

Molecular analysis integrating different pathways associated with androgen-independent progression in LuCaP 23.1 xenograft

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After therapeutic hormone deprivation, most prostate cancer (PrCa) cells develop androgen-independent (AI) growth. PrCa is highly heterogeneous and multifocal, suggesting that several molecular processes or pathways may be contributing to AI. The human LuCaP 23.1 xenograft model retains clinical hallmarks of PrCa, including heterogeneous growth, PSA production, androgen-responsiveness and progression to AI. In this work, we studied the effect of androgen depletion (castration) on the growth of LuCaP 23.1 xenografts. A total of 100 nude mice were implanted and analysed for their growth profiles before and after castration. By 11 and 15 weeks, tumours were harvested and assessed for molecular marker expression specific for PrCa. Prior to castration we found 37 fast growing (FG) tumours ($948.9 \pm 76.9 \text{ mm}^3$) and 63 slow growing (SG) tumours ($229.6 \pm 18.4 \text{ mm}^3$), a previously undescribed result for this PrCa model. Quantitative RT-PCR showed that in comparison to SGs, FGs contained high HER1, uPA and thymidilate synthase (TS) expression with low levels of 5 α -reductase 2 mRNA. All FG tumours progressed rapidly to AI growth 5 weeks after castration (FG-P). In SG castrated tumours, 66% of tumours (SG-P) showed retarded progression (by 12 weeks) to AI, whereas 34% responded to castration (SG-R). Molecular analysis permitted us to define distinct molecular profiles integrating different pathways associated with AI progression. FG-P, and a subgroup of SG-P tumours, presented significantly high levels of peptidylglycine α -amidating monooxygenase (PAM), HER1, HER2, TS, and uPA mRNA, all of which correlated with AR expression. The second subgroup of SG-P tumours showed overexpression of the antiapoptotic gene Bcl-2. A third subgroup of SG-P tumours showed significant expression of hypoxia-related gene (adrenomedullin) after castration. This work permitted to define distinct molecular profiles related to different AI growth in the LuCaP 23.1 xenograft.

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Introduction

Prostate cancer (PrCa) is the most common noncutaneous cancer acquired by men in North America (Parker *et al.*, 1996). Withdrawal of androgens is the mainstay of therapy for progressive advanced PrCa and causes regression of androgen-dependent (AD) tumours (McLeod, 2003). Despite an initial regression upon androgen withdrawal, long-term response to endocrine treatment is often poor (Denis and Griffiths, 2000). The molecular mechanisms underlying AI progression are poorly understood, in part, due to the difficulty in obtaining sufficient tumoral material throughout AI progression. It is clear, however, that multiple signalling pathways are involved in PrCa progression (Feldman and Feldman, 2001).

During AI, PrCa cells can survive with low levels of androgens (Jenster *et al.*, 1999; Feldman and Feldman, 2001) or by increasing 5 α -reductase activity type 2 (5 α -R2) for conversion of testosterone to dihydrotestosterone (Ross *et al.*, 1998). Members of the Epidermal Growth Factor-Receptor family (HER1, HER2) can activate AR by ligand-independent mechanisms. For example, recent studies have shown that HER1 and HER2 can induce AR phosphorylation and expression of the target gene PSA, possibly occurring through the MAP kinase or Akt pathways (Craft *et al.*, 1999; Yeh *et al.*, 1999; Wen *et al.*, 2000; Solit *et al.*, 2002). Clonal expansion of cells with neuroendocrine (NE) differentiation also plays a significant role in the development of AI PrCa (Abrahamsson, 1999) as several NE markers, including adrenomedullin (AM) and peptidyl-glycine α -amidating monooxygenase (PAM) (Rocchi *et al.*, 2001). The antiapoptotic gene Bcl-2 increases after castration in LNCaP xenografts with Bcl-2 antisense oligonucleotide treatment delaying AI progression (Gleave *et al.*, 1999; Miyake *et al.*, 1999). Overexpression of the plasminogen activator urokinase (uPA), its

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receptor (uPAR) and plasminogen activator inhibitor type 1 (PAI-1), are found in invasive PrCa (Gaylis *et al.*, 1989; Hollas *et al.*, 1992; Helenius *et al.*, 2001).

In this study, we describe tumoral growth as a function of androgen depletion (castration) in the LuCaP 23.1 human PrCa xenograft model. The LuCaP 23.1 model is a reproducible and clinically relevant model that includes growth heterogeneity, androgen-sensitivity, PSA-producing and NE features (Ellis *et al.*, 1996; Liu *et al.*, 1996). Therefore, this model is highly suitable for studying different molecular markers associated with androgen depletion and AI progression. Molecular analysis of the tumours using quantitative RT-PCR permitted us to correlate specific molecular expression with the growth profiles of each tumour population. Previous studies have implicated several major signalling pathways, as represented in our study by key molecular markers, associated with AI. These include genes associated with: (1) androgen receptor signalling (AR, 5 α -R type 2); (2) tyrosine kinase receptors (HER1, HER2) activation; (3) apoptosis (Bcl-2); (4) NE differentiation (PAM, AM) and (5) pyrimidine synthesis related cell proliferation (TK1, TK2, TS) and invasive capacity (uPA). The LuCaP 23.1 xenograft is a suitable model for studying individualistic therapeutic approaches based on each type of AI PrCa. The subdivision of noncastrated LuCaP 23.1 xenograft tumours into slow (SG) and fast growing (FG) populations is a novel observation that could provide earlier determination of which tumours will be successful in androgen ablation therapy and which ones will fail.

