

# Cytochrome P450 3A5 is highly expressed in normal prostate cells but absent in prostate cancer

S Leskelä<sup>1</sup>, E Honrado<sup>2</sup>, C Montero-Conde<sup>1</sup>, I Landa<sup>1</sup>, A Cascón<sup>1</sup>, R Letón<sup>1</sup>, P Talavera<sup>3</sup>, J M Cózar<sup>4</sup>, A Concha<sup>3</sup>, M Robledo<sup>1</sup> and C Rodríguez-Antona<sup>1</sup>

<sup>1</sup>Hereditary Endocrine Cancer Group and <sup>2</sup>Human Genetics Group, Human Cancer Genetics Programme, Spanish National Cancer Center (CNIO), C/Melchor Fernández Almagro, 3, 28029 Madrid, Spain

<sup>3</sup>Department of Anatomic Pathology and Tumor Bank and <sup>4</sup>Department of Urology, Hospital Virgen de las Nieves, Avenida Constitución 100, 18012 Granada, Spain

(Correspondence should be addressed to C Rodríguez-Antona; Email: cristina.rodriguez-antona@cniio.es)

## Abstract

Testosterone is essential for the growth and function of the luminal prostate cells, but it is also critical for the development of prostate cancer, which in the majority of the cases derives from luminal cells. Cytochrome P450 3A (CYP3A) enzymes hydroxylate testosterone and dehydroepiandrosterone to less active metabolites, which might be the basis for the association between CYP3A polymorphisms and prostate cancer. However, it is unknown whether the CYP3A enzymes are expressed at relevant levels in the prostate and which polymorphisms could affect this tissue-specific CYP3A activity. Thus, we measured CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNA in 14 benign prostatic hyperplasias and ten matched non-tumoral/tumoral prostate samples. We found that CYP3A5 mRNA in non-tumoral prostate tissue was 10% of the average amount of liver samples, whereas the expression of the other CYP3A genes was much lower. Similarly to liver, CYP3A5\*3 polymorphism decreased CYP3A5 mRNA content 13-fold. CYP3A5 protein was detected in non-tumoral prostate microsomes by western blot, and immunohistochemistry (IHC) localized CYP3A5 exclusively in the basolateral prostate cells. In contrast to the normal tissue, IHC and RT-PCR showed that tumoral tissue lacked CYP3A5 expression. In conclusion, prostate basolateral cells express high levels of CYP3A5 which dramatically decrease in tumoral tissue. This finding supports an endogenous function of CYP3A5 related to the metabolism of intra-prostatic androgens and cell growth, and that polymorphisms affecting CYP3A5 activity may result in altered prostate cancer risk and aggressiveness.

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## Introduction

Prostate cancer is the second leading cause of cancer death in men, and both genetic and environmental factors have been shown to be important for the development of this disease (Novelli *et al.* 2004). More than 95% of the prostate tumors derive from luminal cells, which grow in an androgen-dependent manner through androgen receptor activation. There are three distinct glandular regions in the human prostate gland: peripheral, central, and transitional zone comprising: 70, 30, and 5% of the normal glandular prostate mass respectively (Villers *et al.* 1991). The peripheral zone is the site of origin of most carcinomas and it is the

most susceptible region to inflammation, the central zone is resistant to both carcinoma and inflammation, while the transitional zone is the site of origin of benign nodular hyperplasia and rarely carcinomas (McNeal 1969, 1978, McNeal *et al.* 1988a,b).

Testosterone is essential for prostate cancer development (Feldman & Feldman 2001) and genetic changes affecting the expression/activity of the enzymes metabolizing androgens can influence prostate cancer progression (Makridakis *et al.* 1997, 1999, Park *et al.* 2006). For this reason, several studies have focused on cytochromes P450 3A (CYP3As) polymorphisms, the rationale being that, in addition to the prominent role of

CYP3A enzymes in the metabolism of over 50% of all clinical drugs (Li *et al.* 1995, Thummel & Wilkinson 1998, Rodriguez-Antona & Ingelman-Sundberg 2006), CYP3A enzymes also metabolize testosterone and dehydroepiandrosterone (DHEA) to hydroxy-metabolites less active and easier to eliminate (Ohmori *et al.* 1998, Kamdem *et al.* 2004, Miller *et al.* 2004). Thus, an alteration in the CYP3A prostate activity could change the local testosterone levels and alter the tissue-specific androgen effects, prostate growth, and cancer development.

