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ORIGINAL ARTICLE

A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes

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GATA-2, a member of the GATA family of transcription factors, is involved in androgen receptor (AR) signaling, however, little is known regarding its role in prostate cancer. Here, we report that GATA-2 is expressed in a substantial proportion of prostate cancers and that high expression of GATA-2 is associated with biochemical recurrence and distant metastatic progression in a validation set of 203 cancers. *In vitro* data show that GATA-2 is directly recruited to the promoter region of the AR upon androgen stimulation of LNCaP prostate cancer cells with 5α-dihydroxytestosterone (DHT) for 24 h. Ectopic GATA-2 expression causes the induction of AR transcript levels under androgen-depleted conditions ($P<0.05$). The expression of the AR target gene, *AZGP1*, is induced upon androgen stimulation and this effect is repressed by GATA-2. In contrast, GATA-2 significantly increases transcript levels of KLK2, which increases further in a time-dependent manner on DHT treatment and in the presence of GATA-2. These results indicate that upregulation of GATA-2 may contribute to the progression to aggressive prostate cancer through modulation of expression of AR and key androgen-regulated genes, one of which, *AZGP1*, is associated with the progression to metastatic disease.
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Introduction

Prostate cancer is the most commonly occurring cancer in men in western countries. Prostate cancer development and progression is highly dependent on androgen signaling, and the androgen receptor (AR) has a critical role in both localized androgen-dependent disease and advanced hormone-refractory prostate cancer (Mohler *et al*., 2004).

The AR, a member of the nuclear receptor superfamily, translocates to the nucleus on hormone binding, where it binds to specific androgen response elements (AREs) (Riegman *et al*., 1991). Together with co-regulators, AR controls transcription of specific target genes (Kato *et al*., 2004; Dehm and Tindall, 2006). Although several AR target genes, such as *PSA* (Borgono *et al*., 2004) and *TMPRSS2* (Lin *et al*., 1999), have been implicated in the growth and differentiation of prostate cancer cells, relatively little is known about the function of AR target genes in driving tumor progression.

Recently, it has been shown that the transcription factors, Oct-1 and GATA-2, have an essential role in the recruitment of AR to chromatin (Wang *et al*., 2007b). Both GATA and Oct DNA-binding motifs are significantly enriched within AR-binding regions in the human genome and the presence of AREs near GATA motifs is a strong predictor of the ability of AR to bind to a specific ARE (Perez-Stable *et al*., 2000; Masuda *et al*., 2005).

The fact that GATA-2 is bound to many AR-binding regions and that the amount bound increases in response to androgen (Wang *et al*., 2007b) suggests a potential role for GATA-2 in prostate cancer progression. GATA-2 belongs to the family of GATA transcription factors, which are important in the regulation of numerous genes and are expressed in a variety of tissues during development (Simon, 1995). The vertebrate GATA proteins are subdivided into two groups, based on sequence homology and expression pattern (Lowry and Atchley, 2000). The first subgroup (GATA-1/-2/-3) is essential for various aspects of hematopoiesis (Weiss and Orkin, 1995), whereas the second group (GATA-4/-5/-6) has been described in the endoderm and cardiovascular development (Nemer and Nemer, 2003). GATA-2 and -3 are the predominant GATA family members expressed in human prostate, but only GATA-2 shows expression in androgen-responsive LNCaP cells (Perez-Stable *et al*., 2000).

In this study, we investigate the role of GATA-2 in prostate cancer. We present results that support a role for GATA-2 in aggressive prostate cancer and show that GATA-2, though not itself regulated by androgen, can influence the regulation of androgen-dependent genes, such as *KLK2* and *AZGP1*.

Results

GATA-2 as a marker of aggressive prostate cancer

Two published data sets (Henshall *et al*., 2003; Rasiah *et al*., 2006) that assessed gene expression in localized

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prostate cancers were analysed using Ingenuity Pathway Analysis (IPA) to extract novel genes that are associated with prostate cancer progression. These data sets comprised genes whose differential expression is predictive of prostate cancer recurrence (Henshall *et al*., 2003), or genes that are up- or downregulated in prostate cancer versus benign prostate tissue (Rasiah *et al*., 2006). Genes that were common to both data sets were used for the analysis, which identified 45 networks comprising 883 genes. By examining related functions and disease, a tool of the Ingenuity Pathway core Analysis, within the 883 identified genes and then refining the gene list to focus on known cancer-related genes, we were able to build a new pathway of functionally related genes. This pathway was comprised of the genes, *GATA-2*, *TFF1*, *BMP7*, *PIK3R1*, *PBX1* and *DAPK1*, which were associated with either risk or protection from prostate cancer recurrence (Table 1). The expression of the transcription factor, GATA-2, was associated with the highest risk of recurrence (hazard ratio = 2.65, 95% confidence interval = 1.57–4.49, $P < 0.01$) within this group of cancer-related genes.

This finding and the published report that GATA-2 transcripts are present in prostate cancer cell lines (Perez-Stable *et al*., 2000) led us to validate the association of GATA-2 expression with prostate cancer recurrence in an independent set of prostate cancer patients with known outcome. Immunohistochemical expression analysis of radical prostatectomy specimens showed a nuclear staining pattern for GATA-2 in prostate cancer epithelium. Of the 203 cancers analysed, 33 (16.3%) showed no staining. Of the patients with positive GATA-2 immunostaining, 61 patients (30%) showed weak (1+) staining, 78 (38.4%) showed moderate (2+) staining and 31 patients (15.3%) showed strong (3+) GATA-2 staining (Figure 1a). GATA-2 staining intensities were not correlated to Gleason scores and pre-operative PSA (data not shown). Hence, these data validate our finding that GATA-2 is expressed at high levels in a proportion of prostate cancers.

