Biomarkers, September–October 2007; 12(5): 541–557

informa
healthcare

Measurement of blood *E2F3* mRNA in prostate cancer by quantitative RT-PCR: a preliminary study

CHRISTODOULOS P. PIPINIKAS[1], SABARINATH B. NAIR[1,2], ROGER S. KIRBY[2], NICHOLAS D. CARTER[1], & CHRISTIANE D. FENSKE[1]

[1]*St George's University of London, Clinical Developmental Sciences, Medical Genetics, London, UK and* [2]*St George's Hospital, Department of Urology, London, UK*

Abstract

The use of serum prostate-specific antigen (PSA) measurements necessitates biopsies for accurate prostate cancer (CaP) diagnosis. Overall efficiency of accurate diagnosis, when PSA levels are used alone, is less than 60%. *E2F3* was evaluated as an alternative biomarker using patient blood samples. Expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and correlated with accurate clinicopathological data. Statistical analysis demonstrated significant differences in *E2F3* expression levels ($p < 0.0001$), and high levels of discrimination (receiver operator curve/area under curve analysis values (AUC) > 0.88), in particular at early stages of disease development, between benign disease and localized CaP. Limited levels of discrimination were observed at the later stages of disease development, between localized and metastatic disease ($p = 0.076$, AUC $= 0.633$). A cut-off point of 0.34 with high specificity for benign disease (92.3%) and sensitivity for CaP diagnosis (81.0%) was identified. At this cut-off point, 85% patients were correctly diagnosed with either malignant or benign disease. This study demonstrates the strength of *E2F3* as a potential marker for discriminating benign and malignant disease, addressing the current limitations of serum PSA measurements.

Keywords: *Prostate cancer,* E2F3, *quantitative RT-PCR, diagnosis, biomarkers*

(Received 12 January 2007; accepted 10 April 2007)

Introduction

Prostate cancer (CaP) is increasingly recognized as a major health problem, being the most prevalent of all human malignancies in most developed countries (Sakr et al. 1993, Grönberg 2003) and the second leading cause of cancer-related deaths exceeded only by lung cancer (Nelson et al. 2003). By 2015, prostate cancer is predicted to become the most common cancer in men worldwide (Parkin et al. 2001).

A series of well-defined stages, including prostatic intraepithelial neoplasia (PIN), organ-confined, locally invasive cancer and androgen-dependent/independent metastases, are indicative of stages of CaP initiation, development and progression (Welsh et al. 2001). CaP diagnosis/prognosis has been based on Gleason score and serum

Correspondence: Christodoulos P. Pipinikas, St George's University of London, Clinical Developmental Sciences, Medical Genetics, Jenner Wing, Cranmer Terrace, London SW17 0RE, UK. Tel: +44 (0)2087250192. E-mail: cpipinik@sgul.ac.uk

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500701391353

prostate-specific antigen (PSA), which despite their value, have certain limitations. The specificity of PSA as a prostate cancer marker is questionable and the subject of ongoing debate (Brawer 2000). Elevated serum PSA levels in approximately 60% of men are not due to CaP but require unnecessary biopsy for definitive diagnosis, while 20% of men with normal serum PSA levels have CaP. In addition, lack of PSA test standardization and inter-laboratory assay variability has also been demonstrated (Dearnaley et al. 1999). Biopsy is invasive and difficult, and results may not necessarily be conclusive (DeMarzo et al. 2003). Therefore, there is an urgent need for the development of new, non-invasive and sensitive diagnostic and prognostic CaP tests. CaP patients harbour heterogeneous tumours that vary in progression rates and knowledge about the genes involved in prostate carcinogenesis is limited (Kumar-Shina & Chinnaiyan 2003). Different molecular mechanisms have been associated with different stages of CaP development and progression, so it is unlikely that only one gene would be involved in controlling these processes (Eder et al. 2004). Recently, much effort has been devoted to the identification of new genes that could serve as reliable early diagnostic/prognostic markers and, where their specific characteristics or functions allow, potential therapeutic targets.

Circulating tumour cells (CTCs) from solid tumours are detectable in patient blood circulation (Hampton et al. 2002) and are considered a 'real-time' biopsy since they represent a surrogate source of tissue (O'Hara et al. 2004). Reverse transcriptase polymerase chain reaction (RT-PCR) is a highly sensitive molecular technique capable of detecting prostate cells gene expression in peripheral circulation, lymph nodes and bone marrow in patients with localized and metastatic disease (McIntyre et al. 2000, Ghossein et al. 2001). Quantitative RT-PCR (qRT-PCR) or real-time PCR can be used to monitor levels of gene expression in patient blood samples.

Insensitivity to antiproliferative signals, such as antigrowth factors, is known to be one of the hallmarks of cancer cells (Hanahan & Weinberg 2000). Such antiproliferative signals are known to be mediated by the retinoblastoma protein (pRB) (Muller et al. 2001), the activity of which is tightly regulated through the action of a complex pathway of cyclin kinases (Adams et al. 2000). Cancer development can result from the disruption of various cell cycle regulatory components, which regulate these kinases, highlighting the importance of the pRB pathway (Hunter & Pines 1994). The retinoblastoma gene (*RB1*), a tumour suppressor gene, has the capacity of interacting with the E2F family of cell cycle regulatory transcription factors via protein–protein interactions (Latchman 1998, Nevin 1998, DeGregori 2002). The E2F family normally stimulates the transcriptional activation of growth-promoting genes, including those encoding DNA polymerase α, thymidine kinase and oncogenes. When pRB and E2F are bound, E2F still binds to its target sites in DNA, but transcriptional stimulation is repressed, which prevents the E2F-mediated transcription of growth-promoting genes (Latchman 1998). During the normal cell cycle of dividing cells, pRB becomes phosphorylated by cyclin-dependent kinases (CDKs), which prevent its binding to E2F. In this situation, transcription is stimulated, and E2F activates the growth-promoting genes necessary for cell cycle progression (Figure 1A). *RB1* gene deletion/mutation or *RB1* transcriptional inactivation results in non-functional pRB, and, therefore, free E2F throughout the cell cycle, and constitutively expressed growth-promoting genes (Figure 1B). Thus, any deregulation of the pathway controlling E2F is believed to be a necessary step in cancer development, emphasizing why E2F appears to be a key regulator of normal cell cycle.

