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A Gene Expression Profiling Approach Assessing Celecoxib in a Randomized Controlled Trial in Prostate Cancer

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Abstract. *Background: We performed a pilot study, looking at the COX-2 inhibitor celecoxib, on newly diagnosed prostate cancer patients in the neo-adjuvant setting using DNA microarray analysis. Patients and Methods: This was a single-blinded, randomized controlled phase II presurgical (radical prostatectomy) 28-day trial of celecoxib versus no drug in patients with localized T1-2 N0 M0 prostate cancer. cDNA microarray analysis was carried out on prostate cancer biopsies taken from freshly obtained radical prostatectomy samples. Results were confirmed by qPCR analysis of a selection of genes. Results: Multiple genes were differentially expressed in response to celecoxib treatment. Statistical analysis of microarray data indicated 24 genes were up-regulated and 4 genes down-regulated as a consequence of celecoxib treatment. Gene changes e.g. survivin, SRP72kDa, were associated with promoting apoptotic cell death, enhancement of antioxidant processes and tumour suppressor function (p73 and cyclin B1 up-regulation). Conclusion: Celecoxib at 400 mg b.i.d. for 4 weeks perioperatively gave rise to changes in gene expression in prostate cancer tissue consistent with enhancement of apoptosis and tumour suppressor function. Given the short time interval for the duration of this study, the data are encouraging and provide a good rationale for conducting further trials of celecoxib in prostate cancer.*

Prostate cancer is the commonest non-dermatological cancer in men in Western countries (1) and novel strategies of management are badly needed. Cyclooxygenase-2 (COX-2) is intimately involved in the development and progression of a variety of malignancies, including prostate cancer. COX-2 has been shown to interact with angiogenic, apoptotic, proliferative, invasive, metastatic and other pathways involved in cancer evolution (2). Chronic inflammation and oxidative stress are key players in COX-2-dependent carcinogenesis. COX-2 has been shown to be an independent predictor of prostate cancer progession following radical prostatectomy (3). Moreover, the outcome of radiotherapy in prostate cancer patients has been shown to be associated with COX-2 expression, whereby high levels were indicative of treatment failure (4). Celecoxib is a selective COX-2 inhibitor (coxib) that appears to have an anticancer effect in numerous *in vivo* and *in vitro* models. Coxibs, most notably celecoxib, also exert their anti-cancer effects *via* COX-2-independent mechanisms including interference with Akt (signal transduction), NF-κB (inflammatory mediator of tumorigenesis) and other mediators of cancer development and progression. The end result of both COX-2-dependent and COX-2-independent actions is an inhibition of cancer at multiple stages (2). Presently, the superior anticancer and cardiovascular safety profiles of celecoxib make it the coxib of choice for clinical trials.

The purpose of this study was to investigate the effects of celecoxib in early prostate cancer. Patients were randomized to 400 mg celecoxib twice daily (*b.i.d.*) *versus* no drug for 4 weeks prior to radical prostatectomy (RP). We initially assessed the effects of the treatment on markers of proliferation, apoptosis and angiogenesis using paraffin-embedded tissue taken from the RP specimens from the patients in this clinical trial (5). We then went on to assess the effects of celecoxib on gene expression profiling of prostate cancer by subjecting samples from drug-treated and control patients to DNA microarray analysis. Our overall aim was, therefore, to conduct a pilot study investigating the effects of celecoxib on the gene expression profiling (GEP) of tumour biopsies taken from patients with localized prostate cancer.

Patients and Methods

Patient selection. The study was a single-blinded, randomized, controlled phase II pre-surgical trial of celecoxib *versus* no drug for 28 days prior to radical prostatectomy (RP) for patients with localized (T1-2 N0 M0) prostate cancer. We used a 2:1 block