The CYP3A subfamily comprises four members: CYP3A4, CYP3A5, CYP3A7, and CYP3A43, which have similar substrate specificities but different expression patterns. CYP3A4 is the predominant form in liver and small intestine (Lin *et al.* 2002). CYP3A5 is also present in liver and gastrointestinal tract but at lower levels and in a bimodal manner due to *CYP3A5\*3*, *CYP3A5\*6*, and *CYP3A5\*7* defective alleles, the last two present only in Africans (Hustert *et al.* 2001, Kuehl *et al.* 2001, Lin *et al.* 2002, Lee *et al.* 2003). CYP3A7 is primarily expressed in fetal stages, but *CYP3A7\*1C* allele triggers its expression in adult stages (Kuehl *et al.* 2001, Burk *et al.* 2002, Sim *et al.* 2005). CYP3A43 mRNA has been detected mainly in the prostate, testis, and liver, but at relatively low levels (Domanski *et al.* 2001, Westlind *et al.* 2001). With respect to the CYP3A prostate expression, Murray *et al.* (1995b) found no CYP3A protein in normal prostate tissue, but they detected expression in 61% of tumor samples. In contrast, Moilanen *et al.* (2007), focusing specifically on CYP3A5, detected CYP3A5 protein in normal and tumoral prostate tissue, but the defective *CYP3A5\*3* allele seemed to have no effect on protein expression. On the other hand, Di Paolo *et al.* (2005) found higher amounts of CYP3A4 than CYP3A5 protein in normal tissue and Zhang *et al.* (2006) found both CYP3A4 and CYP3A5 proteins in most tumoral tissues analyzed. With respect to the mRNA content, CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNAs have been detected in normal and tumoral prostate tissue in different works using different techniques (Finnstrom *et al.* 2001, Stamey *et al.* 2001, Westlind *et al.* 2001, Koch *et al.* 2002). Despite the unclear expression profile and role of the CYP3A enzymes in prostate, several studies have shown an association between different *CYP3A* single nucleotide polymorphisms (SNPs), mainly *CYP3A4\*1B*, and prostate cancer risk and/or aggressiveness, suggesting that CYP3A enzymes may play an important role in prostate cell growth (Rebbeck *et al.* 1998, Paris *et al.* 1999, Plummer *et al.* 2003, Loukola *et al.* 2004, Bangsi *et al.* 2006). However, the high degree of ethnic-specific linkage disequilibrium among all *CYP3A4*,

*CYP3A5*, and *CYP3A7* SNPs prevents the identification of the relevant enzyme that could be involved in intra-prostatic androgen metabolism and cancer development (Thompson *et al.* 2004, 2006).

Thus, this study was aimed at identifying the CYP3A enzyme(s) expressed at relevant levels in normal and tumoral prostate tissue and the mechanisms affecting its expression. For that purpose we used quantitative RT-PCR, western blot, and IHC analysis in a panel of normal and matched tumoral prostate samples.

## Materials and methods

### Human samples

The study included 14 benign prostatic hyperplasia (BPH) tissues and ten matched non-tumoral/tumoral prostate tissues, both frozen and paraffin embedded. Specimens were obtained from the Tumor Bank of the Department of Pathology, University Hospital Virgen de las Nieves, Granada. Twenty-five additional tumoral prostate paraffin sections were obtained from the Department of Pathology from the Hospital of Leon. The specimens were selected from radical prostatectomy by an expert pathologist. Hematoxylin- and eosin-stained sections were examined by a pathologist to determine the percentage of cancer cells in the tumor samples. The Gleason score and T stage of the frozen samples are shown in Table 1. The paraffin samples from the Hospital of Leon had Gleason scores between six and nine and the T stage was T2 or T3 in all cases. In addition, seven liver samples were used for RT-PCR mRNA quantification.

### DNA isolation and genotyping

DNA was isolated from the frozen tissue samples using Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. *CYP3A5\*3* polymorphism (rs776746, 6986A>G, the reference sequence corresponds to accession number NG 000004.2) was analyzed by PCR using specific primers (Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>) and direct sequencing (sequencer 3730, Applied Biosystems, Foster City, CA, USA). The PCR was initiated by an initial step for 2 min at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C, and completed with 7 min at 72 °C.