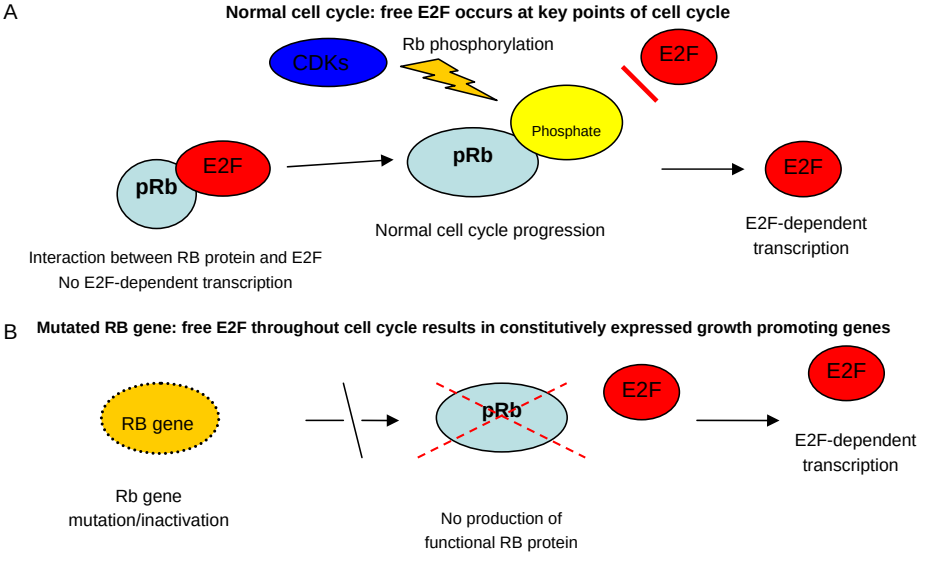

Figure 1. Interactions between the retinoblastoma gene product (pRb) and the E2F cell cycle regulatory transcription factor. Red line indicates prevention of pRb binding to E2F. Adapted from Latchman et al. (1998).

Recent immunohistochemical studies have demonstrated that the *E2F3* gene, a member of the E2F family of cell cycle regulatory transcription factors, is frequently amplified and overexpressed in invasive bladder cancer (Oeggerli et al. 2004) and that there is a strong correlation between *E2F3* expression and tumour stage and grade (Feber et al. 2004). Furthermore, tissue microarray (TMA) studies on CaP have demonstrated high nuclear *E2F3* staining levels, suggesting a role for *E2F3* in CaP development and progression (Foster et al. 2004). In addition, *E2F3* directly modulates the expression of genes, such as *c-MET* and *EZH2*, whose upregulated expression has been shown to be associated with disease progression to end-stage, hormone-refractory metastatic CaP (Bostwick & Foster 1999, Varambally et al. 2002).

The aim of this study was to evaluate *E2F3* as a potentially accurate and sensitive marker for the early diagnosis of CaP. This was done by optimizing and validating the accuracy and reproducibility of the *E2F3* qRT-PCR assay and using this validated protocol to investigate *E2F3* mRNA gene expression levels in blood samples taken from patients with different stages of disease development/progression.

Materials and methods

Patient recruitment and study groups

One hundred patients attending the uro-oncology clinic at St George's Hospital (London, UK) were recruited on the basis of diagnosis by prostate biopsies and transurethral resection of the prostate (TURP). Blood samples were obtained following fully informed consent. The research was carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was obtained from the Wandsworth Local Research Ethics Committee.

In addition, blood samples were collected from ten healthy male control individuals (age range 20–35 years) following informed consent.

Patient data collected include age of patient, date of blood sampling, date of histology, PSA value at time of blood sampling, PSA value at time of initial histological diagnosis, follow-up PSA values, histology results (including Gleason scores), bone scan/computed tomography (CT)/magnetic resonance imaging (MRI) results and treatment status. The data were stored on a secure purpose-built Access database. This ensured accurate correlation between research and clinical data.

All patients had at least one transrectal ultrasound (TRUS)-guided octant prostate biopsy. Patients were classified into distinct groups based on clinical diagnosis and histopathological information as well as radiological information (bone scans and CT/MRI scans). The NEOM group (no evidence of malignancy, $n=26$) included those patients whose biopsy results were negative for cancer and patients diagnosed with benign prostatic hyperplasia (BPH) for which they consequently underwent channel TURP. The LocCaP group ($n=50$) included those patients with biopsy-proven prostate adenocarcinoma but no clinical and/or radiological evidence of metastatic disease (none had undergone radical prostatectomy at the time of blood sampling). This group included patients undergoing surveillance ($n=26$) and those on treatment ($n=21$). Detailed treatment information for three patients in this group was not available. The MetCaP group ($n=24$) included patients who demonstrated evidence of widespread disease (mainly bone metastasis). Bone metastases were diagnosed in 22 patients by bone scan and in two patients by CT/MRI scan. Of the 24 patients, exact treatment details were available for only 12, with six on hormonal treatment and six undergoing no treatment at the time of blood sampling. Details of groups are given in Table I.

Table I. Patient group details.

	Serum PSA (ng ml^{-1})								
	Gleason score (GS)		At histological diagnosis			At blood sample collection			
Patient group	Range	*n*	Range	*n*	Overall median	Range	*n*	Overall median	Age (years) (median)
NEOM ($n=26$)	2–4		≤4	5		≤4	9		
	5–7	No GS.*	4.1–10	14	6.2	4.1–10	13	5.35	60
	8–10		≥10.1	7		≥10.1	4		
LocCaP ($n=50$)	2–4	3	≤4	5		≤4	20		
	5–7	42	4.1–10	19	12.85	4.1–10	13	7.35	72
	8–10	5	≥10.1	26		≥10.1	17		
MetCaP ($n=24$)	2–4	0	≤4	0		≤4	6		
	5–7	11	4.1–10	1	244	4.1–10	2	27.6	71
	8–10	13	≥10.1	23		≥10.1	16		

*No evidence of malignancy (NEOM) patient group, no GS due to negative biopsy results. PSA, prostate-specific antigen; LocCaP, localized prostate cancer; MetCaP, metastatic CaP.

Histological examination was available from the time of initial patient attendance at the uro-oncology clinic. Patients were monitored clinically using only serum PSA levels on subsequent visits; significant changes in clinical symptoms or increases in serum PSA levels dictated further histological examination. The mean number of days between histological analysis and blood sampling for our research was 145 for the NEOM group, 212 for the LocCaP group and 483 for the MetCaP group.

Only patients for whom accurate clinical and histopathological information was available were included in the study. This was to enable accurate correlation of measured gene expression levels with diagnosis, allowing correct assessment of the future application of the marker in a stand-alone molecular technique.