Kaplan–Meier estimates of cumulative recurrence-free survival were derived for prostate cancer patients whose radical prostatectomy specimens exhibited strong staining (3+) compared with those for which GATA-2 staining was absent, weak or moderate (0, 1+ and 2+, respectively). Strong GATA-2 expression was associated with biochemical (PSA) recurrence (hazard ratio = 1.69, 95% confidence interval = 1.02–2.80, $P = 0.043$ (Figure 1b). We also carried out similar statistical analyses for the outcome of distant metastatic progression. We found that strong GATA-2 expression was associated with a higher risk of distant metastatic progression (hazard ratio = 3.00, 95% confidence interval = 1.00–8.94, $P = 0.0493$) (Figure 1c). Hence, high levels of GATA-2 are associated with aggressive disease in a validation set of prostate cancers.

Table 1 Association with recurrence for the cancer-related genes in a network identified by Ingenuity Pathway Analysis

Gene name	*HR (95% CIs)*	P
GATA-2	2.65 (1.57–4.49)	<0.01
TFF1	1.31 (1.07–1.61)	<0.01
BMP7	1.41 (1.15–1.75)	<0.01
DAPK1	0.38 (0.20–0.70)	<0.01
PBX1	2.03 (1.08–3.84)	<0.01
PIK3R1	0.42 (0.23–0.75)	<0.01

Abbreviations: HR, hazard ratio; CIs, confidence intervals.

High GATA-2 expression is not associated with GATA-2 mutations

Recent studies indicate that GATA-3 has a high frequency of mutations in a subset of estrogen receptor-α-positive breast cancers (Usary *et al*., 2004). We hypothesized that GATA-2 may be similarly mutated in prostate cancer. Therefore, we assessed the frequency of mutations in the zinc-finger domain 1, the zinc-finger domain 2 and an AR-binding site in the untranslated region downstream of the transcription start site of *GATA-2* (Supplementary Figure S1) in patients with strong GATA-2 expression ($n = 10$) compared with a control group ($n = 10$), which showed no detectable GATA-2 expression by immunohistochemistry. Using site-specific PCR and direct sequencing, we did not detect any heterozygote or homozygote mutation within any of these regions in the strong expressing patient cohort compared with the control patients, suggesting that high GATA-2 expression is not correlated with a high frequency of mutations in prostate cancer.

GATA-2 binds directly to the AR promoter

The sites for AR recruitment are selectively enriched in GATA DNA-binding motifs (Wang *et al*., 2007b). Analysis of the promoter region of AR (Supplementary Figure S2) using the software programs Gene2promoter and MatInspector within the Genomatix portal predicted three potential GATA-2-binding sites downstream of the transcription start site (Figure 2a).

On the basis of these results, we next assessed whether GATA-2 is recruited directly to the promoter region of the AR. We therefore carried out quantitative chromatin immunoprecipitation (ChIP) assays using a GATA-2 antibody or IgG. Furthermore, we tested whether increasing androgen levels can influence the level of recruitment of GATA-2 to the AR promoter. For these experiments, we used the prostate cancer cell line, LNCaP, an established model for examining the effects of androgens after hormone depletion (Wang *et al*., 2007a, b). These cells express endogenous AR (Horoszewicz *et al*., 1980) and low levels of GATA-2 (Perez-Stable *et al*., 2000), which we confirmed independently by evaluating GATA-2 mRNA and protein levels (data not shown). The PSA enhancer was used as a positive control as it has been reported previously that GATA-2 binds to GATA motifs within this region (Perez-Stable *et al*., 2000), and that expression of the PSA enhancer increases in a time-dependent manner on androgen stimulation (Wang *et al*., 2005).