Results

Tumour growth profile of LuCaP 23.1 xenograft

Figure 1 indicates tumour populations as a function of time postimplantation and postcastration. FG tumours were categorized as greater than 500 mm³

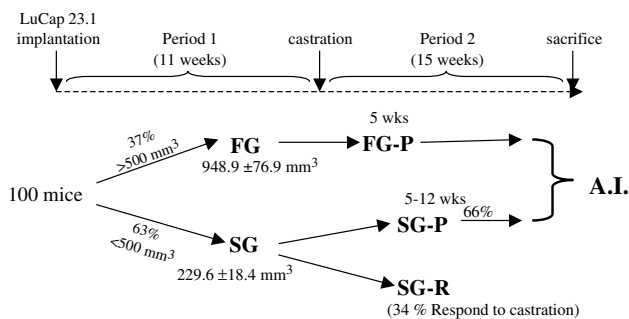


Figure 1 Flow chart of LuCaP 23.1 xenograft tumour populations before (period 1) and after castration (period 2). Prior to castration, two populations are observed including fast growing (FG) and slow growing (SG) tumours. Subsequently, FG tumours (FG-P) and 66% of SG (SG-P) progress to androgen independence (AI), whereas 34% of slow growing tumours (SG-R) respond to castration

(948.9 ± 76.4 mm³) while SG tumours were less than 500 mm³ (229.6 ± 18.4 mm³). In total, 63% of mice responded as SG tumours while the remaining 37% responded as FG tumours. After castration, SGs either responded to castration (34% of tumours) or progressed to androgen independence (66%).

Tumour growth rate and PSA index at the end of periods 1 and 2

Tumour growth rate (TGR) and PSA index were analysed at the end of periods 1 and 2. With a constant tumoral volume implanted ($V_i = 25 \text{ mm}^3$), 37% of tumours reached a volume at castration time (V_c) above 500 mm³ and were named as FG tumours while the tumours that reached a V_c less than 500 mm³ (63%) were designed as SG tumours (SG). A significant relationship between the FG and SG growth rates and the parental donor tumour specimen implanted was not detected (data not shown). During period 1, the TGR mean (Figure 2a) was 37.9 ± 3 for FG group versus 9.1 ± 0.7 for SG group ($P < 0.0001$) and PSA index (PSA value as a function of tumour volume) (Figure 2b) was 5.2 ± 0.3 for FG group versus 4.2 ± 0.3 for SG group ($P = 0.03$). Castration induced a significant decrease in TGR for all subgroups: from 37.9 to 1.7 for FG-P, 9.1 to 3 for SG-P and from 9.1 to 0.8 for SG-R. Similarly, PSA index decreased after castration for all subgroups: from 5.2 to 1.6 for FG-P, from 4.2 to 2.2 for SG-P and from 4.2 to 0.3 for SG-R. Interestingly, comparison of TGRs and PSA index before and after castration demonstrated that all tumours present an initial response to castration. Serum PSA levels detected in the FG group at castration time ($3493.39 \pm 510.02 \text{ ng/ml}$) were significantly higher than the SG group ($1318.48 \pm 187.39 \text{ ng/ml}$; $P < 0.001$) and were strongly correlated with the tumoral volume ($r^2 = 0.866$) (data not shown). The mean of TGR and PSA index were calculated with equal number of mice in each of the groups SG ($n = 63$), FG ($n = 37$), SG-P ($n = 18$), SG-R ($n = 9$) and FG-P ($n = 8$).

Response to castration

Mice bearing SG and FG tumours ($n = 42$) were randomly isolated during period 1 of analysis at 11 weeks postimplantation. Tumour volume and serum PSA were evaluated each week after castration (Figure 3). All FG tumours ($n = 8$) presented a weak response to castration, followed by fast progression to AI growth by 5 weeks after castration. At killing time, PSA was $3200 \pm 272.27 \text{ ng/ml}$ (Figure 3a). The SG group ($n = 27$) showed two different responses to castration. Some SG-P tumours ($n = 18$) had a significant initial response to castration (Figure 3b), followed by AI growth indicated by rising PSA ($1554 \pm 824 \text{ ng/ml}$ at killing). Conversely, the SG-R tumours ($n = 9$) showed a significant PSA and volume decrease (Figure 3c) at killing time ($30.27 \pm 12.79 \text{ ng/ml}$) as compared to castration time (1133.46 ± 315.77). Seven xenograft tumours (two FG and five SG tumours) presented a biphasic biological response after castration and were not