Blood collection, RNA extraction and cDNA synthesis

Patient whole blood samples of 10 ml were collected in EDTA tubes (BD Vacutainer®, BD, Oxford, UK) and stored on ice during transportation in order to avoid any possible RNA degradation. Total RNA was extracted within 30 min of blood sampling in quadruplicate using RNAzol™ (Biogenesis Ltd, Oxford, UK) and on-column DNAse treatment was carried out using the RNeasy Micro kit (Qiagen, Crawley, UK) according to the manufacturer's protocols. All RNA samples were stored at −70°C. Two microlitres of total RNA/quadruplicate were reverse transcribed into first-strand cDNA using SuperScript™ II and oligo(dT) $_{12-18}$ primer according to the manufacturer's protocol (Invitrogen, Paisley, UK). Negative controls for the RT reaction (to identify any potential reagent contamination) were set up as above, but omitting the mRNA (no template control, NTC). In addition, in order to assess the possibility of any genomic DNA contamination after DNase treatment, negative controls containing mRNA but omitting the RT enzyme were also set up (no amplification control, NAC). All cDNA was stored at −20°C prior to PCR amplification.

Primer design

Gene-specific primers for the gene of interest (GOI), *E2F3* (accession no. NM_001949; sense, AAT ATG GCG TAG TAT CTC CG; antisense, CTT CCC AAA CAT ACA CCC AC) and the housekeeping gene (HKG), *GAPDH* (accession no. NM_002046; sense, TGC ACC ACC AAC TGC TTA; antisense, GGA TGC AGG GAT GAT GTT C) were designed on the basis of their published genomic sequences (all primer sequences are given in the 5′-3′ direction). Primers were designed homologous to DNA sequences in different exons such that subsequent amplification would identify the presence of any genomic DNA, demonstrated by a larger PCR product.

RT-PCR

E2F3 primer specificity was verified using RT-PCR and mRNA extracted from LnCaP (lymph node metastatic prostate carcinoma, androgen-sensitive) and PC3 (bone metastasis of grade IV prostate adenocarcinoma, androgen-insensitive) prostate cancer cell lines. Following cDNA synthesis, PCR was carried out using an initial denaturation step (95°C for 15 min) followed by 40 cycles of amplification (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min) and a final elongation step (72°C for

6 min). RT-PCR was also carried out using primers for *GAPDH* to confirm mRNA integrity and to identify false-positive/negative results. *GAPDH* amplification was carried out using an initial denaturation step (95°C for 15 min), followed by 35 cycles of amplification (95°C for 1 min, 60°C for 1 min, 72°C for 1 min), and a final elongation step (72°C for 6 min).

Relative quantitative RT-PCR reaction assays

Relative qRT-PCR was carried out for *E2F3* and *GAPDH* (primers as before) using the LightCycler™ (Roche Diagnostics, Mannheim, Germany) and SYBR® Green I. The qRT-PCR reaction included the following reagents: 1.0 μl cDNA and 2.0 μl LightCycler FastStart™ DNA Master reaction mix (containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix with dUTP, SYBR Green I dye and 10 mM $MgCl_2$), 1.6 μl $MgCl_2$ and a final primer concentration of 0.3 μM *E2F3* and 0.5 μM *GAPDH*. The final volume was adjusted with DEPC-treated H_20 to 20 μl. After loading the reactions into the glass capillaries, samples were incubated for 10 min at 95°C to activate the hot-start Taq DNA polymerase enzyme. Reactions were denatured at 95°C for 10 s followed by 40 cycles of amplification at 58°C (*E2F3*) and 60°C (*GAPDH*) for 5 s. Extension was conducted at 72°C for 16 s.

As SYBR Green I is a double-stranded specific DNA intercalating dye that emits fluorescence once bound to DNA (Morrison et al. 1998), it is necessary to confirm the specificity of the qRT-PCR assays and to distinguish between specific and non-specific PCR products and primer dimers. Therefore, melting curve analysis was carried out, following quantitative amplification, at 58°C (*E2F3*) and 60°C (*GAPDH*) for 10 s. *E2F3* and *GAPDH* products were electrophoresed on 1% agarose gels to confirm melting curve analysis results.

Relative quantitative RT-PCR data analysis

The use of the relative quantification method requires that the PCR efficiencies of all genes under investigation are similar and as close to 100% as possible to ensure exponential amplification of the template. Therefore, it is necessary to evaluate the amplification efficiencies for both the target and housekeeping gene over a wide dynamic range of the kinetic PCR assay. This can be measured by preparing serial dilutions of the template and amplifying for both genes. The resulting crossing point (Cp) values are plotted as a function of $\log_{[10]}$ template concentration (Ginzinger 2002). The slope (S) of the resulting standard curve plotted after the end of the quantification cycle is a direct indicator of the PCR efficiency and is given by the following equation (Rasmussen 2001):

$$E = [10^{(-1/\text{slope})}] - 1$$

Levels of *E2F3* and *GAPDH* mRNA were calculated by the construction of calibration curves using serial 10-fold dilutions of purified *E2F3* PCR product and *GAPDH* plasmid, respectively. Relative quantification was calculated as a ratio of levels of target molecule divided by levels of *GAPDH* (*E2F3*/*GAPDH*). The units used to express relative quantitative mRNA levels are arbitrary. Standard dilutions ($n = 3$; covering the whole range of the *E2F3* and *GAPDH* standard curves) were included in each patient sample run, to allow accurate calculation of the amplification efficiencies within each individual experiment itself, thus accounting for any matrix

effect (i.e. day-to-day or sample-to-sample variation) and enabling more accurate relative quantification. This also enabled accurate calculation of the interassay variation between the different runs. All results described in the study refer to relative quantitative data of *E2F3* gene expression levels normalized to *GAPDH*.

Intra- and interassay variations

The accuracy and reproducibility of the assays were determined by investigating the intra-assay (test precision) and inter-assay (test variability) variation (CV%). Intra-assay variation was investigated in triplicate repeats within one LightCycler run. Inter-assay variation was evaluated in separate runs ($n > 10$) performed on different days using different LightCycler FastStart™ DNA Master reaction mixes.

Statistical analysis

Statistical analyses were carried out using SYSTAT 11 (Systat Software Inc., 2004) and Analyse-it™ (General and clinical laboratory statistics version 1.73). Results from the different patient groups were analysed using initial descriptive statistics and ANOVA to calculate levels of significance (p-values) in differences in individual relative gene expression levels between patient groups. An alternative non-parametric method (Kruskal–Wallis rank sum test) was also used to confirm these differences. Recent reports have demonstrated that *GAPDH* mRNA levels are not constant (Warrington et al. 2000, Szabo et al. 2004). Therefore, using the same approach, *GAPDH* was also investigated to determine if gene expression levels vary with different stages of disease progression.