### RNA isolation and real-time quantitative RT-PCR

RNA was isolated from the frozen tissue using TRI reagent (Molecular Research Center Inc., Cincinnati,

**Table 1** Characteristics of the human prostate samples

| Prostate sample | Age (years) | Tissue             | Gleason score | T stage <sup>a</sup> | Relapse <sup>b</sup> | CYP3A5 genotype |
|-----------------|-------------|--------------------|---------------|----------------------|----------------------|-----------------|
| 112             | 63          | BPH                | –             | –                    | –                    | *1/*3           |
| 113             | 66          | BPH                | –             | –                    | –                    | *3/*3           |
| 114             | 70          | BPH                | –             | –                    | –                    | *1/*3           |
| 116             | 64          | BPH                | –             | –                    | –                    | *3/*3           |
| 118             | 76          | BPH                | –             | –                    | –                    | *3/*3           |
| 120             | 59          | BPH                | –             | –                    | –                    | *1/*3           |
| 121             | 77          | BPH                | –             | –                    | –                    | *3/*3           |
| 122             | 80          | BPH                | –             | –                    | –                    | *3/*3           |
| 123             | 74          | BPH                | –             | –                    | –                    | *3/*3           |
| 124             | 62          | BPH                | –             | –                    | –                    | *1/*3           |
| 125             | 70          | BPH                | –             | –                    | –                    | *3/*3           |
| 126             | 68          | BPH                | –             | –                    | –                    | *3/*3           |
| 127             | 76          | BPH                | –             | –                    | –                    | *3/*3           |
| 002             | 61          | T+ NT <sup>c</sup> | 3+3           | T2C                  | N (22)               | *3/*3           |
| 019             | 69          | T+ NT              | 3+3           | T3B                  | Y (19)               | *3/*3           |
| 049             | 66          | T+ NT              | 3+4           | T3A                  | Y (18)               | *3/*3           |
| 060             | 61          | T+ NT              | 3+3           | T3A                  | N (29)               | *3/*3           |
| 074             | 69          | T+ NT              | 3+4           | T3A                  | Y (16)               | *3/*3           |
| 137             | 67          | T+ NT              | 3+3           | T2B                  | N (25)               | *3/*3           |
| 143             | 53          | T+ NT              | 3+2           | T2B                  | N (14)               | *1/*3           |
| 146             | 61          | T+ NT              | 3+4           | T3A                  | N (23)               | *3/*3           |
| 148             | 71          | T+ NT              | 3+3           | T2B                  | N (23)               | *3/*3           |
| 169             | 51          | T+ NT              | 3+3           | T2B                  | N (14)               | *3/*3           |

<sup>a</sup>Tumor clinical classification corresponds to the 6th edition of the AJCC/UICC TNM classification of malignant tumors.

<sup>b</sup>After surgery, PSA levels below 0.1 mg/ml indicate no relapse (N) and PSA levels above 0.1 mg/ml indicate relapse (Y). The months of follow-up or time to relapse respectively are shown in brackets.

<sup>c</sup>T stands for tumoral and NT stands for non-tumoral tissue in the matched samples.

OH, USA). One microgram of the total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and an oligo dT14 primer following manufacturer's instructions. All the normal tissue samples as well as all carcinomas were from the peripheral zone, while the benign hyperplasia samples were from the transitional zone.

The amounts of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNAs were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems), using specific primers and probes (Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>) at a final concentration of 0.9 and 0.2  $\mu$ M respectively, and the Universal Master Mix (PE Applied Biosystems). The amplification conditions consisted of an initial step at 95 °C for 10 min, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Standard curves were generated with serial 1/10 dilutions of a pool of seven liver cDNAs. For CYP3A5 mRNA quantification, a pool of two CYP3A5\*1/\*3 liver cDNAs were used for the standard curve construction and a pool of four CYP3A5\*3/\*3 liver cDNAs were included for quantification. Normalization was carried out with the internal standard  $\beta$ -glucuronidase (GUS). Negative controls were present

in all series of PCR and all assays were carried out in triplicates.

### Subcellular fractionation of human prostate tissues

Five BPH samples were used to obtain microsomal fractions (samples 112, 113, 114, 118, and 124). The tissues were homogenized with a glass homogenizer in four volumes of ice-cold 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose and protease inhibitors. The resulting homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C, followed by centrifugation at 100 000 *g* for 1 h at 4 °C. The pellet was washed and resuspended in 0.1 M PBS, pH 7.4 with 10% glycerol and protease inhibitors (Roche). Protein content was measured by Bio-Rad protein assay (Bio-Rad laboratories). In all cases we obtained sufficient amount of microsomal fraction to perform immunoblot analyses (in average 1.6 mg microsomal protein/g of starting material).