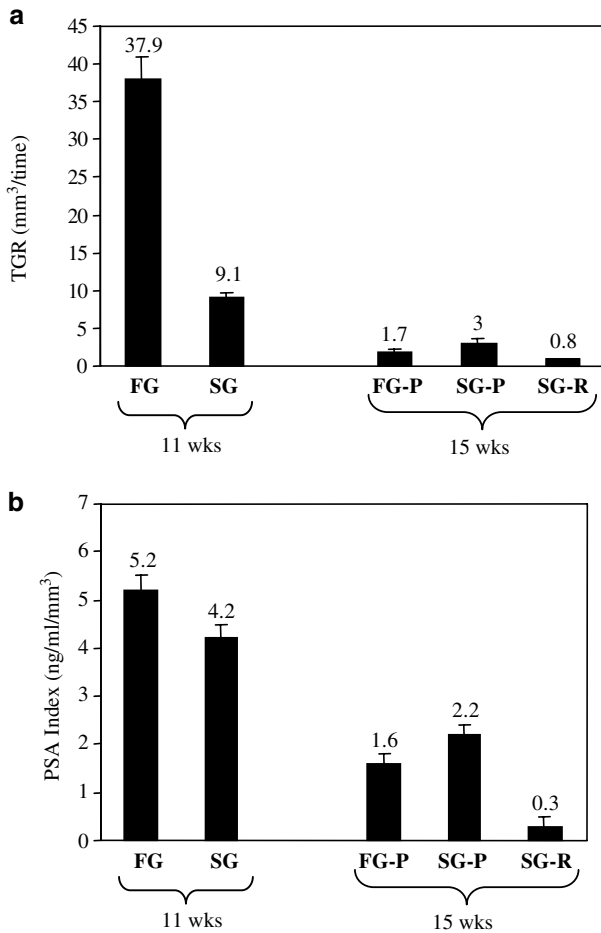


Figure 2 (a) Tumour growth rate TGR and (b) PSA index at castration (11 weeks) and killing time (15 weeks) in LuCaP 23.1 xenograft tumour populations. After the first period of analysis, mice bearing FG and SG tumours ($n=42$) were castrated with tumour volume and serum PSA analysed weekly during 15 weeks. At the end of the second period of analysis, TGR (mm³) was defined as V_c/V_s (where V_c =volume at castration and V_s =volume at killing). PSA index (defined as the PSA secretion in serum per unit of tumoral volume, ng/ml/mm³) was calculated in each group of tumours before and after castration. The mean of TGR and PSA was calculated with unequal number of mice in each of the following groups: SG ($n=63$), FG ($n=37$), SG-P ($n=18$), SG-R ($n=9$) and FG-P ($n=8$)

included as part of our total analysis. These animals were a minority and were statistical outliers (data not shown).

Molecular analysis at completion of period 1

Mean expression levels of HER1, TS and uPA were significantly higher in FG tumours ($n=17$) as compared to SG tumours ($n=7$) with an increase of 7.5-, 2.3- and 4.8-fold, respectively ($*P\leq 0.05$; Figure 4c, f, j). Conversely, SG tumours showed a 17-fold increase in 5 α -R2 mRNA level as compared to FG tumours ($**P\leq 0.01$; Figure 4b). Mean expression of other parameters was not significantly different between FG and SG tumours.

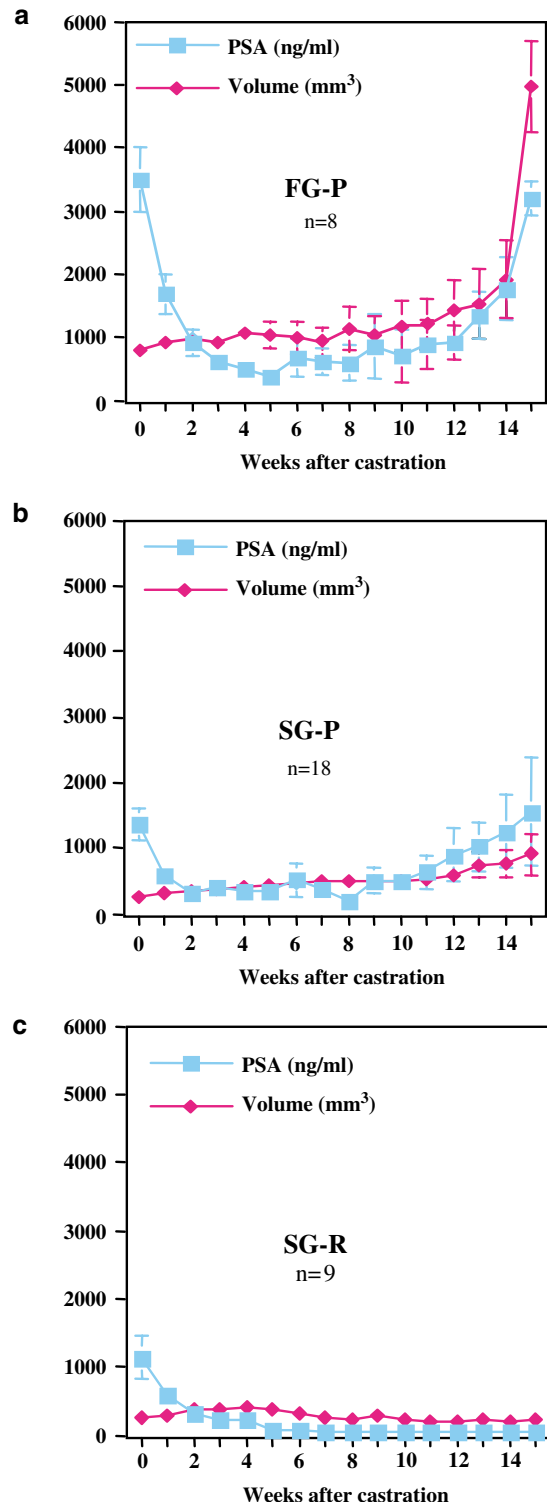


Figure 3 Tumour growth profiles and PSA levels were analysed during 15 weeks after castration. Tumours were defined as progressive (FG-P, LG-P) if serum PSA increased above nadir levels (100 ng/ml) with increased/stable tumour volume or responding (LG-R) if values were less than 100 ng/ml with decreasing/stable tumour volume. (a) All (8/8) FG-P tumours progressed to AI growth 5 weeks after castration, (b) 18/27 SG-P tumour became progressive while (c) 9/27 tumours responded (SG-R) to castration