A receiver operator characteristic (ROC) curve analysis was carried out to determine the accuracy of the test in differential diagnosis of individual patient groups (i.e. maximum discriminatory power). The accuracy was measured by the area under the ROC curve (AUC). An area of 1.0 indicates that the test has a maximum discriminatory power to classify correctly those with and without the disease; an area of 0.5 represents no discrimination. The ability of the *E2F3* qRT-PCR assay to discriminate accurately between benign and malignant disease in an unsupervised manner (i.e. no previous clinicopathological detail) was also investigated by combining the LocCaP and MetCaP (malignant group) and NEOM and BPH (benign group).

Results

Confirmation of primer specificity by RT-PCR

E2F3 gene expression was detectable in both CaP cell lines, LnCaP and PC3, using RT-PCR. Specificity of both primer pairs was determined by analysing RT-PCR products by agarose gel electrophoresis, which resulted in a single product of the desired length (320 bp). RT-PCR products were sequenced in both directions to confirm the correct identity of the amplified products. RT-PCR analysis of *E2F3* expression in normal male control individuals did not demonstrate any product. The presence of intact, high quality RNA in these samples was demonstrated by the amplification of *GAPDH*.

Quantitative RT-PCR amplification efficiencies and linearity

Following the log conversion of the serial dilutions of standards and the generation of standard curves (Cp vs. $\log_{[10]}$ template concentration), qRT-PCR amplification efficiencies were calculated from the given slopes using the LightCycler software. Both investigated genes demonstrated high and comparable amplification efficiencies (*E2F3*, 91.3%; *GAPDH*, 92.5%). In addition, high assay linearity was demonstrated for both *E2F3* and *GAPDH* with correlation coefficient (R^2) of 0.9987 and 0.9991, respectively (Figure 2).

Intra- and inter-assay variations (CV%)

Intra-assay CV was 2.53% for *E2F3* and 0.97% for *GAPDH*. Inter-assay variation was 14.45% (*E2F3*) and 5.1% (*GAPDH*). The mean intra- and inter-assay variations were found to be realistic over the wide dynamic range demonstrated by both qRT-PCR assays and within the expected range previously reported (Pfaffl 2004).

Melting curve analysis

In addition to confirmation of primer specificity by RT-PCR, melting curve analysis, carried out at the end of the quantitative run, demonstrated the amplification of a single product with a sharply defined narrow peak corresponding to a melting temperature of 84°C for *E2F3* (Figure 3A) and 88.6°C for *GAPDH*. No primer dimers were generated (in the presence of template) during the 40 cycles of the qRT-PCR amplification assay. All male control samples gave negative RT-PCR results for *E2F3*. However, the more sensitive technique of qRT-PCR was also carried out on these samples to determine whether this was due to non-expression of *E2F3* or whether expression was below the levels of detection using this method. Melting curve analysis did not demonstrate the expected PCR product (Figure 3B), thus further confirming that there is no *E2F3* expression in normal male control samples.

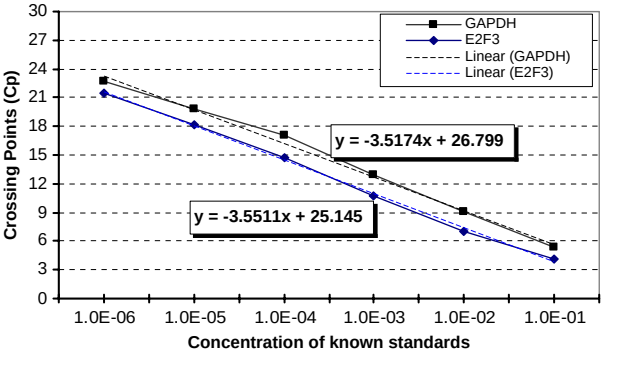

Figure 2. Linear regression analysis demonstrating a linear relationship for both *E2F3* and *GAPDH* standard dilutions over 6 logs of magnitude (range of quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay) with similar slopes and correlation coefficients indicating comparable amplification efficiencies (*E2F3*, $y = -3.5511x + 25.145$, $R^2 = 0.9987$; *GAPDH*, $y = -3.51737x + 26.799$, $R^2 = 0.9991$).

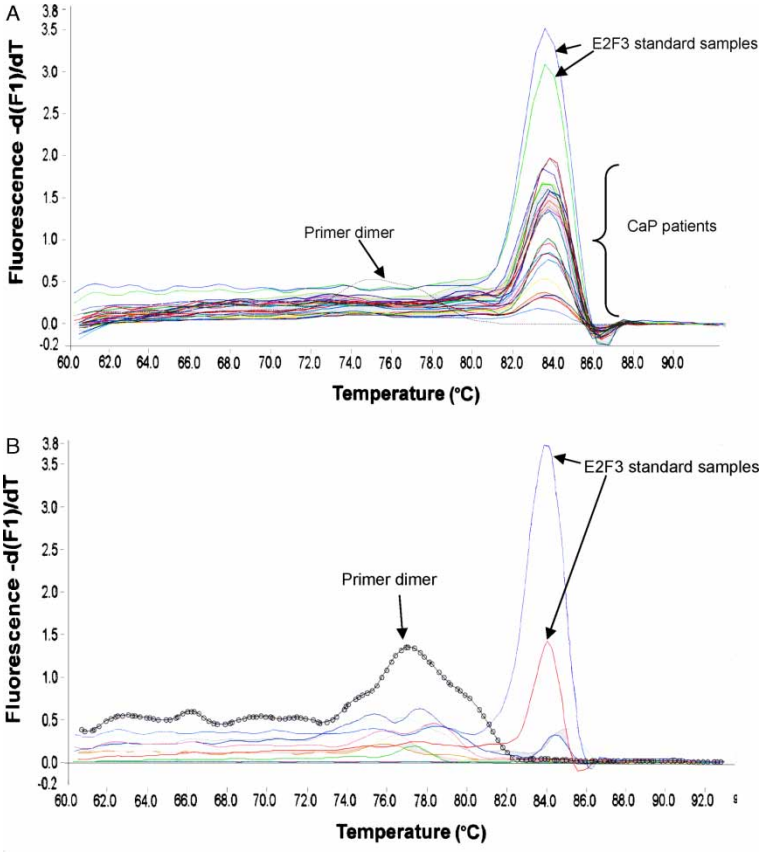

Figure 3. Melting curve analysis of *E2F3* quantitative reverse transcription polymerase chain reaction (qRT-PCR) product on patients diagnosed with metastatic prostate cancer (CaP) and male control individuals. The melting of the PCR product of interest is easily identified as a sharp peak and distinguished from non-specific binding products, which tend to melt at much lower temperatures and over a broader range. A major peak formed at 84°C, corresponding to the *E2F3* product was present in all patients. The dotted line indicating the formation of primer dimers corresponds to the NTC sample. (A) Melting curve analysis on normal male individuals failed to demonstrate the presence of a corresponding peak (B), thus confirming the findings of conventional RT-PCR that *E2F3* is cancer-specific. The two *E2F3*-specific peaks correspond to the highest and lowest standard dilutions included in the run to enable accurate quantification of gene expression.

