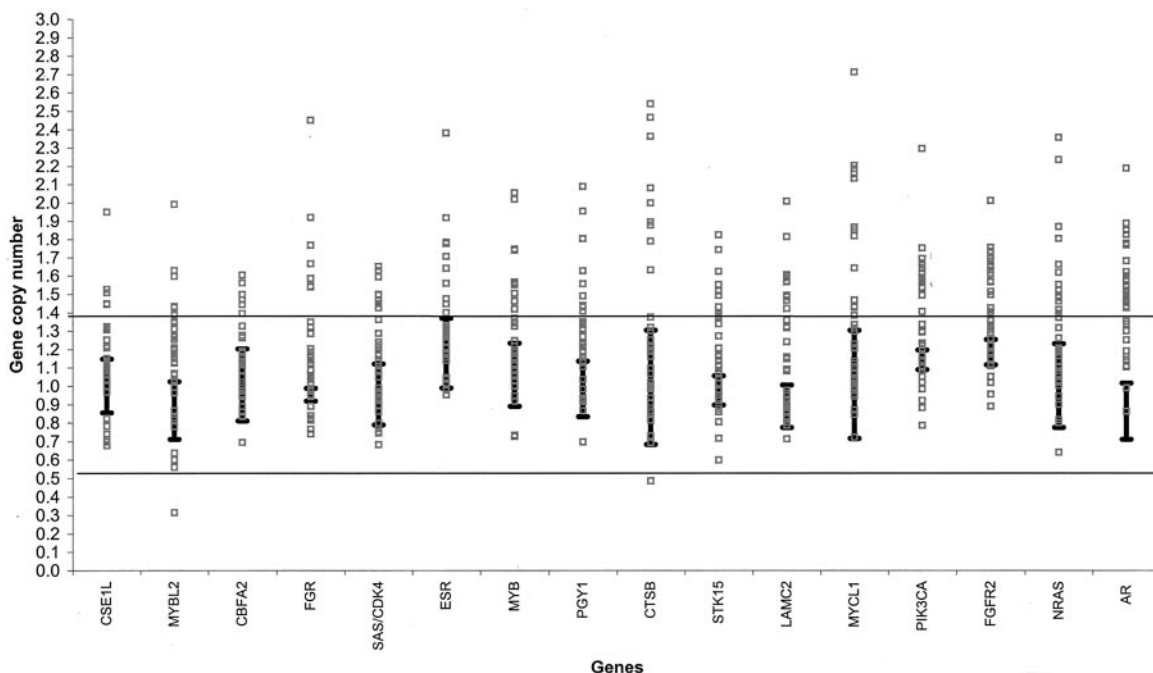


Fig. 1 The copy number of each gene for each tumor is indicated by a square, and the average copy number for each gene calculated from the normal colon controls is indicated by a circle. The error bars on the circles denote the SD, and the horizontal lines on the graph at 0.378 and  $-0.51$  are the 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  $\geq 15\%$  of hormone-sensitive tumors, and 24 genes were ampli-

fied in  $\geq 15\%$  of hormone-escaped tumors (Table 4). *AR* (63% pre-hormone and 67% post-hormone; only gene on X chromosome), *FGFR2* (50% pre-hormone and 44% post-hormone; only gene on chromosome 10), *PI3K* (39% pre-hormone and 50% post-hormone), and *NRAS* (47% pre-hormone and 47% post-hormone) 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 number 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