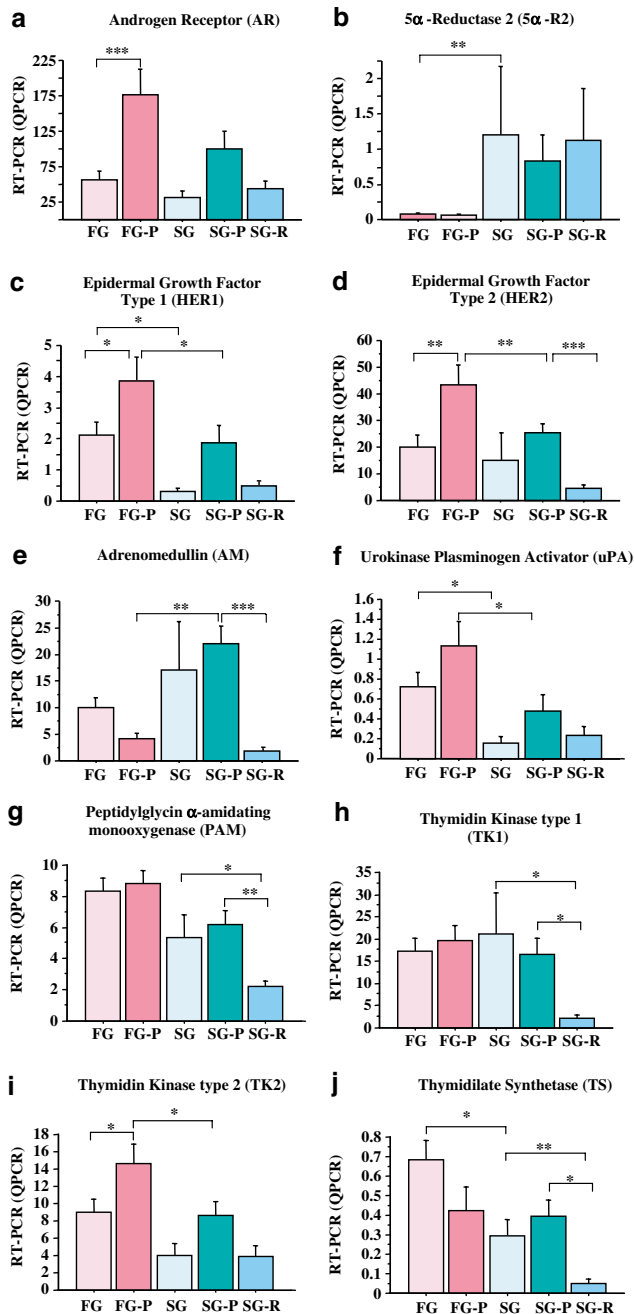


Figure 4 Molecular profiles by quantitative real time PCR in each group of LuCaP 23.1 xenograft before and after castration. RT-PCR estimations of relative mRNA are shown for (a) AR, (b) 5 α -R2, (c) HER1, (d) HER2, (e) AM, (f) uPA, (g) PAM, (h) TK1, (i) TK2 and (j) TS to 18S rRNA in LuCaP 23.1 xenografts. Data are presented as the mean \pm s.e.m. *, ** and *** differ between two groups ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively) by Student's *t*-test

Molecular analysis at the end of the 2nd period

Comparison between progressive tumours after castration show that FG-P tumours have significantly higher expression of HER1 ($*P \leq 0.05$), HER2 ($**P \leq 0.01$), TK2 ($*P \leq 0.05$) and uPA ($*P \leq 0.05$) than SG tumours

SG-P (Figure 4c, d, f, i). Differently, AM expression is significantly higher in SG-P than FG-P ($**P \leq 0.01$; Figure 4e). Comparison between tumours before and after castration reveals that castration induced a significant downexpression of PAM ($*P \leq 0.05$), TK1 ($*P \leq 0.05$) and TS ($**P \leq 0.01$) in responder tumours (SG-R) as compared to the precastration group (Figure 4g, h, j). Comparison between SG-P and SG-R revealed that SG-P tumours express significantly higher levels of PAM ($**P \leq 0.01$), AM ($***P \leq 0.001$) and HER2 ($***P \leq 0.001$), associated with a significant increase of proliferative enzyme TK1 ($*P \leq 0.05$) and TS ($*P \leq 0.05$), than SG-R tumours (Figure 4d, e, g, h, j). Although not statistically significant, Bcl-2 expression increases in SG-P tumours (2.2 ± 0.89) versus SG-R tumours (1 ± 0.55 ; data not shown). Comparison between FG and FG-P show that castration induced a significant upregulation of AR ($***P \leq 0.001$), HER1 ($*P \leq 0.05$), HER2 ($**P \leq 0.01$) and TK2 ($*P \leq 0.05$) (Figure 4a, c, d, i). Significant marker expression ratios between FG and SG and LG/LG-P, LG/LG-R and LG-R/LG-P tumours are expressed in Table 1.