Quantitative E2F3 expression profiling in benign and malignant prostate cancer

Investigation of the use of *GAPDH* as the housekeeping gene for data normalization resulted in no significant differences in gene expression levels between the different patient groups. Therefore, *GAPDH* was identified as being a suitable housekeeping gene for qRT-PCR data normalization in prostate cancer.

Relative quantitative *E2F3* gene expression levels were calculated in blood RNA samples taken from patients with localized CaP (LocCaP, $n=50$), metastatic CaP (MetCaP, $n=24$) and with no evidence of malignancy (NEOM, $n=26$), including those diagnosed with BPH. Samples were analysed in quadruplicate. All LocCaP and MetCaP patients were positive for *E2F3*. Of the 26 NEOM patients analysed by qRT-PCR only 20 were positive. The six negative-*E2F3* patients were, however, positive for

GAPDH, indicating that *E2F3* expression levels were below the limit of detection in these samples.

Using qRT-PCR, levels of *E2F3* expression were found to be low in the NEOM patient group (mean 0.24, median 0.157) and significantly upregulated in the LocCaP patient group (mean 4.70, median 1.48). *E2F3* levels in the MetCaP patient group (mean 1.70, median 0.58) were lower than that of the LocCaP group but significantly higher than those in the NEOM group (Figure 4). Statistical data analysis is summarized in Table II.

Statistical analysis using the multicomparison test showed that there are highly significant differences in *E2F3* expression between all pair-wise comparison groups (NEOM vs. LocCaP, $p=0.0001$; NEOM vs. MetCaP, $p=0.0003$; benign vs. malignant, $p=0.0001$) with the exception of that between LocCaP vs. MetCaP ($p=0.076$). Similar results were obtained using the Kruskal–Wallis rank sum test.

Based on accurate and detailed treatment information and follow-up PSA measurements, the MetCaP group was further divided into two subgroups: treatment responders (MetCaP_R, $n=6$) and treatment non-responders (MetCaP_NR, $n=9$) to investigate whether *E2F3* gene downregulation at the metastatic stage may be the result of treatment. No statistically significant differences in gene expression levels between the two subgroups were demonstrated ($p=0.369$). Similarly, no significant differences were found between the LocCaP patient group and the MetCaP_R ($p=0.145$) or MetCaP_NR ($p=0.278$).

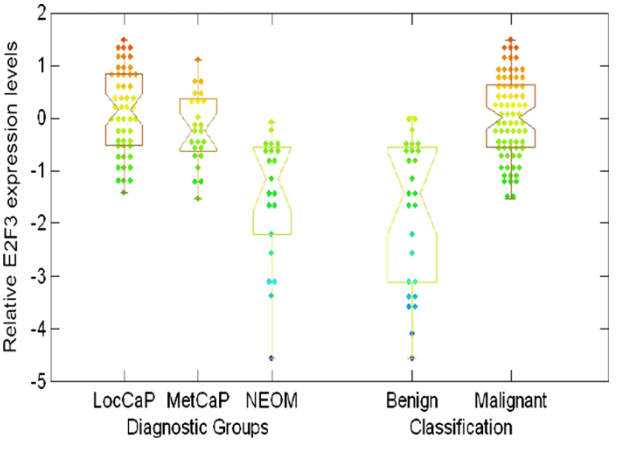

Figure 4. A combined box plot and symmetrical dot density plot showing distribution of relative *E2F3* gene expression levels (*E2F3*/*GAPDH*) in the three different patient diagnostic groups and according to benign or malignant prostatic disease, plotted in logarithmic scale (to show differences between patient groups). Boxes are notched (narrowed) at the median and return to full width at the lower and upper 95% confidence interval values. Note that the six patients with no evidence of malignancy (NEOM), with levels of *E2F3* below the limits of detection, have also been included in the classification box plots. To enable their inclusion in statistical analysis, values equivalent to a 10-fold reduction of the lowest measured level of *E2F3* in the NEOM patient group was taken and a ratio to individual *GAPDH* measurements calculated. LocCaP, localized prostate cancer; MetCaP, metastatic CaP.

Table II. *E2F3* mRNA expression levels*.

Patient group	$n^{\dagger}$	Mean	95% CI of mean	Median	95% CI of median
NEOM	20	0.24	0.097 to 0.37	0.157	0.0234 to 0.317
LocCaP	50	4.70	2.75 to 6.65	1.48	0.66 to 3.49
MetCaP	24	1.70	0.54 to 2.86	0.58	0.24 to 2.33

*The values are corrected for *GAPDH* expression and are unitless ratios.
†RNA was extracted in quadruplet from each patient. We underline the reliability of the method given its resistance to outliers since we calculate the average of the four different measurements for every patient.
NEOM, no evidence of malignancy; LocCaP, localized prostate cancer; MetCaP, metastatic CaP.

E2F3 *expression and inflammation*

E2F3 was also analysed in patients with high levels of serum C-reactive protein (CRP) ($n = 10$), to investigate the influence of the inflammatory network in gene expression levels. The mean and median *E2F3* gene expression levels in the CRP group (mean 0.198, 95% confidence interval (CI) 0.108–0.29; median 0.158, 95% CI of median 0.086–0.39) were similar to those obtained in the NEOM group (Table II). Using a non-parametric test (Kruskal–Wallis), significant differences in *E2F3* gene expression levels were found between CRP and LocCaP ($p < 0.0001$) and CRP and MetCaP ($p < 0.0152$). No statistically significant difference was observed between the NEOM and CRP patient group comparison ($p = 0.5467$). These results indicate that, although *E2F3* expression is present in CRP patients, the levels of expression are minimal and they do not contribute to the upregulation observed during disease progression from the NEOM patient group to either the LocCaP or MetCaP patient group.