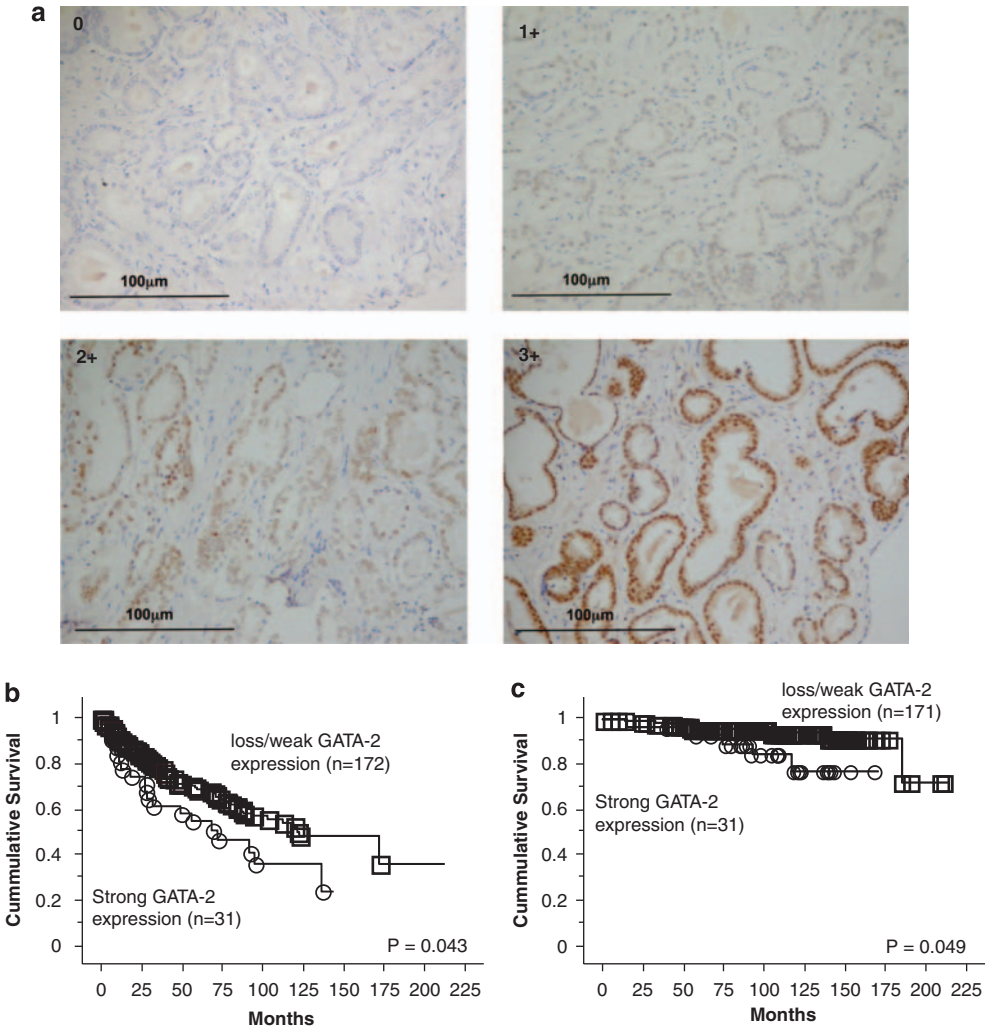

Figure 1 Association of GATA-2 with prostate cancer outcome. (**a**) Images of paraffin-embedded prostate tissue obtained during radical prostatectomy and immunostained for GATA-2 showing the spectrum of staining in this study; Gleason grade 2 prostate cancer showing complete loss of GATA-2 immunostaining; Gleason grade 3 prostate cancer showing 1+ intensity immunostaining; Gleason grade 4 prostate cancer showing 2+ intensity immunostaining; and Gleason grade 3 prostate cancer showing 3+ intensity immunostaining. Original magnification ×200. (**b**) Kaplan–Meier survival curve showing biochemical recurrence for patients showing low or no GATA-2 expression (0, 1+ and 2+) versus patients showing high (3+) GATA-2 expression. (**c**) Kaplan–Meier survival curve showing outcome of distant metastatic progression for patients with low or no GATA-2 expression (0, 1+ and 2+) versus patients showing high (3+) GATA-2 expression. One patient was lost in follow-up.

Our results confirmed that GATA-2 protein is bound directly to the PSA enhancer region, but minimal binding of GATA-2 to the AR promoter in hormone-free conditions was observed (Figure 2b). We next treated the cells with 100 nm 5α-dihydroxytestosterone (DHT) for 12 and 24 h to assess the recruitment of GATA-2 to the AR promoter and PSA enhancer in the presence of high levels of AR (Wang *et al*., 2005). GATA-2 recruitment to the PSA enhancer was enhanced on androgen stimulation and GATA-2 recruitment to the AR promoter increased in a time-dependent manner with a substantial increase in binding observed at 24 h after DHT treatment. Hence, GATA-2 is recruited directly to the AR promoter and therefore implicates a role in the transcriptional control of AR.

GATA-2 expression causes upregulation of AR mRNA and protein levels

On the basis of the ChIP results, we next examined whether GATA-2 expression influences AR expression levels in the presence or absence of androgens. For these experiments, we established LNCaP cells expressing tetracycline-inducible GATA-2. Lower levels of endogenous GATA-2 were detected in cells not treated with doxycycline, DHT or in the control cell line that was established with the vector alone, and the induction of GATA-2 occurred within 24 h after doxycycline treatment (Figure 3a). Androgen stimulation showed no effect on GATA-2 mRNA and protein expression levels in the established cell lines (Figures 3b and c). No change in cell growth in the GATA-2-expressing cell line was observed (data not shown).

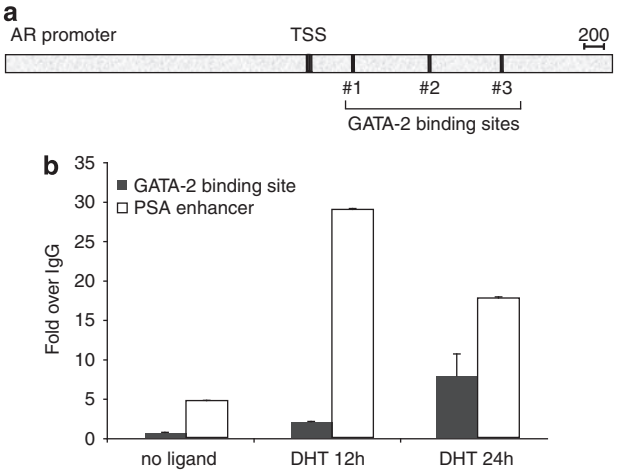

Figure 2 GATA-2 is directly recruited to the androgen receptor (AR) promoter region. (**a**) Schematic representation of predicted GATA-2-binding sites within the AR promoter region (TSS, transcription start site). (**b**) LNCaP cells were cultured in hormone-depleted medium for 48 h and then treated with 5α-dihydroxytestosterone (DHT) for 12 and 24 h. Chromatin immunoprecipitation analysis was carried out with indicated antibodies or control IgG. DNA precipitates were analysed by real-time PCR using primer spanning the PSA enhancer and indicated GATA-2-binding region within the AR promoter. The data were normalized against the input control. Normalized data of two independent experiments were averaged and represented as the fold over IgG control with error bars representing s.d.