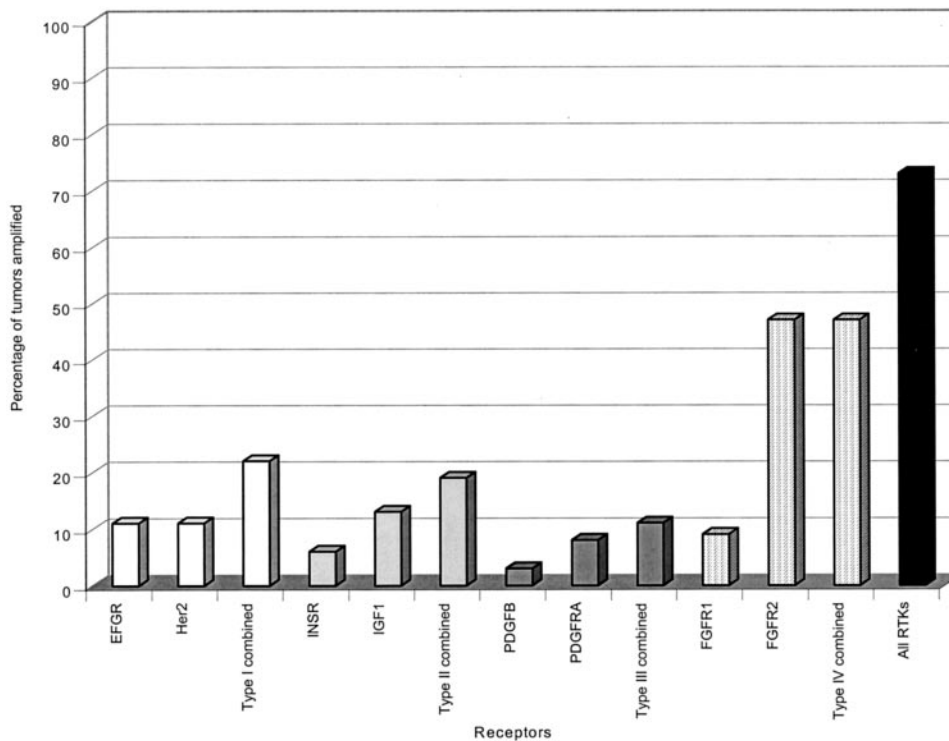


Fig. 2 The total percentage of tumors amplified for cell surface RTK are shown in Fig. 1. Each family [type I (*EGFR* and *HER2*), type II (*INSR* and *IGF1*), type III (*PDGFB* and *PDGFRA*), and type IV (*FGFR1* and *FGFR2*)] is shown for each individual family member and the family in total.

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  $\sim 73\%$  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<sup>HER2</sup> is associated with poor prognosis (31, 32, 34), and we have demonstrated by FISH that *HER2* amplification rates increase with grade in prostate tumors.<sup>3</sup>

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

<sup>3</sup> J. Edwards, personal communication.

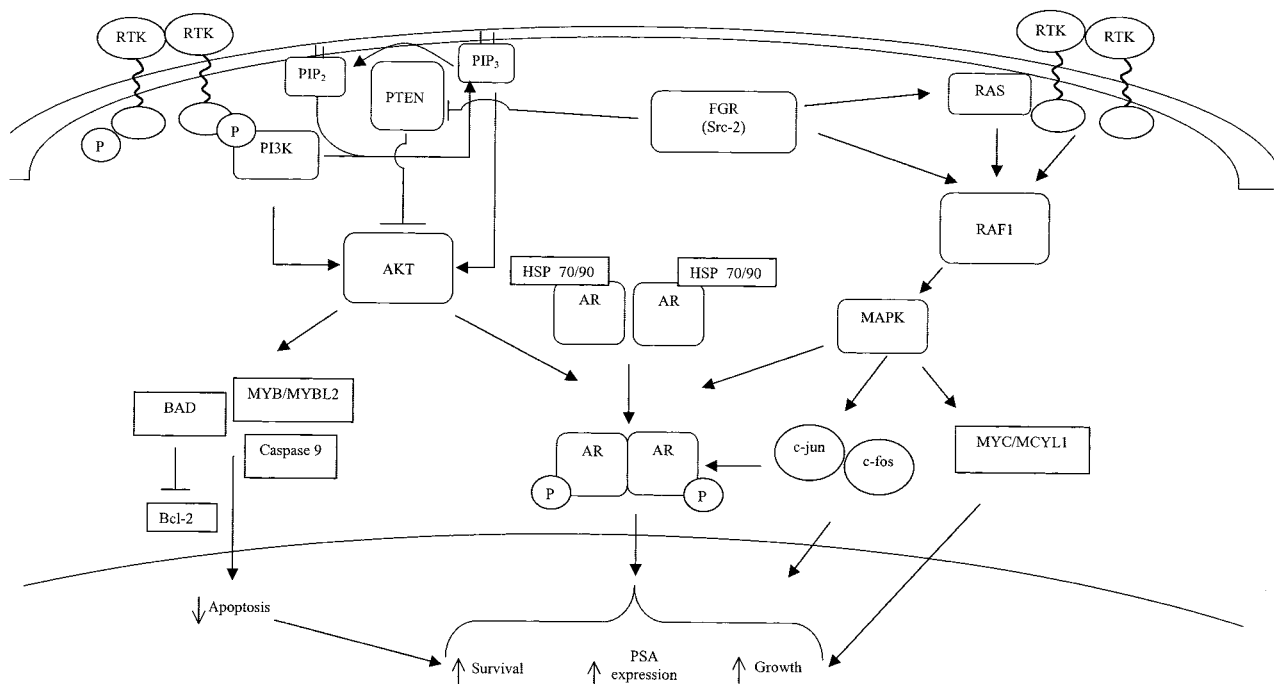


Fig. 3 Interactions between the Src-2/RAS/RAF/MAPK and Src-2/PI3K/Akt pathways and related transcription factors in the hormone-resistant prostate cancer cell. Activation of both these pathways may result in phosphorylation and, hence, activation of the AR in the absence of androgens.