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randomization, hence the difference in numbers of controls and treated patients. The power calculation was based on this and the use of a 2:1 randomization schedule meant we had greater numbers of treated patients and thus needed fewer patients in the study to reach the desired power. Ethical approval was obtained prior to starting the study and all patients recruited to the trial gave their informed, signed consent. Patients who had decided to undergo RP as their management option were eligible. Patients taking NSAIDs or other coxibs were excluded, as were those with a significant past history of these medications. Patients on hormonal manipulation and 5-ARIs (*e.g.* finasteride) were also excluded, because of the uncertain effects these drugs have on prostate tumour biology, especially given the recent evidence from the Prostate Cancer Prevention Trial (6). Patients with cardiovascular risk factors were also excluded. Baseline clinical data are indicated in Table I. Although there is no evidence that COX-2 inhibitors affect cardiovascular risk when taken for periods of less than one year, and certainly not for a period of 28 days, patients with cardiovascular risk factors were also excluded. No patient in our trial suffered an adverse cardiac event. All patients had Gleason score 6 (3+3) or 7 (3+4) disease and clinically localized prostate cancer (T1c-T2c N0 M0); the celecoxib and control groups were therefore matched in terms of histological grade and stage. There was also no significant difference in PSA values based the two arms. These baseline clinical data are indicated in Table I.

Table I. *Baseline clinical comparison of control versus celecoxib-treated patients.*

Parameter	Control group N=13	Celecoxib-treated N=27	Median difference (95% CI and interquartile range)
PSA	8.06 (3.47)	8.25 (4.62)	0.197 (–2.30 to 2.69)
*Pathological stage	2.07 (0.26)	2.10 (0.31)	0.03 (–0.14 to 0.21)
+Gleason sum	6.53 (0.74)	6.33 (0.71)	–0.20 (–0.66 to 0.26)

Values shown are the means with the standard deviation shown in parentheses. *All patients had clinical stage T1c-T2c N0 M0 disease; +All patients had Gleason sum 6/7.

Sample collection. Prostatic tissue was collected at the time of surgery for RP (38 laparoscopic, 7 open procedure for the entire sample series). The prostate was removed from the body prior to the vesico-urethral anastomosis being performed then sectioned vertically and needle/punch biopsies were taken of the peripheral zone of the prostate in six areas: left apex, right apex, left midzone, right midzone, right base, and left base. Biopsies were then rapidly sectioned transversely into three roughly equal parts. The middle portion was placed in RNAlater® and sent for subsequent DNA microarray analysis. The mean time from prostatectomy to RNAlater® (Qiagen Ltd., Crawley, UK) submersion for sample preservation was ≤15 minutes. Only those biopsy sections that were histologically confirmed as cancer were used for cDNA microarray analysis.

GEP using cDNA microarray analysis of prostate cancer biopsies. Freshly collected core biopsies were rapidly frozen down to –80˚C for short-term storage prior to extraction. The frozen biopsies in RNAlater™ solution were thawed on ice and preservation fluid aspirated. RNA was isolated using the RNeasy method (mini-column procedure; Qiagen Ltd.) with sample lysis using a TissueLyser (Qiagen Ltd.). The quality of the resulting RNA was checked with an Agilent 2100 Bioanalyser using an RNA NanoLabchip® according to the manufacturer's instructions (Agilent Technologies UK Ltd, Stockport, Cheshire, UK). The RNA was diluted with RNase-free water and quantified using the Nanodrop spectrophotometer (Nanodrop Technologies Inc.). Due to the quantity necessary for the microarray analysis and stringent requirements for quality, RNA from some samples were not suitable for full analysis and hence, there were 8 control patients and 12 celecoxib-treated patients biopsies used in this analysis.

Labelling reactions were carried out with 3-5 μg total RNA using the ChipShot Pronto system (Corning Life Sciences, Schiphol, The Netherlands) according to the manufacturer's instructions. A reference labelling reaction was also prepared with 5 μg total Universal Human Reference RNA (Stratagene Europe, Amsterdam, The Netherlands) and used as an internal control for the normalization of the microarray data. Cy®3 (test sample) and Cy®5 (reference) were obtained from GE Healthcare (Amersham, Buckinghamshire, UK) and used in conjunction with the ChipShot kit. The amount of Cy®3 and Cy®5 incorporation (frequency of incorporation; FOI) in the cDNA was quantified using the Nanodrop Spectrophotometer. Printing of the 3000 Human Cancer oligo subset V2.0 (Operon Biotechnologies GmbH, Germany) was carried out using UltraGAPS-coated glass microarray slides (Corning B.V. Life Sciences, The Netherlands). The 70mer oligonucleotide probes were dissolved in Pronto cDNA/Long Oligo Spotting Solution (Corning) and spotted onto a glass slide with a Qarray2 microarray spotter (Genetix, UK) and ScoreCard controls (GE Healthcare). Pre-hybridization, hybridization and washing of the microarrays were carried out according to manufacturer's instructions (Pronto Microarray hybridization kit; Corning). Cy3 and Cy5 fluorescent signals were detected with an Affymetrix 428 laser scanner and the raw TIFF images were analysed for spot signal quantification and quality evaluation with Blue Fuse software V3.1 (BlueGnome, Cambridge, UK). The generated data files were then exported into R (version 2.5.0; http://www.R-project.org), normalised (Block-median followed by Across Array Bioconductor LIMMA package (7, 8), 'bad' spots (determined by BlueFuse V3.1, BlueGnome) extracted and analysed using Rank Product analysis (9, 10), a technique chosen for its robustness against biological variance such as that caused by tumour and/or patient experimental designs (9). Genes with a 'pfp' (probability of false prediction) value (9, 10) less than 0.15 were selected as being significantly up- or down-regulated.