Molecular analysis of pre- and postcastrated tumours by principal component analysis (PCA)

PCA projections are along the orthogonal axis (x , y , z) of molecular parameters (QPCR units) of period 1 and 2 tumours (FG, SG, FG-P, SG-P and SG-R) (Figure 5a–d). Projection of parameters into the principal factorial plan (Figure 5a, c) permitted definition of four clusters of correlated parameters. PAM expression is highly correlated to HER1, HER2, uPA and AR ($0.6 < r^2 < 1$). A second cluster is represented by AM, which is independent of the other parameters. A third group is represented by Bcl-2, which is strongly expressed in the z -axis (not shown). Lastly, a fourth group is represented by 5 α -R2 far from the first cluster of parameters. Projection of the FG and SG tumours into the principal factorial plan is represented in Figure 5b. FG tumours are homogeneously located in the same area of the plan, whereas SG tumours are heterogeneously distributed, showing three different subgroups. The first subgroup overexpress 5 α -R2, the second overexpresses AM while the third subgroup is linked to apoptotic pathway (Bcl-2), principally located on the z -axis (not shown). Projection of the tumours FG-P, SG-P and SG-R into the principal factorial plan is represented in Figure 5d. FG-P tumours are homogeneously located in the same area of the plan, whereas SG-P tumours are heterogeneously distributed, showing three different subgroups. The first subgroup expresses the same parameters (AR, HER1 and HER2) as FG-P and is distributed in the same area of the PCA. The second SG-P subgroup overexpresses AM, while the third SG-P subgroup was linked to apoptotic pathway (Bcl-2), and these are principally located on the z -axis (not shown). SG-R tumours show high levels of 5 α -R2, uncorrelated with other parameters that play a role in AI progression.

Table 1 Quantitative evaluation by RT-PCR (QPCR) of molecular parameters showing significant trends before (a) castration (FG and SG) and (b) after castration (FG, FG-P, SG, SG-P and SG-R)

Marker name	FG tumours (> 500 mm ³) versus SG tumours (< 500 mm ³)			
<i>(a) Marker profiles before castration</i>				
HER1	2.2 → 0.3*			
uPA	0.76 → 0.16*			
TS	0.67 → 0.29*			
5 α -R2	0.07 → 1.2**			
	Markers of late AI progression (QPCR)	Tumours responding to castration (QPCR)	Androgen dependent response (QPCR)	
Marker name	SG versus SG-P	SG versus SG-R	SG-R versus SG-P	
<i>(b) Marker profiles after castration</i>				
HER2	NS	NS	4.5 → 25***	
PAM	NS	5.3 → 2.1*	2.1 → 6.1**	
AM	NS	NS	1.9 → 22***	
TS	NS	0.2 → 0.05**	0.05 → 0.3*	
TK1	NS	21 → 2*	2 → 16*	

NS means nonsignificant and *, **, *** differ between the groups ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively) by student's *t*-test.

Discussion

The LuCaP 23.1 xenograft was chosen for its capacity to retain major clinical hallmarks of human PrCa: heterogeneous growth, PSA production, androgen-responsiveness and AI recurrence after castration. This model permitted us to evaluate different molecular profiles of markers potentially associated with serum PSA levels and tumour volumes. Temporal analysis before castration shows two molecular profiles based on PSA index and TGR. One tumour group was FG while the other was SG, marking the first documentation of two statistically, different growth rates in the LuCaP 23.1 xenograft prior to castration. Previously, Bladou *et al.* (1996) reported that in this model, changes in serum PSA were higher than tumoral volume variations after androgen withdrawal. The same observation is found in this study for FG-P, SG-P and SG-R groups. Upon castration, significantly lower PSA levels were detected indicating that at the time of castration, these tumours were androgen sensitive. Androgen withdrawal induced three different responses: (1) an early AI growth (during the first 5 weeks postcastration; FG-P); (2) late AI growth (between 5 and 15 weeks; SG-P) and (3) prolonged response (SG-R). These results confirm the heterogeneity in tumour response to castration as previously reported (Ellis *et al.*, 1996; Liu *et al.*, 1996).

Tumour growth was correlated with expression of molecular markers linked to established pathways of AI progression including androgen receptor (AR) regulated pathways and metabolism (AR, 5 α -R2), heregulin tyrosine kinase receptors (HER1, HER2), NE markers (PAM, AM), apoptotic response (Bcl-2) and invasive capacity (uPA). While some of these markers did not show statistical differences, several did as a function of the LuCaP 23.1 subpopulations. For example, no difference in AR expression was observed at the end of period 1 in FG and SG groups. These results are

consistent with previous reports by Jenster *et al.* (1999), who report that AR does not show significant differences between clinical stages and hormone-responsiveness in human PrCa. (Jenster *et al.*) It is interesting that all postcastrated FG-P tumours and a subgroup of SG-P tumours were associated with higher AR expression. In accordance with this result, studies have indicated the capacity of PrCa cells to survive in low levels of residual androgens (Jenster *et al.*, 1999). In fact, overexpression or amplification of AR is reported in 30% of AI tumours in response to low androgen levels (Visakorpi *et al.*, 1995; Koivisto *et al.*, 1997). These data show that AR is directly involved with AI PrCa acquisition, supporting data from chromatin immunoprecipitation studies using LNCaP xenograft tumours (Zhang *et al.*, 2003).