### Immunoblot analysis

Prostate microsomal proteins were separated by 9% SDS-PAGE using the Mini-PROTEAN III electrophoresis cell (Bio-Rad) and transferred to

polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore, Billerica, MA, USA). Equal loading of proteins was verified by Ponceau S staining. The membranes were blocked and then incubated with an antihuman CYP3A5 antibody (ab22692, Abcam, Cambridge, UK) following manufacturer's instructions. This is a commercial polyclonal antibody raised against a synthetic hexapeptide including the C-terminal of CYP3A5 and reacting exclusively with CYP3A5, effective both for western blot and immunohistochemistry (IHC). After washing, the membranes were incubated with a goat anti-rabbit (DAKO, Copenhagen, Denmark) secondary antibody, and the corresponding horseradish peroxidase signal was visualized using SuperSignal Femto substrate (Pierce, Rockford, IL, USA) and BiomaxLight membranes (Kodak). The CYP3A5 content was determined from standard curves derived from human CYP3A5+P450 reductase Supersomes (BD Biosciences, San Jose, CA, USA). The detection limit under these conditions was of 0.025 pmol CYP3A5/mg microsomal protein.

### Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections were immunohistochemically stained using a polyclonal anti-CYP3A5 antibody (1:20000, ab22692, Abcam). Immunohistochemical staining was performed by the DAKO Envision system (DAKO) with a heat induced, antigen retrieval step. Sections from the paraffin-embedded tissue were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker and finally proteinase K was added for 10 min at room temperature. For the frozen tissues 5 µm sections were cut with a cryostat, dehydrated in 70% ethanol over night, fixed in acetone for 10 min, and stained with the same antibody and dilution used for the paraffin-embedded tissue, without antigen retrieval. Kidney samples were used to optimize the signal detection and the specificity of the signal was assured by following the same immunohistochemical staining procedure but without adding CYP3A5 antibody (Fig. 3E). The results were analyzed by two experienced pathologist (E H and A C) and cytokeratin 34βE12 was used as a marker for prostate basolateral cells.

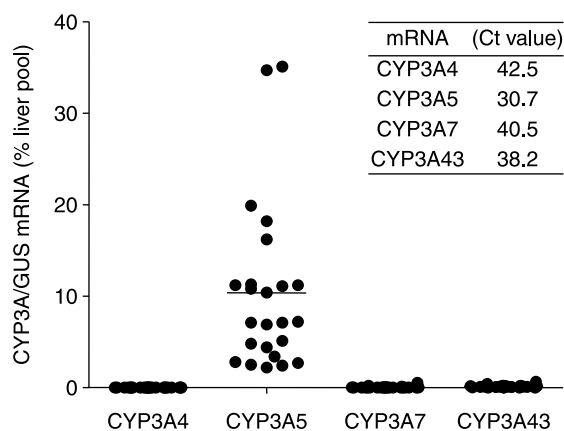
## Results

### CYP3A5 is expressed at high levels in normal prostate tissue and its expression is influenced by CYP3A5\*3 polymorphism

We used quantitative RT-PCR to measure the CYP3As mRNA content in 24 non-tumoral prostate tissues and

compared it with the expression of a pool of livers, which is the tissue with the highest CYP3A content. There were no significant differences in the CYP3As expression between the 14 BPH (from the transitional zone) and the ten non-tumoral (from the peripheral zone) prostate samples, suggesting that the CYP3A expression is not dependent on the prostate zone. Thus, the 24 tissues were analyzed together. We found that CYP3A5 mRNA content in prostate was the highest (10% hepatic levels), while CYP3A4, CYP3A7, and CYP3A43 mRNA content was much lower (0.0004, 0.05, and 0.15% of the liver respectively), and that more than seven PCR amplification cycles separated CYP3A5 content from the other CYP3A isoform (Fig. 1). The prostate CYP3A5 content represented from 7.9 to 69% of the hepatic levels, depending on the liver used for comparison. For CYP3A4 and CYP3A7 the maximum values were below 1%, while for CYP3A43, due to a very low CYP3A43 content in one of the livers up to 5.4% could be observed. Nevertheless the main CYP3A enzyme in prostate was CYP3A5.