Induction of GATA-2 resulted in a significant (two-fold, $P<0.05$) increase of AR mRNA and protein expression when compared with the control cell line or when cells were not treated with doxycycline. Androgen stimulation did not alter these effects on AR protein levels (Figure 3c) but had an inhibitory effect on mRNA levels within 12 h when compared with doxycycline-treated cells ($P<0.005$) (Figure 3b). This inhibitory effect was partly released after 24 h treatment with DHT ($P<0.05$). No significant change between GATA-2 expressing and control cells was observed on 12 and 24 h of treatment with DHT. Hence, GATA-2 protein expression stimulates AR mRNA expression levels, indicating that AR is a target gene of GATA-2.

GATA-2 effects the expression of the AR target genes, AZGP1 *and* KLK2

AZGP1 is a strong predictor of outcome in prostate cancer (Henshall *et al*., 2006) and its expression is increased in differentiated adipocytes (Bing *et al*., 2004) at a time point when GATA-2 expression is switched off. In addition, low or absent AZGP1 expression correlates with prostate cancer progression (Henshall *et al*., 2006), which is an opposite effect to GATA-2 expression as reported here. A recent study reported AZGP1 mRNA expression in LNCaP cells and the presence of putative AREs within the AZGP1 promoter region (Nelson *et al*., 2002). Hence, we first confirmed that AZGP1 expression levels are altered by androgen stimulation. Using quantitative real-time PCR, we could show that AZGP1 mRNA levels are increased in LNCaP cells in a time-dependent manner on treatment with DHT (Figure 4a).

We next assessed the effect of GATA-2 expression on AZGP1 mRNA levels in LNCaP cells expressing inducible GATA-2. High levels of GATA-2 had no significant effect on relative AZGP1 mRNA levels compared with the control cell line or cells not treated with doxycycline, although there was a trend to decreased AZGP1 levels (Figure 4b). Subsequently, we examined whether androgen stimulation of cells expressing high levels of GATA-2 had an effect on AZGP1 mRNA expression. Control cells showed an increase in AZGP1 expression on DHT treatment as shown in previous experiments ($P<0.05$). Surprisingly GATA-2-overexpressing cells showed a reduced induction of AZGP1 mRNA expression on androgen stimulation.

Furthermore, we examined the effects GATA-2 might have on the known AR target gene, *KLK2*. *KLK2* has been used for the detection of prostate cancer (Young *et al*., 1992) and it has been reported that KLK2 mRNA and protein expression levels increase on androgen stimulation (Wang *et al*., 2006). Induction of GATA-2 expression under hormone-depleted conditions resulted in a significant increase in KLK2 transcript levels when compared with the control cell line ($P<0.005$) or cells not treated with doxycycline (Figure 4b). Androgen stimulation caused an even further stimulation of KLK2 expression when combined with GATA-2 overexpression compared with cells just expressing basal GATA-2 levels.

Taken together, these results indicate that AZGP1 is androgen-dependent, and expression on androgen stimulation is repressed by GATA-2. In contrast, KLK2 transcript levels are increased because of GATA-2 expression under androgen-depleted conditions. Hence, GATA-2 can have either inhibitory or stimulatory effects on AR target gene expression.

Discussion

Despite the importance of the AR signaling cascade in prostate cancer, the precise mechanism of androgen action remains unclear. Here, we report that GATA-2 is directly recruited to the promoter region of the AR on androgen stimulation. We show also that ectopic GATA-2 expression causes the induction of *KLK2*, a known AR target gene, and AR transcript levels under androgen-depleted conditions. The presence of high levels of GATA-2 enhances the increase of KLK2 mRNA after androgen treatment. In contrast, although the expression of the androgen-dependent gene, *AZGP1*, is induced on androgen stimulation, this effect is repressed by GATA-2.

Androgen-mediated gene expression involves the binding of hormone to AR, which results in its nuclear translocation and binding to specific AREs in regulatory regions of downstream target genes leading to gene transcription (Roche *et al*., 1992). Although our data are consistent with a role for GATA-2 as a cofactor