However, the 5 α -R2 gene is significantly under-expressed in FG tumours as compared to SG tumours, suggesting a poor differentiation in the FG group. Previously reports have indicated that low expression of 5 α -R2 isoform is associated with overexpression of 5 α -R1 in undifferentiated AI PrCa (Negri-Cesi *et al.*, 1999). Furthermore, Delos *et al.* (1998) demonstrated that overexpression of 5 α -R1 is correlated with lack of 5 α -R2 in AI PrCa cell lines.

FG tumours express significantly higher levels of HER1 survival factor, associated with higher expression of proliferative and cell invasion markers TS and uPA, respectively. At the time of killing, a significant HER1 expression increase was observed in FG-P and a subgroup of SG-P tumours. Previous results have shown that prostatic tumour cells overexpress tumour growth factor α (a ligand of HER1), EGF and HER1 (Turkeri *et al.*, 1994; Dahiya *et al.*, 1996), suggesting the possibility of epithelial autocrine stimulation (Scher *et al.*, 1995). In our study, expression of HER2 was not different in either precastrated FG or SG groups. However, after castration we found a significant increase

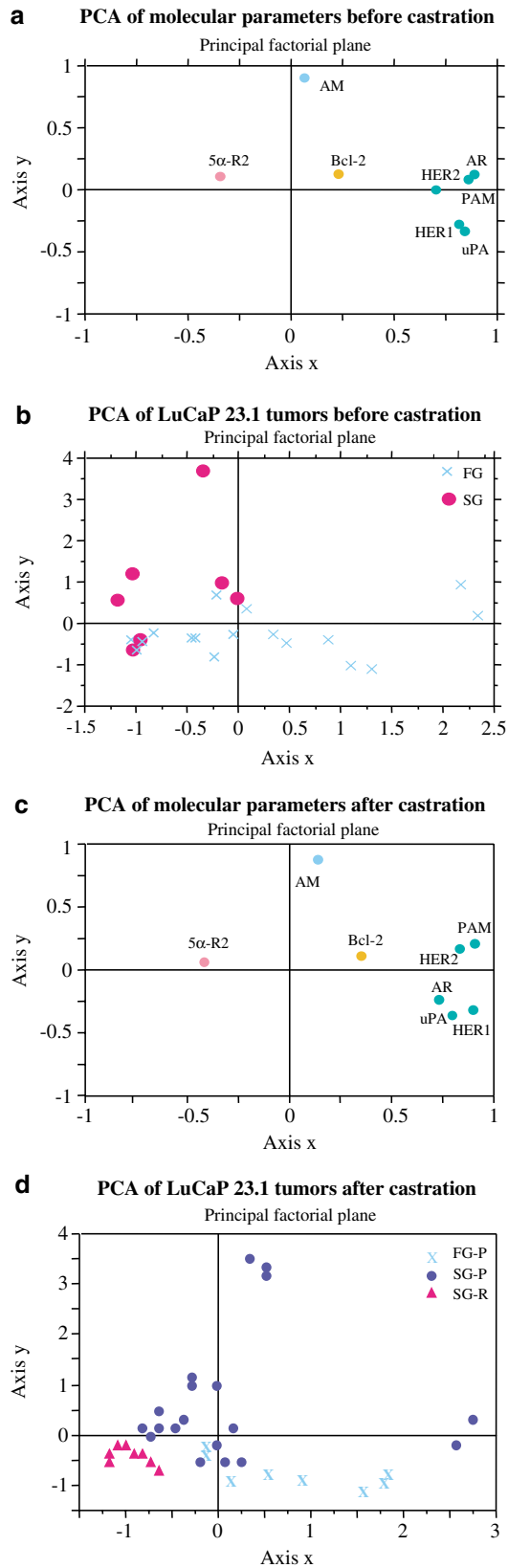


Figure 5 Molecular Analysis of tumours by principal component analysis (PCA) projected along the two orthogonal axes with (a, c) projection of molecular parameters and (b, d) pre- and post-castrated tumours into the principal factorial plan

(by a factor 2) in HER2 expression in FG-P and SG-P subgroups. While there is conflicting results regarding the prognostic significance of HER2 in PrCa (Craft *et al.*, 1999; Scher, 2000) reported an overexpression of HER2 during AI in LAPC human PrCa xenograft. Scher (2000) also described an overexpression of HER2 in metastatic and hormone-refractory PrCa.