CYP3A5 showed a 16-fold inter-sample variability in the 24 prostate cases studied. In the liver, CYP3A5 expression is influenced to a large extent by CYP3A5\*3 polymorphism which, with a 90% frequency in Caucasians, creates a cryptic consensus splice site in intron 3. CYP3A5\*3 results mainly in splice variant



**Figure 1** CYP3A5 mRNA is expressed at relevant levels in prostate normal tissue. The mRNA content of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 was measured by quantitative RT-PCR, as described in Materials and methods section, in 10 normal and 14 BPH prostate tissues. For CYP3A5 the primers used measured the total amount of CYP3A5 mRNA (correctly plus alternatively spliced mRNA). The amount of CYP3A mRNA was normalized with the GUS mRNA content in each sample. The quantification was performed using a pool of seven liver cDNAs and the results are expressed as percentage of the liver expression. The average number of PCR cycles needed to amplify each isoform above the threshold ( $C_t$ ) is shown in the insert.

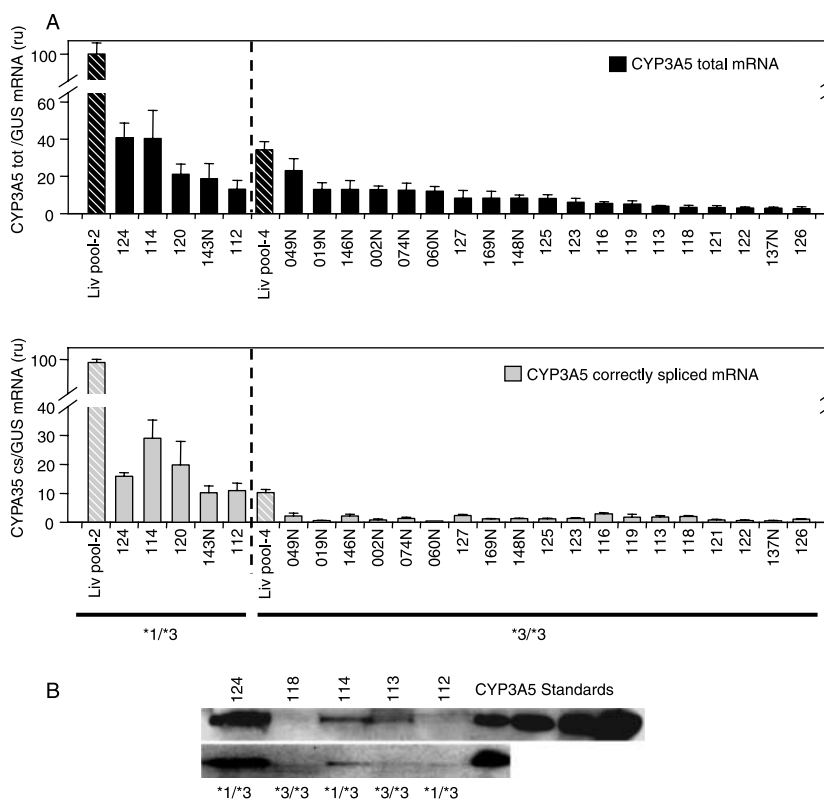
mRNAs that contain a premature termination codon (amino acid 102) and only a small amount of correctly spliced *CYP3A5* mRNA and protein is produced. Thus, to determine the influence of *CYP3A5*\*3 in prostate, we performed *CYP3A5* RT-PCR analysis using liver samples with different *CYP3A5* genotypes and quantified both the correctly spliced and the total (correctly spliced plus alternatively spliced) *CYP3A5* mRNAs. Similar to liver, *CYP3A5*\*3/\*3 prostate samples had lower *CYP3A5* mRNA content than those *CYP3A5*\*1/\*3 (3.3- and 13-fold lower total and correctly spliced *CYP3A5* mRNA in prostate compared with 2.8- and 10-fold difference in the liver samples respectively; Fig. 2A). The *CYP3A5* total mRNA content of the prostate samples was 25% of the average amount in liver samples with the same *CYP3A5* genotype (Fig. 2A upper panel), while for correctly spliced mRNA it was 15% (Fig. 2A lower panel).