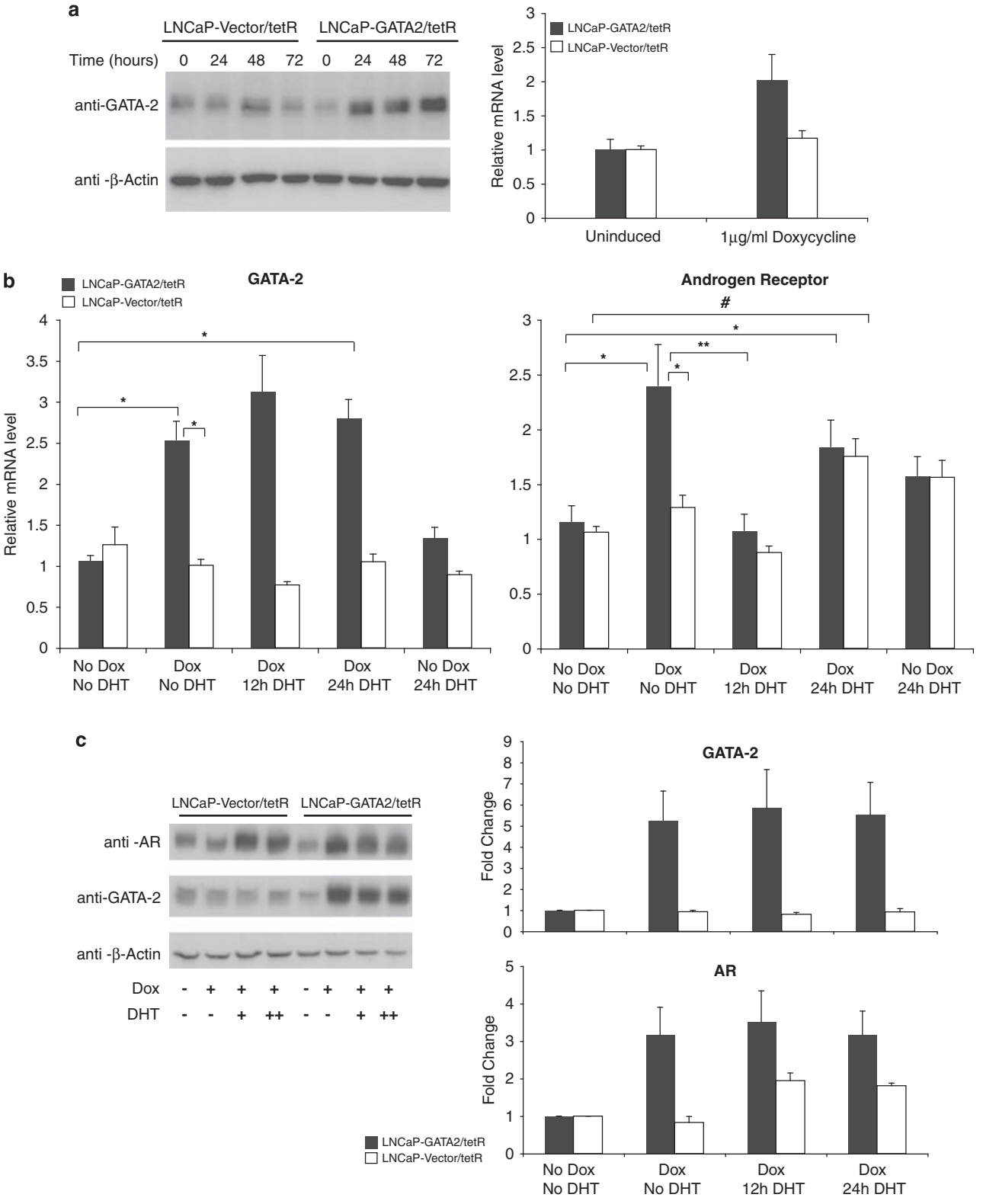

Figure 3 GATA-2 expression in the prostate cancer cell LNCaP. (**a**) LNCaP cells were stably transfected with either pcDNA4/TO-GATA-2-HA or empty vector. GATA-2 expression was induced with 1 μg/ml doxycycline for 24, 48 or 72 h and presented as immunoblot and relative mRNA levels using indicated antibodies and primer. (**b**) LNCaP cells were cultured in hormone-depleted medium for 48 h and then treated with 5α-dihydroxytestosterone (DHT) for 12 and 24 h. cDNA were analysed by real-time PCR using indicated primer GATA-2, an androgen receptor (AR) (*$P<0.05$, **$P<0.005$, $^{\#}P=0.052$). Results are shown as one representative of three individual experiments with error bars representing s.d. (**c**) Immunoblot and densitometric analyses of GATA-2 and AR protein levels after induction of GATA-2 and DHT treatment. Results are shown as the mean of the fold change of three individual experiments with error bars representing s.d.