Real-time quantitative PCR. cDNA was made using the ImPromII reverse transcription system (Promega, Southampton, UK). Real-time quantitative PCR was performed using the Stratagene QPCRMX3005P thermal cycler (Stratagene Europe). Reactions were performed in a 20 μl volume with 5 pmol primers and 4 mM $MgCl_2$ using Brilliant SYBR Green QPCR Master Mix reagent (Stratagene). For survivin, the protocol was 10 min 95˚C for activating the hot start Taq polymerase, then 20 s at 95˚C, 20 s at

60˚C and 20s at 72˚C for 45 cycles; for p73, 95˚C for 5 min then 40 cycles of 94˚C 30 s, 60˚C for 30 s, 68˚C for 2 min; for cyclin B1, 95˚C for 5 min then 40 cycles of 95˚C for 20 s, 60˚C for 60 s. For SRP72kDa, the protocol was 95˚C for 10 min, then 35 cycles of 95˚C for 60 s, 62˚C for 2 min and 72˚C for 3 min. All analyses were set up in duplicate and were also repeated on at least 2 separate occasions. Relative expression of genes was normalized to that of actin and gene expression in each sample calculated as $2^{-\Delta\Delta Cr}$.

Primer sequences were designed using Primer 3 software and supplied by Invitrogen (Paisley, UK). For survivin: forward: 5'ACCAGGTGAGAAGTGAGGGA-3', reverse: 5'-AACAGTAG AGGAGCCAGGGA-3'; for cyclin B1: 5'-CTCCTGTCTGGTG GGAGGA-3', reverse 5'-CTGATCCAGAATAACACCTGA-3'; for P73: forward: 5'-TCTTTCGAGGGTCGCATCTG-3', reverse: 5'-TCC CGGTAATGGTCTTCATCAG-3'; for SRP72kDa: forward: 5'-GTCG AACAGAGGAGGCTTTG-3', reverse: 5'-CGGGACTTTGGGACTG TAAA-3'; for β actin forward: 5'-GCATCCACG AAACTACCTTC-3', reverse: 5'-CAGGAGGAGCAATGATCTTG-3'.

Statistical analysis. For comparison of all the q-PCR data obtained, we used the SPSS 12.0.1 program. Comparison of means was carried out using the one-way ANOVA or equality of means test, as appropriate. Differences were considered significant if a *p*-value of 0.05 or less was obtained.

Results

Baseline clinical parameters obtained for control and celecoxib-treated patients. Data are shown in Table I. *Microarray analysis and Q-PCR validation:* For the microarray part of the study we used a cut-off of ± 50% change in expression relative to the control arm. Table II lists the up-regulated genes. The data obtained revealed a list comprising 24 genes that showed statistically significant (see methods) >1.5-fold increments between celecoxib-treated and control untreated patients. A variety of genes with different functional significance were up-regulated in the celecoxib-treated patient group. Figure 1 shows the subsequent q-PCR evaluation of 3 up-regulated genes that were on the list (*cyclin B1, p73* and *SRP72kDa*). The data help to verify the microarray analysis with levels of significance for the variation between control and celecoxib-treated group as being significant or very close to significant for *SRP72kDa,* where a high standard deviation for the levels of mRNA obtained in the celecoxib-treated patient biopsies was seen. Table III lists the down-regulated genes. A further list comprising 3 genes showed significant <0.5-fold decrease in celecoxib treated *versus* control patients. Due to our previous observation that *survivin* mRNA and protein were markedly down-regulated in COX-2-expressing human prostate cancer LNCaP cells (11), we also considered this gene in our subsequent q-PCR evaluation in the present study. The mRNA levels proved to be significantly reduced in patients from the celecoxib-treated group *versus* controls (see Figure 1).