To further characterize the expression of *CYP3A5* in prostate, we performed a western blot analysis using a

*CYP3A5*-specific antibody. As shown in Fig. 2B, *CYP3A5* protein was detected in only some BPH microsomal fractions. Although there was not a strong correlation between *CYP3A5* mRNA and protein content, the strongest signals corresponded to *CYP3A5*\*1/\*3 samples. The amount of *CYP3A5* protein in sample 124 (with the highest expression) was of 0.15 pmol *CYP3A5* per mg of total microsomal protein, which is much lower than *CYP3A5* hepatic levels. However, this data should be referred to the prostate cells expressing *CYP3A5*, thus, it is important to determine *CYP3A5* localization.

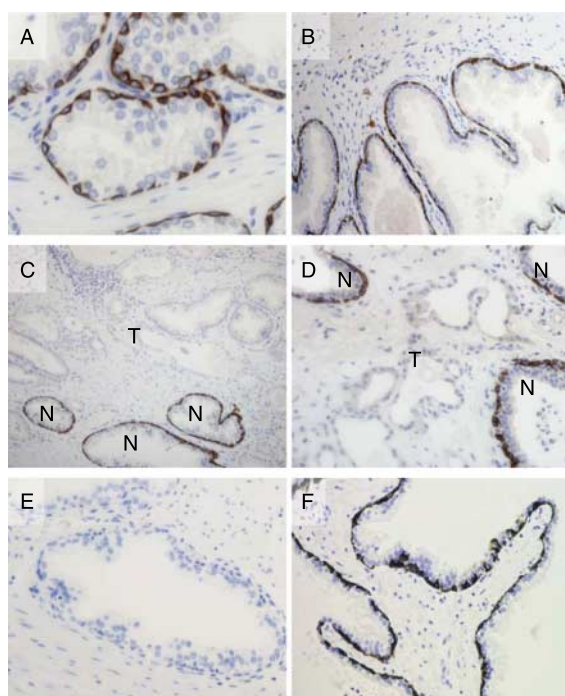
### **CYP3A5 is expressed exclusively in the basolateral cells of the prostate**

IHC was used to determine the localization of *CYP3A5* in the prostate tissue. Both in frozen and paraffined tissues, *CYP3A5* staining was exclusively localized in the basolateral cells in the 24 non-tumoral samples



**Figure 2** *CYP3A5* mRNA and protein content in human prostate. (A) The amounts of total and correctly spliced *CYP3A5* mRNA were quantified in 24 prostate samples and two liver pools using specific primers: the black bars correspond to total (tot) *CYP3A5* mRNA while the gray bars correspond to correctly spliced (cs) mRNA. The *CYP3A5* genotype of the samples is shown below as '\*1/\*3' (five prostate samples and one pool of two livers) and '\*3/\*3', (19 prostate samples and 1 pool of 4 livers). The liver pools are shown with dashed bars. Relative units (ru); liver pool (Liv pool). (B) *CYP3A5* protein expression was analyzed by western blotting in BPH microsomes (samples 112, 113, 114, 118, and 124) using a *CYP3A5*-specific antibody and commercial *CYP3A5* Supersomes were used as standards for quantification. Two independent experiments are shown. The *CYP3A5* genotype of each prostate sample is indicated below.

analyzed, specifically in the cytoplasmatic region, as expected for a microsomal protein (Fig. 3A and B). In contrast, stromal and luminal cells did not express CYP3A5. The prostate basolateral cell marker cytokeratin 34βE12 was used to confirm CYP3A5 basal localization (Fig. 3F). The effect of *CYP3A5\*3* was not evident with the standard IHC conditions. However, when using lower amounts of the antibody, a difference in the intensity of the CYP3A5 signal could be appreciated between *CYP3A5\*1/\*3* and *CYP3A5\*3/\*3* samples (Supplementary Fig. 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). This data implies that since CYP3A5 protein and mRNA quantifications were performed using extracts from total prostate tissue (including stromal, luminal, and basolateral cells), but only the basolateral



**Figure 3** CYP3A5 protein detection by immunohistochemistry in prostate non-tumoral and tumoral tissue. Immunohistochemical staining of CYP3A5 was performed in paraffin sections as described in Materials and methods section. In the non-tumoral tissue (A and B) CYP3A5 was localized in the prostate glands, specifically in the basolateral cells of *CYP3A5\*1/\*3* (143N, A) and *CYP3A5\*3/\*3* (169N, B) samples. Immunohistochemical staining of CYP3A5 in paraffin sections of tumoral prostate samples (C and D) showed that CYP3A5 was absent in the tumoral areas (T) and CYP3A5 signal was localized only in the surrounding normal tissue (N). The tumor samples analyzed were: 143T (C), 169T (D). (E) shows the same immunohistochemical staining procedure in 143N but without CYP3A5 antibody and (F) corresponds to an IHC staining using the basolateral marker cytokeratin 34βE12. Original magnification  $\times 20$ , except for A with  $\times 10$  and for B with  $\times 40$ .