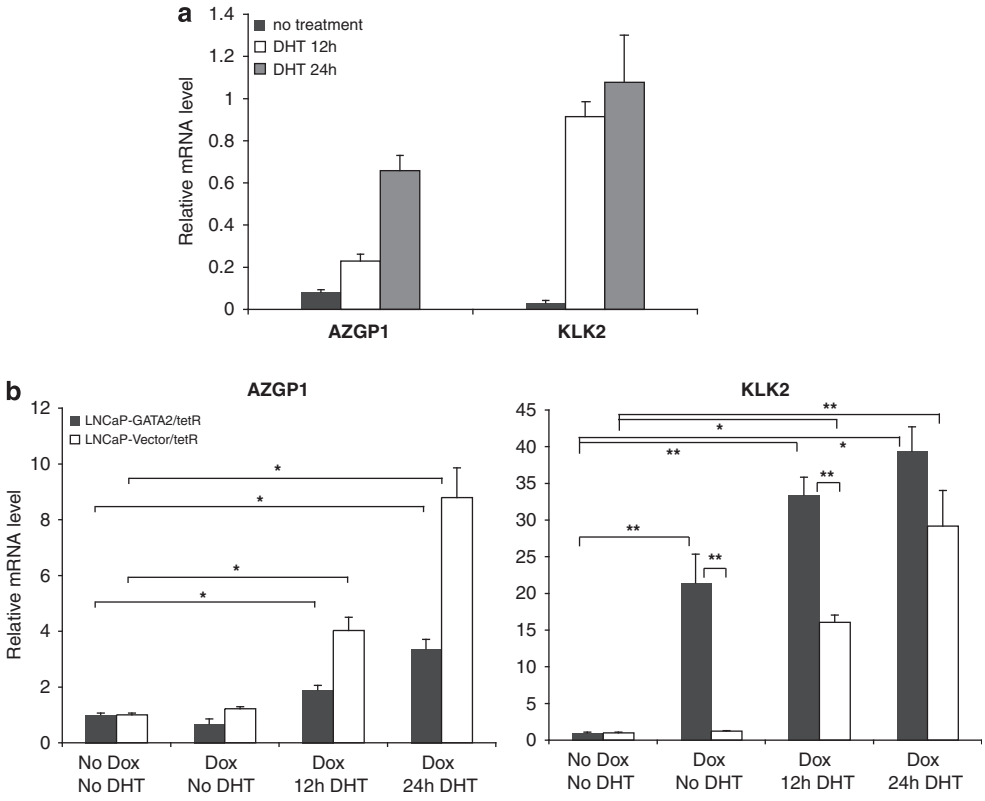

Figure 4 AZGP1 stimulation by androgen. (**a**) LNCaP cells were cultured in hormone-depleted medium for 48 h and then treated with 5α-dihydroxytestosterone (DHT) for 12 and 24 h. cDNA was analysed by real-time PCR using indicated primer of AZGP1 and KLK2. (**b**) Stable expressing GATA-2 or vector cells were cultured in hormone-depleted medium for 48 h and then treated with DHT for 12 and 24 h. cDNA was analysed by real-time PCR using indicated primer of AZGP1 and KLK2 (*$P<0.05$, **$P<0.005$). Results are shown as one representative of three individual experiments with error bars representing s.d.

supporting AR-activated transcription, our finding that the significant increase in KLK2 and AR expression is enhanced by high GATA-2 levels in the absence of androgens, indicates that GATA-2 is involved in a novel androgen independent mechanism of transcriptional activation of AR-regulated genes. A role for GATA-2 in the mediation of AR-regulated genes is supported by a recent study that shows that GATA-2 co-operates with the transcriptional cofactor, Oct1, in the regulation of the androgen target genes, *PSA* and *TMPRSS2*, with GATA-2 likely to act upstream of Oct1 recruitment (Wang *et al*., 2007b). Besides Oct1, the cofactor CBP is downregulated on androgen treatment of LNCaP prostate cancer cells (Heemers *et al*., 2009). Future studies would need to assess the functional role of CBP in mediating AR-dependent transcription, as well as the identification of new cofactors of GATA-2 in a genome-wide screen for GATA-2-binding sites.

Recruitment of GATA-2 to the AR promoter region supports the idea that GATA-2 is regulating AR target genes through transcriptional control of AR, which is further supported by GATA-2-mediated upregulation of AR mRNA expression. In recent years, a growing number of studies show a functional relationship between steroid hormone receptors and members of the GATA family (Eeckhoute *et al*., 2007; Magklara and Smith, 2009). For example, the role of the transcription factor, GATA-3, in estradiol signaling requires a direct positive regulation of estrogen receptor-α by GATA-3 itself (Eeckhoute *et al*., 2007). Furthermore, a synergism between the progesterone receptor (PR) and GATA-2 has recently been described, in which PR, GATA-2 and their associated co-regulators participate in a complex to stabilize DNA looping to promote transcription of PR-regulated genes (Magklara and Smith, 2009).

Here, we show that GATA-2 modulates key androgen-regulated genes. It is possible, as described for GATA-3 in estradiol signaling, that the role of GATA-2 in mediating AR target genes requires a direct positive regulation of AR. This would explain AR transcript upregulation caused by GATA-2. This hypothesis is strengthened by the repressive effect GATA-2 has on AZGP1 expression on androgen stimulation, as well as the fact that we did not identify GATA-2 motifs within the promoter region of AZGP1 (data not presented).

Using the analysis tool, Ingenuity Pathway Analysis, we found GATA-2 to be within a novel network of cancer-related genes, in which GATA-2 is associated with a high risk of recurrence. We report for the first time that GATA-2 is expressed in the malignant epithelium in a significant proportion of prostate cancers. High expression levels of GATA-2 are associated with biochemical recurrence and distant metastatic progression. As only 15% of patients showed

strong GATA-2 staining, it is probable that there are other factors contributing to progression in an AR-depleted environment. However, this does not discount the potential contribution of GATA-2 to regulate AR target genes with known roles in metastatic prostate cancer. The putative ability of GATA-2 to regulate the expression of AR target genes in a minimal or depleted androgen environment has major implications in clinical prostate cancer, particularly in advanced disease, where the majority of patients with metastatic disease are treated with androgen-ablation therapies. The result is commonly the growth of a population of prostate cancer cells that have adapted to a low androgen environment and continue to depend on signaling through the AR to proliferate. Although altered AR signaling in these cells is proposed to occur through several mechanisms that include changes in expression of coactivators and corepressors, the data presented here support a putative role for changes in GATA-2 expression in altered AR signaling. This conclusion is supported by data from a prostate cancer xenograft model of androgen-independent disease that suggest a trend of increasing GATA-2 expression levels from an androgen-dependent to an androgen-independent status (Hendriksen *et al*., 2006). Future study would need to assess GATA-2 levels in human prostate cancer metastases and to confirm a functional role for GATA-2 in a model of androgen-independent disease.