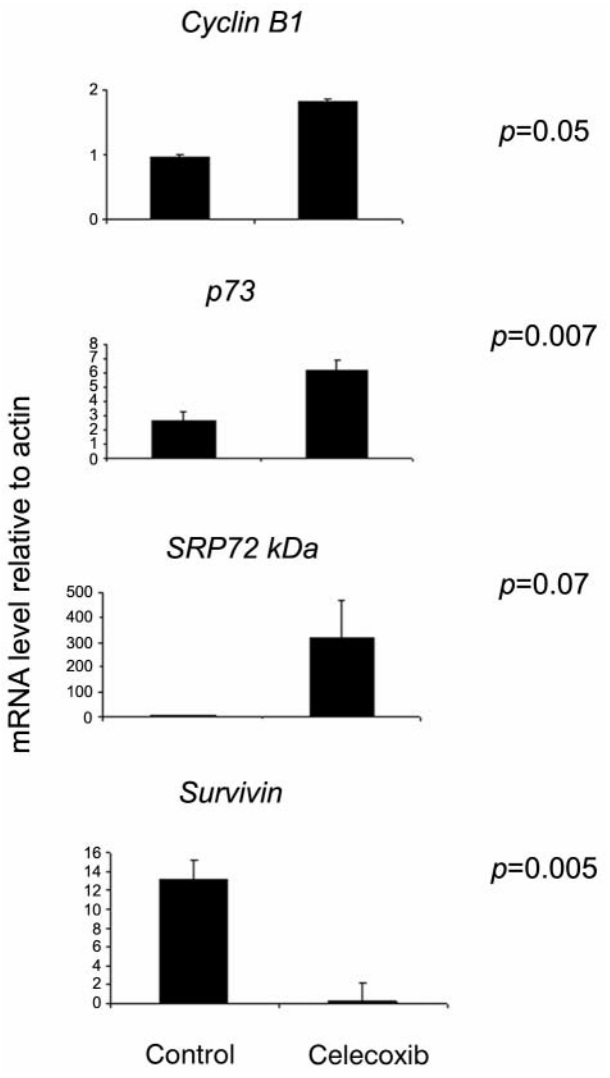

Figure 1. *Quantitative PCR data obtained for control, untreated prostate cancer patients and celecoxib-treated patients. All assays were carried out at least 3 times and levels of statistical significance were calculated using SPSS software with p-values of 0.05 and below being considered significant.*

Discussion

In order to investigate the effects of celecoxib on GEPs, we analysed biopsies from patients treated with celecoxib as well as control patients. After statistical processing, we compiled a list of 24 up-regulated and 3 down-regulated genes in celecoxib-treated *versus* control arm patients. In addition, to the gene list, we looked at the anti-apoptotic factor *survivin* as this was shown to be a very sensitive indicator of the effects of celecoxib in COX-2-expressing prostate cancer cells in our previous studies (11). Due to the stringent filtering criteria used (spot quality, statistical

Table II. *Genes statistically significantly up-regulated in celecoxib-treated patients.*