cells contribute to CYP3A5 expression, the actual CYP3A5 levels of the basolateral cells must be higher than the calculated 15–25% of hepatic mRNA levels and 0.15 pmol/mg microsomal protein.

### Tumoral prostate tissue lacks CYP3A5 expression

When we investigated the expression of *CYP3A5* in the ten matched non-tumoral samples by IHC, we found that in all of them there was a complete lack of CYP3A5 protein in the tumoral areas of the tissue and that only the surrounding areas containing non-tumoral normal glands showed CYP3A5 staining (Fig. 3C and D). The lack of CYP3A5 expression in the tumor is consistent with its basolateral localization, since prostate tumors lack basal cells. To confirm this, we analyzed the expression of the basolateral marker 34βE12 and found it absent, similarly to CYP3A5, in the prostate cancer regions (data not shown). The lack of CYP3A5 expression in the tumoral areas was confirmed in 25 additional prostate cancer samples (data not shown). Analysis of the ten matched non-tumoral/tumoral samples by RT-PCR confirmed the IHC results, and we found that CYP3A5 mRNA was significantly lower in the tumoral tissue (in average 61- and 19-fold difference for total and correctly spliced CYP3A5 mRNA respectively; Supplementary Table 2). The detection of some CYP3A5 mRNA by RT-PCR in the tumoral samples can be easily explained by the contribution of contaminating non-tumoral tissue (see Fig. 3C and D). Therefore, the CYP3A5 RT-PCR and the IHC data are in full agreement.

### Discussion

Contradictory and incomplete results have been reported with respect to the expression of the CYP3A enzymes in prostate tissue (Murray et al. 1995a, Finnstrom et al. 2001, Stamey et al. 2001, Koch et al. 2002, Di Paolo et al. 2005, Zhang et al. 2006, Moilanen et al. 2007). In this work, we clearly show that of the four human CYP3A enzymes only CYP3A5 had a relevant expression in prostate, with mRNA contents similar to liver (Fig. 1). Specifically, when comparing the CYP3A5 mRNA content of liver samples with prostate samples with the same *CYP3A5* genotype, the prostate samples showed about 20% of the hepatic levels (Fig. 2A). Among the prostate samples included in this study, there was a large CYP3A5 mRNA variation, mainly caused by *CYP3A5\*3* polymorphism, which introduces an alternative splicing site that in the liver decreases the amount of full-coding transcript and

ultimately causes low CYP3A5 protein expression (Kuehl *et al.* 2001). In this work, *CYP3A5\*1/\*3* prostate samples had 13-fold higher amounts of correctly spliced mRNA than *CYP3A5\*3/\*3* samples and, although the number of samples analyzed was small and the correlation with mRNA levels was not strong, the highest CYP3A5 protein content also corresponded to *CYP3A5\*1/\*3* prostate samples (Fig. 2B). Recently, Moilanen *et al.* (2007) detected CYP3A5 protein in the prostate, but surprisingly *CYP3A5\*3/\*3* samples showed similar or even higher CYP3A5 protein content than *CYP3A5\*1/\*3* samples, and by IHC they found CYP3A5 protein both in luminal and basolateral cells and both in non-tumoral and tumoral prostate tissues. In contrast, in this work we found that CYP3A5 was exclusively expressed in the basolateral cells of the non-tumoral tissue (Fig. 3A and B), while absent in the tumor (Fig. 3C and D). This data were fully supported by the quantitative RT-PCR analysis in the matched non-tumoral/tumoral samples (Supplementary Table 2). The differences between this and other studies are probably caused by the use of antibodies with low specificity (e.g., CYP3A5 IHC signal by Moilanen *et al.* was also localized in the nuclei) which is not in agreement with the microsomal localization of CYP3A5 (compare with Fig. 3A) and in the case of mRNA by the use, in some studies, of non-quantitative PCR methods.