PAM and AM expressions have been shown to be related to NE phenotype and AI status in prostate cancer cell lines (Rocchi *et al.*, 2001). In this study, PAM and AM expressions were not found to correlate with tumoral progression before castration, but to correlate with AI growth after castration. By 15 weeks postcastration, FG-P tumours that progress rapidly to AI growth express the highest PAM level. PAM is strongly correlated with HER1 and HER2 expression in FG-P and a subgroup of SG-P tumours, suggesting an intriguing possibility of HER1 regulation by PAM and, therefore, NE activity. Small bioactive neuropeptides like bombesin and endothelin-1 have been shown to stimulate growth and invasiveness of human PrCa through uPA expression (Nelson and Carducci, 2000a, b). Neuropeptide biological activity can be PAM activated and Neutral EndoPetidase 24-11 inactivated (Papandreou *et al.*, 1998; Rocchi *et al.*, 2001). Our report indicates that AM neuropeptide correlates with tumours shown to progress to castration (SG-P). These results are in accordance with other studies showing the role of NE differentiation in AI PrCa progression (Papandreou *et al.*, 1998; Rocchi *et al.*, 2001). Together, these data suggest that AM could serve as a reliable predictor for those tumours that will fail androgen ablation treatment. Interestingly, SG-P tumours advancing slowly to AI growth express the highest level of Bcl-2 mRNA, as compared to FG-P and SG-R tumours. This is suggestive of an antiapoptotic role in response to androgen withdrawal. These results are in accord with previous studies using the LuCaP 23.1 xenograft describing overexpression of Bcl-2 in AI tumours (Liu *et al.*, 1996). We further found that fast growing tumours (FG and FG-P tumours) expressed significantly higher levels of uPA than slow growing tumours (SG and SG-P/R). Related to this, other studies have shown castration-induced upregulation of uPA, emphasizing the potential role of serine protease family in the acquisition of hormone refractory PrCa (Lyon *et al.*, 1995; Xing and Rabbani, 1999; McGowen *et al.*, 2000). HER1 overexpression was associated both with FG and FG-P tumours and, therefore, could serve as predictive markers for early AI progression, possibly meriting testing in human translational research. Androgen-independent FG-P tumours and a subgroup of SG-P tumours overexpress HER1, HER2 and AR, highlighting the potential cross-talk between the AR and tyrosine kinase signal transduction pathways, possibly providing a mechanism contributing to AI PSA production (Culig *et al.*, 1994). Angiogenic pathways could also be involved in AI PC progression (Huss *et al.*, 2001). Buttyan *et al.* (2000) have shown that castration induces a significant reduction of prostatic blood

flow with a large loss of epithelia due to hypoxic conditions. Our study showed that a subgroup of SG-P tumours is linked with a significant overexpression of a hypoxic-related gene, AM, after castration. Interestingly, AM contains hypoxia inducible factor-1 (HIF-1) response element in its promoter region (Zudaire *et al.*, 2003).

Our work indicates that growth of PrCa tumour subpopulations can display statistically significant changes in expression of markers representative of androgen-regulated signalling pathways. The LuCaP 23.1 xenograft is a useful model to study how malignant cells can hijack the endocrine system and develop alternative prosurvival signalling pathways. The motivation for studying the heterogeneous growth patterns present in the LuCaP 23.1 model is in identification of valid therapeutic options for treatment of individualistic forms of AI PrCa. As a result, translational clinical research is in progress in order to evaluate expression of HER1, HER2, AR, 5 α -R2, PAM, AM, TK1, TK2, TS, uPA and Bcl-2 at different stages of human PrCa and correlate biological patterns with the castration response. Given that cancer is a heterogeneous disease, evaluating molecular profiles of signature genes associated with castration and AI has the potential to reveal novel targets for therapeutic intervention. While correlative in nature, our study promotes the notion that an understanding that identification of gene clusters and their interactions, may facilitate the determination of which tumours will fail androgen ablation therapy and which will respond.

Materials and methods

Animal protocol

balb/cByJ-nu/nu athymic male mice (5 weeks old) were obtained from IFFA CREDO (L'Abresle, France) and housed in sterile cages with laminar flow hoods in a temperature-controlled room with a 12 h light/12 h dark schedule. Animals were fed autoclaved chow and water *ad libitum*. Institutional guidelines for the proper and human use of animals in research were followed. LuCaP 23.1 xenograft samples were obtained from a series of mice previously xenografted at Dr Robert Vessella's Laboratory, Department of Urology, University of Washington, Seattle. LuCaP 23.1 xenografts were maintained by successive passages (14–20), as previously described (Ellis *et al.*, 1996). Animals ($n = 100$) were ectopically implanted over the right shoulder with LuCaP xenograft tumours (volume implanted, $V_i = 25 \text{ mm}^3$) under general anaesthesia.

LuCaP 23.1 xenograft growth analysis was based on two time points including before castration (11 weeks) (period 1) and after castration (15 weeks) (period 2). At 11 weeks postimplantation, 42 mice were castrated and 24 were killed by neck dislocation after blood sampling (100 μl). Tumours were removed, snap-frozen in liquid nitrogen and stored at -80°C . Postcastration tumour responses were observed by measuring weekly tumour volumes and PSA levels for 15 weeks. At the end of the second period (11 weeks postimplantation and 15 weeks postcastration), all animals were killed.

Tumour volume analysis

Volumes were calculated using the formula of an ellipsoid (volume = length \times width \times height \times 0.5236). ($V = L \times l \times h$) expressed in mm^3 (Janik *et al.*, 1975). We defined implanted tumour volume as V_i , tumour volume at the time of castration as V_c and tumour volume at killing time as V_s . For each period, TGR was calculated with the formula: %TGR = $V_{\text{end}}/V_{\text{start}}$ (Landström *et al.*, 1994). TGR was defined as V_c/V_i for period 1 (precastration) and V_s/V_c for period 2 (postcastration period).

PSA index determination

PSA determination was performed weekly for the two constant periods in all mice. Blood samples were obtained (100 μl) by tail vein bleeding, and serum was recovered and stored at -20°C . Serum PSA concentration was measured using the automated IMX immunoassay system (Abbott, France). The PSA index was defined as the PSA secretion in serum per unit of tumoral volume (ng/ml/mm^3).