Gene accession number	Gene description	Ratio of expression celecoxib *vs.* control	Probability of false prediction	Ontology
AK056837	Ribosomal proteinL13a	1.88	0.09	Catalysis of protein synthesis, component of ribosomal 60S subunit
NM_000889	Integrin beta 7	1.87	0.05	Receptor involved in immune developmental synapse formation
NM_006082	Tubulin, alpha, ubiquitous	1.81	0.13	Protein component of tubulin (along with beta tubulin) to form microtubule
NM_000612	Insulin-like growth factor -2	1.81	0.13	Paracrine growth factor, epigenetically regulated in prostate cancer
NM_002734	Protein kinase, cAMP-dependent, alpha	1.81	0.10	cAMP regulation; growth factor promoting cell growth and transformation
NM-006947	Signal recognition particle 72kDa	1.77	0.08	Signalling pathway involving endoplasmic reticulum
NM_003836	Delta-like 1 homolog (drosophila)	1.75	0.12	Member of epidermal growth factor-like homeotic protein family, immune regulatory function
X58529	Immunoglobulin heavy constant mu	1.74	0.13	Immune modulatory function
NM_000023	Sarcoglycan, alpha (50 kDa dystrophin associated glycoprotein)	1.72	0.11	Critical to the stability of muscle fibre membranes and linking of actin to ECM
NM_031966	Cyclin B1	1.71	0.13	Cell cycle regulation, tumour suppressor role
NM_005581	Lutheran blood group (Auberger b antigen included)	1.70	0.12	Member of immunoglobulin superfamily and receptor for lamin
NM_006256	Protein kinase-C-like 2	1.70	0.13	Essential regulator for entry into mitosis and exit from cytokinesis
NM_005427	Tumor protein p73	1.69	0.12	p53-related, tumour suppressor role
NM_006325	RAN, member *RAS* oncogene family	1.69	0.12	GTP-binding protein, cell cycle and DNA synthesis regulation
NM_004494	Hepatoma-derived growth factor (high mobility group protein 1-like)	1.69	0.10	Mitogenic and DNA-binding activity, plays a role in cellular proliferation and differentiation
NM_014977	KIAA0670 protein/acinus	1.68	0.13	Involved in signal transduction involving AKT and susceptibility to caspase-mediated apoptosis
NM_001013	Ribosomal protein S9	1.67	0.11	Encodes a protein that comprises the 40S ribosome subunit
NM_003299	Tumor rejection antigen (gp96) 1	1.66	0.10	Involved in signal transduction, protein folding and degradation
NM_013282	Ubiquitin-like, containing PHD and RING finger domains	1.66	0.12	Member of the RING-finger type E3 ubiquitin ligases, uses histone deacetylase in gene regulation, cell cycle role in G1/S transition
NM_001961	Eukaryotic translation elongation factor 2	1.66	0.12	Essential factor for protein synthesis, promoting GTP-dependent protein translocation
NM_006164	Nuclear factor (erythroid-derived 2)-like 2	1.63	0.13	Encodes basic leucine zipper transcription factors
NM_002512	Non-metastatic cells 2, protein (NM23B)	1.57	0.10	Metastasis suppressor function
NM_005845	ATP-binding cassette, sub family C (CFTR/MRP) member 4	1.57	0.11	Androgen-regulated gene, ATP-binding cassette transporter family member
NM_000224	Keratin 18	1.54	0.13	Type 1 intermediate filament chain component.

significance as opposed to fold change alone) for the microarray data analysis, certain genes may not have been identified. We aimed to look at the *survivin* gene in our patient dataset and so performed q-PCR analysis including other genes that had been identified as being up-regulated in the celecoxib-treated prostate cancer patients.

A hallmark of cancer cells *versus* normal cells is uncontrolled cell division as a consequence of dysregulated

Table III. *Genes statistically significantly down-regulated in celecoxib-treated patients.*

Gene accession number	Gene description	Ratio of expression celecoxib *vs.* control	Probability of false prediction	Ontology
NM_000062	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	0.47	0.001	Involved in regulation of the complement cascade
NM_053025	Myosin, light polypeptide kinase	0.44	0.002	Facilitates myosin and actin interaction, tumour-promoting function
NM_003186	Transgelin	0.38	0.001	Transformation and shape change sensitive protein in fibroblasts, function unclear

cyclins and cyclin-dependent kinases (CDKs). CDKs are necessary for proper progression of the cell cycle through mitosis. A study by Gomez *et al*. (12) established a positive correlation between cyclin B1 protein and apoptotic cell death. Stable overexpression of cyclin B1 in LNCaP and PC-3 cells induced a chemosensitizing effect and conversely an SiRNA approach was shown to decrease apoptosis. In the present study, we were able to demonstrate a clear increase in *cyclin B1* in prostate cancer tissue taken from celecoxib-treated patients, an effect confirmed using q-PCR analysis. Our data are suggestive of a proapoptotic effect orchestrated by induction of *cyclin B1* along with other factors attributable to celecoxib administered to prostate cancer patients.