Because CYP3A5 protein is exclusively localized in the basolateral cells, and these represent 2% of the total prostate cells (Liu *et al.* 1997) about 7.5 pmol CYP3A5/mg microsomal basolateral protein can be estimated, which is similar to the 37 pmol found in *CYP3A5\*1/\*3* livers (Liu *et al.* 1997, Westlind-Johnsson *et al.* 2003). Similarly, if we take into account that only the basolateral cells contribute to CYP3A5 expression, the CYP3A5 mRNA levels in the prostate basal cells would be ten times higher than that in the liver cells. The difference between the prostatic and hepatic CYP3A5 mRNA and protein contents could indicate a tissue-specific CYP3A5 posttranscriptional regulation. In any case, the high CYP3A5 prostatic expression suggests that CYP3A5 may play a relevant function in the prostate and, since the prostate is not a tissue relevant for drug metabolism, this function must be related to the metabolism of prostatic endogenous CYP3A5 substrates, such as androgens (Ohmori *et al.* 1998, Miller *et al.* 2004). In other tissues CYP3A5 has also been shown to play an important endogenous function, and *CYP3A5\*3* has been shown to influence the systolic blood and pulse pressure, presumably by altering CYP3A5-mediated glucocorticoid metabolism (Kreutz *et al.* 2005). Thus, by oxidation of testosterone and DHEA in the basolateral prostate cells,

CYP3A5 could control their entrance into the luminal cells which grow in a hormone-dependent manner (Masai *et al.* 1990). In fact, androgens up-regulate CYP3A5 expression in human prostate, suggesting an autoregulatory loop to control testosterone exposure (Moilanen *et al.* 2007). Consequently, polymorphism affecting CYP3A5 activity could alter the oxidation of androgens in the basolateral cells and ultimately luminal prostate cell growth function and prostate cancer development (Parnes *et al.* 2005, Zhenhua *et al.* 2005).

In line with this idea, several publications have associated *CYP3A* SNPs with prostate cancer risk and aggressiveness (Rebbeke *et al.* 1998, Paris *et al.* 1999, Plummer *et al.* 2003, Loukola *et al.* 2004, Zhenhua *et al.* 2005, Bangsi *et al.* 2006). However, most studies have focused on *CYP3A4\*1B*, which does not result in major changes in CYP3A4 expression (Rodriguez-Antona *et al.* 2005) and is not expressed in the prostate (Fig. 1). The *CYP3A* locus shows a high degree of linkage disequilibrium (Kuehl *et al.* 2001, Lee *et al.* 2003), and ~80% of Caucasians carrying *CYP3A4\*1B* are simultaneously *CYP3A5\*1* (Wojnowski *et al.* 2002). In Asians, in which *CYP3A4\*1B* is absent and, thus, is not a confounding factor, *CYP3A5\*1/\*1* men had a 0.23-fold lower risk of developing a low-grade prostate cancer and a 0.31-fold lower risk of developing localized prostate cancer than *CYP3A5\*3/\*3* men (Zhenhua *et al.* 2005). In Africans, in addition to *CYP3A5\*3*, there are two other common functional *CYP3A5* polymorphisms which have not been taken into account in any of the association studies carried out so far: *CYP3A5\*6*, which creates an alternative splicing site similarly to *CYP3A5\*3*, and *CYP3A5\*7* which has a single nucleotide insertion causing a frameshift and early stop codon (with 13 and 10% frequency respectively; Hustert *et al.* 2001, Kuehl *et al.* 2001, Lee *et al.* 2003). Therefore, the association studies in Africans can only be complete when all three *CYP3A5* defective alleles are considered together and compared with the wild-type *CYP3A5\*1* allele. Undoubtedly, further studies are needed to confirm the association of *CYP3A5* functional SNPs with prostate cancer.

In conclusion, we have shown that only *CYP3A5* out of the four *CYP3A* genes is expressed at high levels in the non-tumoral prostate tissue, specifically in the basolateral cells and that this expression does not occur in the tumors. This data reveal an important endogenous role of CYP3A5 in the prostate and association studies between prostate cancer and *CYP3A* polymorphisms indicate that this function must be related to the metabolism of intra-prostatic androgens and regulation of luminal cell growth. Furthermore, this data suggest that future prostate cancer association

studies on CYP3A genes should focus on CYP3A5 functional polymorphisms such as CYP3A5\*3, \*6, and \*7 which could be directly associated with prostate cancer risk and aggressiveness.

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