Diagnostic validity of E2F3 *assay*

Using relative *E2F3* gene expression levels, ROC curves were plotted in order to evaluate the discriminatory power of *E2F3* to distinguish accurately between different stages of disease as well as between benign and malignant disease (as measured by the AUC). In addition, using the different cut-off values derived from the ROC analysis, the maximum specificity and sensitivity of the *E2F3* qRT-PCR assay to detect CaP in an unsupervised manner (i.e. no previous clinicopathological data) was investigated. The six negative-*E2F3* NEOM patients were also considered when calculating sensitivities and specificities. The discriminatory power of the *E2F3* assay was found to be maximum when comparing NEOM with LocCaP, as indicated by an AUC of 0.908 ($p < 0.0001$). Our blood-based assay detected levels of *E2F3* mRNA expression above an optimal cut-off point of 0.34, in 41 of 50 LocCaP patients and only in two of 26 NEOM patients, demonstrating an overall sensitivity of 82.0% and a specificity of 92.3%. The overall efficiency of the assay at this cut-off point was 85.5% (65/76). A similar discriminatory power was also demonstrated between NEOM and MetCaP, given by an AUC of 0.880 ($p < 0.0001$). Using the same cut-off point of 0.34, the sensitivity was found to be 79.2% (19/24) for accurately diagnosing metastatic CaP while specificity was the same as before. When *E2F3* was evaluated as a discriminatory factor of benign and malignant disease, the AUC was 0.899 ($p < 0.0001$).

Using the same cut-off point (0.34), the sensitivity and specificity were 81.1% (60/74) and 92.3%, respectively; approximately 85% of patients were correctly

Table III. ROC/AUC analysis and diagnostic validity.

	ROC/AUC analysis			Diagnostic validity			
	AUC	AUC p-value	95% CI of AUC	Sensitivity (%)	Specificity (%)	Efficiency (%)	Cut-off (≥)
NEOM/LocCaP	0.908	<0.0001	0.840–0.977	82.00	92.3	85.5	0.34
NEOM/MetCaP	0.880	<0.0001	0.777–0.982	79.2	92.3	86.0	
LocCaP/MetCaP	0.633	0.03	0.496–0.769	Limited discriminatory power			
Benign/malignant	0.899	<0.0001	0.831–0.968	81.1	92.3	84.0	0.34

Level of significance was set as 0.05. NEOM, no evidence of malignancy; LocCaP, localized prostate cancer; MetCap, metastatic CaP.

classified as true positives (malignant cancer) or true negatives (benign disease). ROC/AUC and diagnostic validity findings are summarized in Table III.

Blood E2F3 *gene expression and serum PSA levels*

The diagnostic potential of *E2F3* in accurately predicting disease stage was compared with the diagnostic utility of serum PSA levels at the cut-off point of 4.0 ng ml^{-1}, currently used, to differentially diagnose NEOM and LocCaP. Lowering the serum PSA cut-off point to 2.6 ng ml^{-1} has been reported to result in more frequent detection of small, organ-confined tumours without over-detecting possibly clinically insignificant ones (Krumholtz et al. 2002). Therefore, the diagnostic validity of serum PSA level at this lowered cut-off point was also compared with *E2F3*.

When considering the currently used 4.0 ng ml^{-1} PSA cut-off point, 30 patients with LocCaP were identified as true positives (i.e. having serum PSA levels >4.0 ng ml^{-1}), while 20 were identified as false negatives (i.e. having serum PSA levels ≤ 4.0 ng ml^{-1}). The sensitivity for the LocCaP diagnosis was 60%. Specificity for the NEOM group was limited to 34.6% as only 9 patients were identified as true negatives (i.e. having serum PSA ≤ 4.0 ng ml^{-1}). An improved sensitivity of 70% was demonstrated for the 2.6 ng ml^{-1} cut-off point, with 35 LocCaP patients correctly classified (35/50). The specificity, however, decreased to 15.4% as only four NEOM patients had PSA Levels below or equal to this cut-off point and the remaining 22 above. Overall diagnostic efficiency of serum PSA measurement at both cut-off points was 51.3% (39/76). Table IV summarizes the results.

Overall, *E2F3* (AUC 0.908) was found to provide a better discrimination for early disease diagnosis when compared to PSA at both cut-off points (AUC 0.557,

Table IV. Comparison of *E2F3* quantitative reverse transcription polymerase chain reaction and serum prostate-specific antigen (PSA) diagnostic performance in early disease diagnosis (NEOM (no evidence of malignancy) vs. LocCaP (localized prostate cancer)).

	AUC	p-value	Cut-off point	Specificity (%)	Sensitivity (%)	Overall efficiency (%)
E2F3	0.908	<0.0001	0.34	92.3	82.0	85.5
PSA (ng ml^{-1})	0.557	0.193	4.0	34.6	60.0	51.3
			2.6	15.4	70.0	

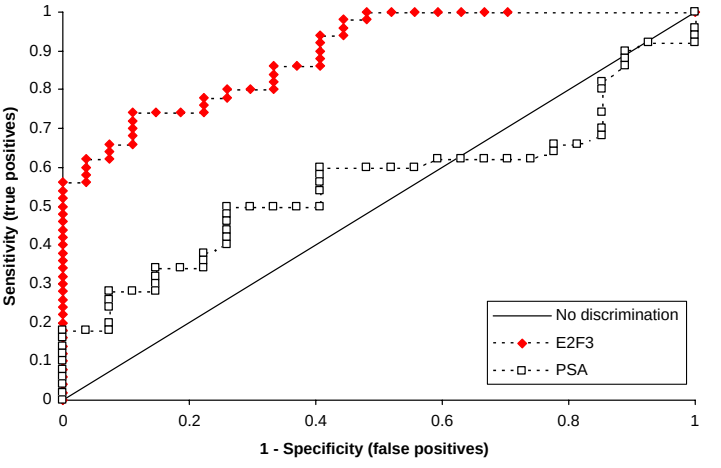

Figure 5. Comparison of prostate-specific antigen (PSA) (black) and *E2F3* (red) ROC curves to discriminate between patients with localized prostate cancer (LocCaP) and no evidence of malignancy (NEOM).

$p = 0.193$, 95% CI of AUC 0.429–0.685). Figure 5 illustrates the direct comparison between the *E2F3* and serum PSA ROC curves.

Conclusions

We have assessed the accuracy, reproducibility and application of a real-time qRT-PCR assay in measuring expression levels of a malignancy related gene marker, *E2F3*, for the early diagnosis and accurate staging of CaP. Measurement of *E2F3* mRNA gene expression levels were demonstrated for the first time in patients with different stages of CaP development and progression using this extremely sensitive, non-invasive molecular technique.

E2F3 expression has been found to be significantly upregulated in LocCaP patients, compared with those with benign disease, indicating a diagnostic application for the early detection of CaP when treatment is more effective. Areas of overlap in serum PSA measurements are frequently observed in the early stages of disease development, making accurate diagnosis difficult without the use of biopsy. An assay using relative qRT-PCR and *E2F3* (normalized to *GAPDH*), as an alternative biomarker, has the potential to eliminate these grey areas and, therefore, avoid unnecessary biopsies. Results are supported by ROC analysis, which demonstrated high diagnostic specificity (92.3%) and sensitivity (81.1%) with an overall efficiency of 85% in discriminating between benign and malignant disease.