Published data evaluating AZGP1 expression in the same patient cohort, showed that low or absent AZGP1 expression is predictive of metastastic spread of prostate cancer (Henshall *et al*., 2006). Hence, an inverse relationship between AZGP1 and GATA-2 expression seems to exist in patients with aggressive disease, and is replicated in our *in vitro* studies, in which ectopic GATA-2 expression was shown to repress androgen-mediated induction of AZGP1 in LNCaP cells. Interestingly, the putative complementarity between AZGP1 and GATA-2 expression described here is analogous to the process of adipocyte differentiation, in which published data support GATA-2 being a key driver of dedifferentiation. During pre-adipocyte to adipocyte transition induced by peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists, levels of GATA-2 are reduced with a concomitant increase in the levels of AZGP1 and PPAR-γ (Tong *et al*., 2000; Bao *et al*., 2005). The pro-differentiating effects of PPAR-γ agonists in primary prostate cells and cell lines are well documented (Xu *et al*., 2003). However, the mechanism by which PPAR-γ induces differentiation in these cells is unknown (Matsuyama and Yoshimura, 2008). Future studies would need to establish the ability of PPAR-γ to regulate GATA-2 and AZGP1 in prostate cancer and a potential role in the dedifferentiation of prostate cancer cells to an aggressive phenotype. Moreover, the ability of GATA-2 and AZGP1 to act in concert as important drivers of metastases with other genes known to be associated with prostate metastasis should be investigated.

In breast cancer, GATA-3 has been reported to have a high frequency of mutations in a subset of estrogen receptor-α-positive tumors (Usary *et al*., 2004), although the link between GATA-3 mutation and high GATA-3 expression remains speculative. Although we hypothesized that an association exists between high GATA-2 expression and mutations, here we show that GATA-2 upregulation is not correlated to mutations. Nothing is known so far about the transcriptional regulation of GATA-2 in prostate cancer, which results in upregulation of GATA-2 in specific cases. One possible mechanism could be changes in signaling pathways in aggressive prostate cancer cells that converge on GATA-2. Although we suspect the PPAR-γ pathway as one possible upstream modulator of GATA-2 function, previous reports show that in zebrafish, the GATA-2 promoter is transcriptionally activated through BMP signaling, a key regulator of metastatic spread in prostate cancer cells (Oren *et al*., 2005). A second possible mechanism could involve the alterations in GATA-2 protein turnover. In hematopoietic cells, the GATA-2 protein degrades rapidly. This degradation is regulated through the ubiquitination-proteasome pathway (Minegishi *et al*., 2005). Patients with aggressive prostate cancer could harbor an alteration within the ubiquitination-proteasome pathway that causes upregulation of GATA-2. Thirdly, post-transcriptional modification such as acetylation may have an important role in the regulation of GATA-2 and its function in prostate cancer, as *in vitro* studies indicate that acetylation influences GATA-2 DNA-binding activity and transactivating potential (Hayakawa *et al*., 2004). As we were not able to show GATA-2 acetylation in LNCaP cells (data not shown), future studies would need to investigate the potential for acetylation to mediate GATA-2 function *in vivo*.

In summary, high GATA-2 expression is predictive of poor outcome in prostate cancer and modulates the expression of key androgen-regulated genes that have potential roles in the transition of prostate cancer cells to an aggressive phenotype. The ability of GATA-2 to influence putative regulators of prostate cancer proliferation and differentiation, such as AZGP1, now requires further investigation to determine the feasibility of targeting GATA-2 as part of a novel therapeutic approach to this disease.

Materials and methods

Patient cohort and tumor samples

The clinical and pathological characteristics of patients were as previously described (Henshall *et al*., 2006). Briefly, the patient cohort consisted of 203 patients who had radical prostatectomy carried out between 1988 and 1998 (median of 1995). Each patient case was represented by a mean of three 1-mm tissue core biopsies taken from each of 203 paraffin-embedded specimens in tissue microarrays. Mean and median follow-up times after surgery were 109.2 and 110 months, respectively. Ninety-one of 203 (44.8%) patients developed biochemical recurrence defined as a serum PSA concentration of $\geqslant 0.2$ ng/ml increasing over a 3-month period after surgery. Mean and median times from radical prostatectomy to biochemical recurrence were 36.7 and 26.5 months, respectively. The presence of distant metastases was determined by bone scan.

Antibodies and primers
Antibodies were anti-GATA-2 (4595) (Cell Signaling Technologies, Danvers, MA, USA); anti-AR (N-20), anti-GST (B-14), anti-GATA-2 (H-116), anti-rat horseradish peroxidase (HRP) (sc-2006) and anti-ZAG (1D4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-mouse-HRP (NA931V) and anti-rabbit-HRP (NA934V) (GE Healthcare, Buckinghamshire, UK); and anti-hemagglutinin (HA) (clone 3F10) (Roche, Mannheim, Germany). Primers for ChIP and mutational sequencing were designed using Primer Express (Applied Biosystems, Carlsbad, CA, USA; Supplementary Table S1).