SRP72kDa was one of the genes up-regulated by 1.8-fold in the celecoxib group. SRP72kDa is essential for protein translocation from the cytoplasm to the endoplasmic reticulum. It has also been shown to undergo post-translational modification during apoptosis (13), and its role in death receptor-mediated processes has recently been reported (14). Hence, SRP72kDa plays a role in protein trafficking which appears to support DR4-mediated apoptosis, associated with the extrinsic pathway of apoptosis. *Nuclear factor (erythroid-derived 2)-like 2* (*NFED2*) was up-regulated in the celecoxib group by 1.6-fold. *NFED2* has been shown to be stimulated by *BRCA* overexpression; BRCA up-regulates the expression of multiple genes involved in the cytoprotective antioxidant response (15). Moreover, these findings may be of relevance to prostate cancer, in addition to inheritable breast cancer with that mutation and consequential dysregulation of *BRCA* is frequently associated, as it is associated with increased risk for the development of this malignancy in susceptible populations (16). *NFED2* is, therefore, an antioxidant response transcription factor and thus preserves genome integrity. Prostate cancer at its various stages can be described as an inflammatory process associated in part with an imbalance of reactive oxygen species (ROS) *versus* antioxidant processes (2). Hence, the effects of celecoxib in promoting antioxidant processes are particularly relavant to the problem of prostate cancer and add further support to its application in this disease setting.

The *p73* tumour suppressor gene was up-regulated by 1.7-fold in the patients treated with celecoxib. In its role as a tumour suppressor, p73 is involved in growth suppression and cell cycle arrest. However, the alternate spliced (truncated) forms of the gene product can undermine or overlap p53 function and have been reported in prostate cancer (17). Although the precise tumour suppressor role for p73 and its interaction with p53 and apoptosis remains unclear, we show a clear induction of wild-type *p73* as a consequence of celecoxib treatment in the present study. A recent report by Beitzinger *et al*. (18) lends further support to the hypothesis that p73 has a tumour suppressor role, independent of the p53 tumour suppressor, and is associated with inhibition of malignant transformation by limiting anchorage-independent growth.

A gene that was shown to be reduced in celecoxib-treated prostate cancer tissue was *myosin light chain kinase*. Inhibition of this enzyme has been shown to reduce the growth of mammary and prostate cancer cells in *in vitro* and *in vivo* models with chemosensitization of cells to the effects of etoposide (19). Thus, the effect of celcoxib has been shown to reduce tumorigenic potential in prostate cancer cells and this has been borne out in a clinical setting in the present study. We have previously shown a very significant reduction of *survivin* in celecoxib-treated COX-2-expressing prostate cancer cells (11) and this is in good agreement with the present study. Survivin is a regulator of apoptosis that plays a significant role in cancer and is expressed in most human tumours whereas in normal differentiated tissues it is more or less undetectable. Krajewska *et al*. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysan *et al*. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabilisation. A report by Yoo *et*

al. (22) described the effects of aspirin (a non-specific-COX-2 inhibitor) in promoting TRAIL-induced apoptosis *via* down-regulation of survivin due to inhibition of E2F-1 binding activity to the *survivin* promoter region.

A study by Febbo *et al*. (23) described a trial involving weekly docetaxel for 6 months in patients with high-risk localized prostate cancer in the neoadjuvant setting, prior to RP. A gene expression profiling approach was also used by these authors to assess the biological effects of the chemotherapy on prostate cancer tissues taken from those patients taken at RP and compared with untreated controls. The magnitude of the gene expression changes seen between the two groups of patients in that particular report was of a similar order to that in the present study, which essentially compared two groups (control and celecoxib-treated) of cancerous tissue, rather than normal adjacent *versus* cancerous tissue, where higher differentials of gene expression have been reported. Nevertheless, the study by Febbo *et al*. (23), taken together with the data described herein, suggest that microarray analysis following neoadjuvant therapy in prostate cancer is feasible and can also form a valuable line of enquiry when exploring the effects of novel targeted therapy. With regard to celecoxib, other clinical studies have shown the beneficial effects in prostate cancer using different endpoints. For example, Pruthi *et al*. (24) demonstrated stabilisation of PSA levels in patients who had been treated with celecoxib for various time periods of up to 18 months, following radiation therapy or radical prostatectomy.

We have successfully completed a randomized clinical trial investigating celecoxib in patients with primary, localized prostate cancer prior to RP. Overall, this study suggests that celecoxib has anticancer effects affecting cell proliferation, promotion of apoptosis, cell communication and integrity, and supports our earlier findings (5, 11). Our results suggest that celecoxib is worthy of further investigation as a novel therapy in prostate cancer.

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