Tumour growth classification during the first period (implantation to castration)

From a constant implanted volume (25 mm^3), tumours were classified as FG tumours if they reached a minimal size of 500 mm^3 and as SG tumours if they reached a size less than 500 mm^3 at the end of the first constant period.

Response to castration during the 2nd period of analysis (15 weeks from castration to killing)

After castration, tumours were defined as responders (R) if their PSA nadir value was less than 100 ng/ml with a decreasing or stable volume. Similarly, tumours were classified as progressive (P) if serum PSA increased above nadir levels with stable or increased tumour volume.

Quantitative RT-PCR analysis

Total RNA was extracted from LuCaP 23.1 xenografts as previously described (Chomczynski and Sacchi, 1987). In total, $2.5 \mu\text{g}$ of total RNA was treated with 30 U ribonuclease-free deoxyribonuclease I (Roche Molecular Biochemical, Germany). RNA was then reverse-transcribed into cDNA as previously described (Rocchi *et al.*, 2001). Amplification of the cDNA was performed using fluorogenic probes for PAM, AM, HER1, HER2, TK1, TK2, TS, uPA, RA, 5 α -R2, Bcl-2 and 18S with the experimental conditions previously described (Rocchi *et al.*, 2001). For each unknown sample, we determined the fluorescent signal (ΔRQ) values for all genes and the results were expressed as fg of each gene per ng of 18S. Each value was expressed in PCR units (QPCR). Primer sequences and Probes for RT-PCR are, respectively, listed for each gene as follows. AR: 5'tga gca gag tgc cct atc cc3', 5'aaa cat ggt ccc tgg cag tc3', 5'cca ctt gtg tca aaa gcg aaa tgg gc3'; AM: 5'tgc cca gac cct tat tgc g3', 5'agt tgt tca tgc tct ggc gg3', 5'aca tga agg gtg cct ctg gaa gcc c3'; 5 α -R2: 5'gaa tgg atc ggc tat gcc ct3', 5'tga ggt aga acc tat ggt ggt gaa3', 5'ttt ctg act ttg ttt cct tgg gct cgg3'; TK1: 5'cca gat tgc tca gta caa gtg c3', 5'tcg cag aac tcc atg atg tc3', 5'ttc tgc aca cat gac cgg aac acc at3'; TK2: 5'gta cca cga tgc ctg ct3', 5'tca acc gta cag atg aca cct ga3', 5'cag ctg acc atg ctg gac agg cat a3'; HER1: 5'ggg aga aaa caa cac cct ggt3', 5'cat tgc ttg gac agc ctt ca3', 5'tgg aag tac gca gac gcc ggc3'; HER2: 5'gct ccc cat atg tct ccc g3', 5'cat ggt tgg gac tct tga cca3', 5'tgc ctg aca tcc acg gtg cag c3'; TS: 5'aga tcc aac aca tcc tcc gc3', 5'cac gtt tgg ttg tca gca gag3', 5'acc ctg tgc gta ttc

ggc atg cag3'; PAM: 5'cac tga ttg gac ggc aga g3', 5'cat cac tag acg tgc cac ca3', 5'ttt tgg tga cct act ggc tgc aa3'; uPa: 5'ctt aac tcc aac acg caa ggg g3', 5'agc ttg tgc caa act ggg gat c3', 5'cac gct tgc tca cca caa cga cat tg3'; Bcl2: 5'agg agc tct tca ggg acg g3', 5'tgc ctg aca tcc acg gtg cag c3', 5'tgc ctg aca tcc acg gtg cag c3'; 18S: 5'cta cca cat cca agg aag gca3', 5'ttt ttc gtc act acc tcc ccg3', 5'cgc gca aat tac cca ctc ccg ac3'.

Statistical analysis

TGR and PSA index were expressed as the mean \pm s.e.m. while RT-PCR results were analysed by Student's *t*-test (Statview 512, Brain Power Inc., CA, USA). $P \leq 0.05$ was considered significant (*); $P \leq 0.01$ (**); $P \leq 0.001$ (***) (NS (nonsignificant)).

Principal component analysis (PCA)

PCA is commonly used to reduce one multicomponent analysis into a geometrical interpretation (Joliffe and Morgan, 1992). In the first plane are projected biological parameters and LuCaP 23.1 tumours are projected into the second plane. PCA reduces the complex information in order to facilitate biological interpretation. Proximity between the parameters relates a statistical significant correlation. Proximity between the tumours means that they express the same parameters. Proximity between the tumour and the parameter means that the biological parameter is highly expressed in the tumour. All

the values are projected close together or in the opposite way with a maximum alignment into the principal plane, whether the correlation between the values is strongly positive or negative, respectively. If the value is well represented into the first principal plane, it is projected close to a circle of unit radius; otherwise it is represented close to the origin of the axis. Statistical calculations were carried out by using Statview 512 (Brain Power Inc., CA, USA).

Abbreviations

AI, androgen independent; AM, adrenomedullin; FG, fast growing; FG-P, fast growing/progressing; 5 α -R2, 5 alpha-reductase 2; PAM, peptidyl α -amidating monooxygenase; PrCa, prostate cancer; SG, slow growing; SG-P, slow growing/progressing; SG-R, slow growing/responding; TK, thymidine kinase; TS, thymidilate synthetase; uPA, urokinase plasminogen activator.

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