Specificity, sensitivity and reproducibility are all critical factors for successful and valid quantification of gene expression levels and should be considered carefully when optimizing the qRT-PCR assay (Bustin 2000). In addition, specific factors such as the amount of the starting material/nucleic acid recovery/purity, possible RNA degradation, differences in cDNA synthesis efficiencies and subjectivity in data normalization/analysis, should also be considered and strictly controlled for accurate quantitative results (Bustin & Nolan 2004). The use of relative expression ratios has the advantage of correcting for such factors influencing the overall performance of the kinetic

reaction (Roche Applied Science Technical Note No. LC 13/2001: Relative quantification). In this type of quantitative analysis, serial dilutions of known positive samples for both the gene of interest (GOI) and the housekeeping gene (HKG) are prepared and used to generate standard curves. The resulting curves are then used for quantification of the GOI and the HKG in each unknown sample. The concentration of the GOI is then divided by the concentration of the HKG in the same sample and the resulting GOI/HKG ratio expresses the amount of the target gene normalized by the HKG (Roche Applied Science Technical Note No. LC 13/2001: Relative quantification).

GAPDH is an ubiquitously expressed gene that has been frequently used as a HKG in qRT-PCR. Recently, the use of *GAPDH* for data normalization has become questionable as gene expression levels have been shown to vary. However, no significant differences in *GAPDH* gene expression levels between the different patient groups were observed in our study. This observation agrees with previous published data demonstrating that *GAPDH* was among the three top-ranked candidates for gene expression studies in colon cancer (Andersen et al. 2004) and among the seven most suitable HKGs for qRT-PCR data normalization in prostate cancer (Ohl et al. 2005).

The correlation of our qRT-PCR results relies on diagnoses based on current clinical techniques and assays that, together with the system of patient recruitment have some limitations. The first is the time interval between diagnosis and blood sampling for qRT-PCR. On first visit to the clinic, patients undergo serum PSA testing and biopsy, resulting in the diagnosis used in our study for correlation of gene expression results. No further biopsy is carried out unless serum PSA levels demonstrate significant and permanent change. Therefore, the diagnosis remains and is within the limits of current techniques. However, given that serum PSA is not an accurate marker for prostate cancer, it cannot be ruled out that the disease in these patients may have progressed to the next stage of development. Secondly, all patient groups have been diagnosed on the basis of biopsy, which relies on correct area of sampling. Whilst this does not result in false positives, false negatives are a possibility. Therefore, any patient with localized cancer may conceivably be misdiagnosed as NEOM. In addition, while metastatic disease is diagnosed on the basis of positive bone scan findings, micrometastases or circulating cells, which increase the risk of metastasis, will be undetectable. Finally, due to the large interval between diagnosis and blood sampling, the possibility of reduced gene expression levels due to treatment cannot be ruled out. Although investigation of any possible treatment effect in *E2F3* gene expression levels in MetCaP patients demonstrated no statistically significant differences, useful conclusions cannot be made at this point due to the limited number of patients. These diagnostic limitations may well account for some of the anomalies identified in our results. It is precisely these limitations that may be potentially minimized by the introduction of this new molecular test.

It is not known whether variations in *E2F3* gene expression levels between the three patient groups reflect differences in numbers of CTCs or are due to their increased metastatic potential. Current research involves enhancement of prostate cancer cells in blood samples of patients with the disease to define the fraction of tumour cells contributing to increased *E2F3* levels. This involves the use of fluorescently activated cell sorting techniques. RNA extracted from the tumour cell fraction and subsequent measurement of gene expression levels by qRT-PCR will determine whether numbers of CTCs correlate with gene expression levels and disease stage/aggressiveness.

E2F3 may be one of several mechanisms involved in prostate carcinogenesis and progression rather than being the end product of CaP. Thus, *E2F3* expression has been shown to be upregulated in patients with localized CaP in this study, with subsequent downregulation at the metastatic stage. This supports the hypothesis of early involvement of *E2F3* in disease establishment and development, with alternative mechanisms and genes playing a role at later stages of disease progression to metastases. It is well documented that several molecular mechanism and distinct sets of genes, representing distinct biochemical pathways, are involved in disease development and progression. These results further highlight the importance of identifying a panel of gene markers, which in combination with *E2F3*, can be used to accurately diagnose the stages of disease development. *E2F3* is part of a control axis (*pRB-E2F3-EZH2*) that may represent an underlying mechanism of prostate carcinogenesis (Foster et al. 2004). Whilst *E2F3* has been shown to be overexpressed in locally invasive CaP with decreased expression levels in MetCaP patients, *EZH2* has been reported to be similarly overexpressed in LocCaP but significantly upregulated further in MetCaP patients (Varambally et al. 2002). Therefore, evaluation of *EZH2* expression levels by qRT-PCR and combining the results with *E2F3* may not only add to accurately distinguishing between benign and malignant disease but also between clinically localized and metastatic CaP.

It is well documented that even after surgery, tumour cells may still be present in blood circulation, often being a possible reason of disease recurrence and/or metastasis; for example, presence of minimal amounts of circulating tumour cells is correlated with poor prognosis in colorectal cancer patients (Schott et al. 1998). This indicates the persistence of circulating cancer cells and may be indicative of minimal residual disease or risk of disease recurrence. Preliminary data using postoperatively obtained blood samples from patients who had undergone radical prostatectomy demonstrated that this technique may also be used to identify any undetected disease after surgery. Increased levels of *E2F3* expression accompanied by possible increase in serum PSA levels (biochemical recurrence) would be indicative of increased risk of disease relapse. However, this is yet to be clinically proven and is the basis of an ongoing study, currently being carried out by this research group.

We have shown the potential of a sensitive real-time qRT-PCR assay in monitoring levels of expression of *E2F3*, a cancer-specific marker. Despite the limitations of current diagnostic procedures described, our data show that *E2F3* is a potential marker for the accurate diagnosis of early stages of prostate cancer, demonstrating high sensitivity, specificity and overall correct diagnostic efficiency. Current diagnostic techniques, questioned regarding their accuracy, have often led to unnecessary surgical procedure (biopsy), which may be avoided using this non-invasive accurate diagnostic molecular technique. Further analysis of additional informative markers, together with the construction of probability plots will lead to the accurate diagnosis of all stages of prostate cancer development (including post-surgery monitoring). This in turn will allow for more timely and effective therapy.

Acknowledgements

This study was supported by grants from the Prostate Research Campaign UK, the Swire Foundation and the Everard Goodman Charitable Foundation. Many thanks to Dr Catherine Corbishley for the use of St George's Healthcare NHS Trust prostatic

disease and radical prostatectomy databases. Additional statistical support and advice was kindly provided by Drs Kostas Konstantinou and Nicky Best. We would also like to acknowledge Dr Suman Rice for reviewing this manuscript.