Construction of expression vector
An expression vector for HA-tagged GATA-2 protein was generated by amplifying the GATA-2-coding region by PCR using pOTB7-GATA-2 (Geneservice Ltd, Cambridge, UK) as template and pOTB7-GATA-2-For/Rev primer (Supplementary Table S1) introducing *Eco*RI and *XhoI* restriction sites, a Kozak sequence and HA tag. The product was cloned into the pGEM-T-Easy system (Promega, Madison, WI, USA), verified by sequencing and further subcloned into pcDNA3.1(+) and the T-REx tetracycline-regulated expression system (Invitrogen, Carlsbad, CA, USA) to generate pcDNA3.1-GATA-2-HA and pcDNA4/TO-GATA-2-HA.

Cell culture and transfection
LNCaP clone FGC (LNCaP) (American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI 1640 containing 10% fetal bovine serum at 37 °C and 5% CO_2. Transfections were carried out using Lipofectamine 2000 (Invitrogen). The T-Rex system (Invitrogen) was used to generate stable GATA-2-expressing LNCaP cells. Cells were transfected with 30 μg pcDNA6/TR and selected with 6 μg/ml Blasticidin S (Calbiochem, San Diego, CA, USA) for 2 weeks. The stable cell line LNCaP-tetR was then transfected with 5 μg of pcDNA4/TO-GATA-2-HA or empty vector and selected (750 μg/ml Zeocin (Invitrogen), 3 μg/ml Blasticidin S) for 2 weeks to generate LNCaP-GATA-2/tetR and LNCaP-Vector/tetR. Stable cell lines were maintained at 50% of the original selection pressure. Cells for drug treatment were hormone-depleted (medium containing 10% charcoal-stripped serum) for 48 h and treated with 100 nM DHT (Wang *et al*., 2007b) for 0, 12 and 24 h. GATA-2 expression was induced before drug treatment using 1 μg/ml doxycycline for 24 h.

DNA and RNA isolation
DNA and RNA were extracted using the Puregene kit (Gentra Systems, Qiagen, Valencia, CA, USA) and the RNeasy mini kit (Qiagen). Details are available in the Supplementary Material and methods.

Quantitative real-time PCR
Total RNA was converted to cDNA using the A3500 Reverse Transcription System (Promega). Quantitative real-time PCR was carried out on 20 ng of RNA using Taqman Gene-Expression Assays (Applied Biosystems, Foster City, CA, USA) for AZGP1 (Hs00426651_m1), GATA-2 (Hs00231119_m1) and AR (Hs00171172_m1). ALAS1 (Hs00167441_m1) was used as reference gene. All quantitative real-time PCR was carried out using the ABI-prism-7900 sequence detection system (Applied Biosystems).

ChIP
Chromatin immunoprecipitation was carried out using the EZ-ChIP Chromatin Immunoprecipitation kit (Upstate, Lake Placid, NY, USA). Details are available in the Supplementary Material and methods.

Immunoblotting
Proteins (20 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Membranes were blocked in either 5% (w/v) bovine serum albumin or skim milk powder in Tris-buffered saline-Tween 20 (0.1%) and probed with primary and the appropriate secondary antibody. Antibody binding was visualized using Western Lightning chemiluminescence reagent plus (Perkin Elmer, Waltham, MA, USA). Details are available in the Supplementary Material and methods.

Immunohistochemistry
Tissue microarray sections (4 μm) were dewaxed in xylene and rehydrated through a graded ethanol series. Antigen retrieval was carried out by heating in 1 × Target Retrieval Solution (pH 6, DAKO, Glostrup, Denmark) for 120 s. After blocking of endogenous peroxidases (5 min, 3% H_2O_2) and nonspecific antibody-binding sites (30 min, Protein Block Solution), the slides were incubated for 2 h with 8.3 μg/ml GATA-2 antibody (4595), followed by 30 min of goat anti-rabbit IgG (HRP-conjugated) (EnVision, DAKO). Antigen was visualized with 3,3′-diaminobenzidine chromogen and counterstained with hematoxylin. Antigen block with the GATA-2 peptide was used as control.

Each core biopsy of the tissue microarray was scored separately. The highest intensity score for an individual patient was recorded as the final score as previously reported (Henshall *et al*., 2006). The intensity of immunostaining was assessed by two independent scorers, who were blinded to the patient outcome. In case of discrepancies, a third scorer was used. Staining intensity was stratified into the categories of absent, weak, intermediate or strong staining intensities (0, 1+, 2+ and 3+).

Databases
Ingenuity Pathway Analysis version 4.0 from Ingenuity Systems (Ingenuity) was used for the analysis of existing data sets. For promoter sequence retrieval, transcription factor-binding site analysis and AR-binding site identification, the Genomatix Software (Genomatix) was used. Statview 4.51 (Abacus Concepts Inc., Berkeley, CA, USA) (PPC) was used for statistical analysis.

Identification of GATA-2 mutations
Genomic DNA of patients was amplified using conventional PCR and specific primer (Supplementay Table S1). The PCR product was purified using the Agengourt Ampure system (Beckman Coulter, Fullerton, CA, USA). Sequencing PCR was carried out using BigDye 3.1 (Applied Biosystem) and the product was purified using the Agencourt CleanSEQ (Beckman Coulter). Results were analysed using FinchTV and NCBI/BLAST (bl2seq).

Statistical analysis
Variables of scored tissue microarrays were analysed as predictors of disease relapse using the Kaplan–Meier method (Kaplan and Meier, 1958) and univariate and multivariable analysis in Cox proportional hazards models (Cox, 1972) as

previously described (Henshall *et al*., 2006). *P*-values for expression data were calculated using a two-tailed *t*-test with two samples assuming unequal variances.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)