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



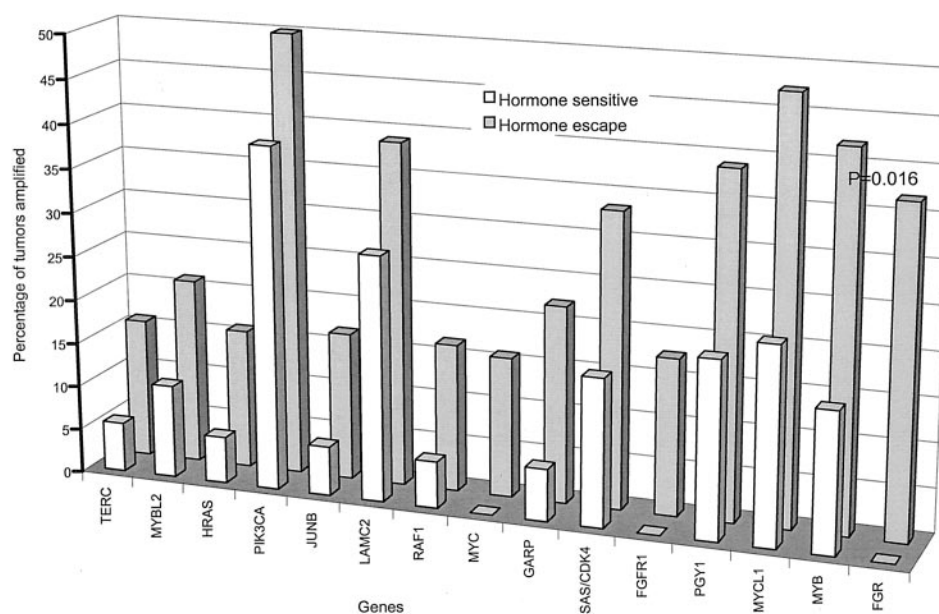


Fig. 4 Percentage of tumors amplified for each individual gene in both the hormone-sensitive (white) and hormone-escaped (gray) tumors. Only genes with an amplification rate increased by  $\geq 10\%$  in the transition from hormone sensitive to hormone escape are shown. The *FGR* (*Src-2*) amplification 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	<i>P</i>
MAPK pathway			
<i>FGR</i> ( <i>Src-2</i> )(control <i>MYCL2</i> )	0% (0/19)	37% (7/19)	0.016
<i>HRAS</i> (control <i>FGF3</i> )	5% (1/19)	16% (3/19)	0.500
<i>RAF1</i> (control <i>TERC</i> )	5% (1/19)	17% (3/18)	0.500
<i>MYC/MYCL1</i> (control <i>MOSF/FGR</i> )	21% (4/19)	63% (12/19)	0.027
<i>FGR/HRAS/RAF1</i>	10% (2/19)	60% (11/19)	0.005
<i>FGR/HRAS/RAF1MYC/MYCL1</i>	31% (6/19)	74% (14/19)	0.024
PI3K pathway			
<i>FGR</i> ( <i>Src-2</i> ) (control <i>MYCL2</i> )	0% (0/19)	37% (7/19)	0.016
<i>PI3K</i> (control <i>TERC</i> )	39% (7/18)	50% (9/18)	0.375
<i>FGR</i> ( <i>Src-2</i> ) and <i>PI3K</i>	0% (0/19)	26% (5/19)	0.046
<i>Akt</i> (no control)	0% (0/19)	0% (0/19)	1.000
<i>MYB/MYB12</i> (control <i>ERS/CSEIL</i> )	26% (5/19)	53% (10/19)	0.180
<i>FGR</i> ( <i>Src-2</i> ) and <i>PI3K/MYB/MYB12</i>	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.

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*Clin Cancer Res* 2003;9:5271-5281.

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