References

Adams MR, Sears R, Nuckolls F, Leone G, Nevins J. 2000. Complex transcription regulatory mechanisms control expression of the E2F3 locus. Molecular and Cellular Biology 20:3633–3639.

Andersen CL, Jensen JL, Ørntoft TF. 2004. Normalisation of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalisation, applied to bladder and colon cancer data sets. Cancer Research 64:5245–5250.

Bostwick DG, Foster CS. 1999. Predictive factors in prostate cancer: current concepts from the 1999 College of American Pathologists Conference on solid tumour prognostic factors and the 1999 World Health Organization second international consultation on prostate cancer. Seminars in Urologic Oncology 17:222–272.

Brawer MK. 2000. Prostate-specific antigen. Seminars in Surgical Oncology 18:3–9.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse-transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25:169–193.

Bustin SA, Nolan T. 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. Journal of Biomolecular Techniques 15:155–166.

Dearnaley DP, Kirby RS, Kirk D, Malone P, Simpson RJ, Williams G. 1999. Diagnosis and management of early prostate cancer. Report of a British Association of Urological Surgeons Working Party. British Journal of Urology 83:18–33.

DeGregori J. 2002. The genetics of the E2F family of transcription factors: shared functions and unique roles. Biochimica et Biophysica Acta 1602:131–150.

DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. 2003. Pathological and molecular aspects of prostate cancer. Lancet 361:955–964.

Eder IE, Bektic J, Haag P, Bartsch G, Klocker H. 2004. Genes differentially expressed in prostate cancer. British Journal of Urology 93:1151–1155.

Feber A, Clark J, Goodwin G, Dodson AR, Smith PH, Fletcher A, Edwards S, Flohr P, Falconer A, Roe T, Kovacs G, Dennis N, Fisher C, Wooster R, Huddart R, Foster CS, Cooper CS. 2004. Amplification and overexpression of E2F3 in human bladder cancer. Oncogene 23:1627–1630.

Foster CS, Falconer A, Dodson AR, Norman AR, Dennis N, Fletcher A, Southgate C, Dowe A, Dearnaley D, Jhavar S, Eeles R, Feber A, Cooper CS. 2004. Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene 23:5871–5879.

Ghossein RA, Bhattacharya S, Coit DG. 2001. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of melanoma-related transcripts in the peripheral blood and bone marrow of patients with malignant melanoma. What have we learned? Recent Results. Cancer Research 158:63–77.

Ginzinger DG. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Experimental Haematology 30:503–512.

Grönberg H. 2003. Prostate cancer epidemiology. Lancet 361:859–864.

Hampton R, Walker M, Marshall J, Juhl H. 2002. Differential expression of carcinoembryonic antigen (CEA) splice variants in whole blood of colon cancer patients and healthy volunteers: implication for the detection of circulating colon cancer cells. Oncogene 21:7817–7823.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100:57–70.

Hunter T, Pines J. 1994. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell 79:573–582.

Krumholtz JS, Carvalhal GF, Ramos CG, Smith DS, Thorson P, Yan Y, Humphrey PA, Roehl KA, Catalona WJ. 2002. Prostate-specific antigen cut-off of 2.6 ng/ml for prostate cancer screening is associated with favourable pathologic tumour features. Urology 60:469–473.

Kumar-Sinha C, Chinnaiyan AM. 2003. Molecular markers to identify patients at risk for recurrence after primary treatment for prostate cancer. Urology 62(Supplement 6B):19–35.

Latchman D. 1998. Gene Regulation: a eukaryotic perspective. UK: Stanley Thornes Ltd, 304–305.

McIntyre IG, Spreckley K, Clarke RB, Anderson E, Clarke NW, George NJ. 2000. Optimization of the reverse transcriptase polymerase chain reaction for the detection of circulating prostate cells. British Journal of Cancer 83:992–997.

Morrison TB, Weis JJ, Wittwer CT. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24:954–962.

Muller H, Bracken AP, Vernell R, Moroni MC, Christians F, Grassilli E, Prosperini E, Vigo E, Oliner JD, Helin K. 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation and apoptosis. Genes and Development 15:267–285.

Nelson WG, De Marzo AM, Isaacs WB. 2003. Mechanisms of disease: prostate cancer. New England Journal of Medicine 349:366–381.

Nevin JR. 1998. Towards an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth and Differentiation 9:585–593.

Oeggerli M, Tomovska S, Schraml P, Calvano-Forte D, Schafroth S, Simon R, Gasser T, Mihatsch MJ, Sauter G. 2004. E2F3 amplification and overexpression is associated with invasive tumour growth and rapid tumour cell proliferation in urinary bladder cancer. Oncogene 23:5616–5623.

O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG, Terstappen LW. 2004. Multigene reverse transcription-PCR profiling of circulating tumour cells in hormone-refractory prostate cancer. Clinical Chemistry 50:826–835.

Ohl F, Junf M, Xu C, Stephan C, Rabien A, Burkhardt M, Nitsche A, Kristiansen G, Loening SA, Radonic A, Jung K. 2005. Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization? Journal of Molecular Medicine 83:1014–1024.

Parkin DM, Bray FI, Devesa SS. 2001. Cancer burden in the year 2000: the global picture. European Journal of Cancer 37:S4–66.

Pfaffl MW. 2004. Quantification strategies in real-time PCR. In: Bustin SA, ed. A–Z of quantitative PCR. La Jolla, CA, USA: International University Line, Chapter 3.

Rasmussen R. 2001. Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid Cycle Real-time PCR, Methods and Applications. Heidelberg: Springer Press. p. 21–34.

Roche Applied Science Technical Note No. LC 13/2001: Relative Quantification.

Sakr WA, Haas GP, Cassin BF, Pontes JE, Crissman JD. 1993. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. Journal of Urology 150:379–385.

Schott A, Vogel I, Krueger U, Kalthoff H, Schreiber HW, Schmiegel W, Henne-Bruns D, Kremer B, Juhl H. 1998. Isolated tumour cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker. Annals of Surgery 227:372–379.

Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, Bernard PS. 2004. Statistical modelling for selecting housekeeper genes. Genome Biology 5:R59.1–R59.10.

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. 2002. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629.

Warrington JA, Nair A, Mahadevappa M, Tsyganskaya M. 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiological Genomics 2: 143–147.

Welsh JB, Sapinoso LM, Su AI, Kern SG, Wang-Rodriguez J, Moskaluk CA, Frierson HF, Jr, Hampton GM. 2001. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer Research 61:5974–5978.