Heliyon

ELSEVIER

*Received:*
20 November 2015
*Revised:*
24 March 2016
*Accepted:*
25 April 2016

Heliyon 2 (2016) e00104

# Hypoxia regulates Notch-3 mRNA and receptor activation in prostate cancer cells

**Armelle Meunier** [a], **Angela Nilda Flores** [b], **Niamh McDermott** [a], **Karla Rivera-Figueroa** [b], **Antoinette Perry** [c], **Thomas Lynch** [d], **Kathrine Røe Redalen** [e], **Laure Marignol** [a,*]

[a] *Radiobiology and Molecular Oncology, Applied Radiation Therapy Trinity, Trinity College Dublin, Ireland*

[b] *Division of International Health, Mount Sinai School of Medicine, New York, USA*

[c] *Cancer Biology and Therapeutics Laboratory, Conway Institute, University College Dublin, Ireland*

[d] *Department of Urology, St James's Hospital, Dublin 8, Ireland*

[e] *Department of Oncology, Akershus University Hospital, Lørenskog, Norway*

* Corresponding author at: Applied Radiation Therapy Trinity, Discipline of Radiation Therapy, Trinity Centre for Health Sciences, St James's Hospital, Dublin.

E-mail address: marignol@tcd.ie (L. Marignol).

## Abstract

The Notch-3 receptor is a recognized key regulator of vascular responses and is increasingly associated with tumorigenesis. Hypoxia-inducible factors activate specific signaling pathways such as Notch in a number of cellular models. This study aimed to evaluate the regulation of Notch-3 by hypoxia in prostate cancer cells. Notch-3 gene and protein expression was established in a panel of aerobic and hypoxic prostate cell lines *in vitro*, the CWR22 xenograft model and RNA extracted from low grade (Gleason score $< = 6$); high grade (Gleason score $> = 7$); non-hypoxic (low HIF, low VEGF); hypoxic (high HIF, high VEGF) patient FFPE specimens. *NOTCH-3* was upregulated in PC3 (3-fold), 22Rv1 (4.1-fold) and DU145 (3.8-fold) but downregulated in LnCaP (12-fold) compared to the normal cell lines. *NOTCH-3* expression was modified following hypoxic exposure in these cells. *NOTCH-3* was upregulated (2.2-fold) in higher grade and hypoxic tumors, when compared to benign and aerobic pools. In the CWR22 xenograft model, Notch-3 expression was restored in castrate resistant tumors. Nuclear translocation of the Notch-3 intracellular domain was no longer detected following

http://dx.doi.org/10.1016/j.heliyon.2016.e00104

exposure of cells to hypoxia but not associated with a change in expression of HES-1. Our data further identifies Notch-3 as a potentially key hypoxic-responsive member of the Notch pathway in prostate tumorigenesis.

Keywords: Medicine, Cell biology

## 1. Introduction

Notch-3 is one of the four receptors of the Notch pathway, whose interaction with associated ligands (Jagged 1 & 2, and Delta-like homologues 1–4) regulates a number of biological functions such as differentiation, proliferation, angiogenesis, apoptosis and stem cell renewal [1, 2, 3, 4]. Notch-3 is emerging as a potential marker of treatment resistance in several cancer types and was identified as a novel exosomal protein in the DU145 prostate cancer cell line [5]. Recognized as a major signaling pathway that is deregulated in cancer [6, 7], the Notch pathway has been proposed to facilitate prostatic tumorigenesis, influence the outcome of anti-cancer hormonal [8, 9] and docetaxel treatments [10] and may be particularly involved in the development of prostate cancer in men with high body mass index [11]. As a result Notch inhibition is being explored for the simultaneous targeting of several cancer properties and represents an attractive novel anti-cancer therapy [12].

Activation, regulation, and degradation of the Notch signal require endosomal trafficking of both ligands and receptors, [13, 14] which may be negatively controlled by the Numb protein, an important cell fate determinant [15, 16]. Cleavage of the Notch receptors following ligand interaction releases the Notch intracellular domain (NICD) to allow its translocation into the nucleus and induction of downstream target genes expression, such as several helix-loop-helix transcription factors named Hairy/Enhancer of split HES and HEY [17, 18]. While accumulated evidence mostly relates to the upregulation of the Notch-1 receptor in prostate tumors, [19, 20] expression of the Notch-3 and Notch-4 receptors in cancer has been less commonly reported, but they appear elevated in glioblastoma, [21] salivary adenoid cystic carcinoma, [22] breast, [23, 24] cervical [25] and ovarian cancer [26, 27]. In prostate cancer patients, Notch-3 expression was positively correlated to Gleason score [4]. The mechanistic significance for the preferential expression of each Notch receptor in tumors however remains to be elucidated.

Tumor hypoxia is a common feature of prostate tumors that is associated with disease progression and treatment resistance [28]. Notch activity has been associated with vascular endothelial growth factor (VEGF) [29] and hypoxia inducible factor 1 alpha (HIF-1α) expression, [30] two well-recognized markers of tumor hypoxia. Many oxygen-responsive genes are regulated by the hypoxia-inducible factor-1 (HIF-1) complex. HIF-1α overexpression, evidenced by increased immunostaining, has been reported in a variety of human cancers


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

and their metastases, including prostate cancer [31, 32]. Evidence of a relationship between the Notch-, androgen- and hypoxia-responsive pathways is accumulating, and could be centrally controlled by the HIF-1α transcription factor [33, 34, 35].

The Notch signaling pathway is essential to the regulation of blood vessel structure [36, 37] and relates to the altered microvasculature and vascular function within tumors (including those of the prostate) [38, 39]. The Notch-3 receptor is particularly involved in the ischemic response of blood vessels [40] and has been proposed to facilitate breast cancer stem-cell survival under hypoxic conditions [23]. The activation of Notch-3 signaling was reported in hypoxic prostate cancer [4]. *In vitro,* exposure of LnCaP prostate cancer cells to hypoxic conditions (2% oxygen) induced Notch-3 receptor activation and compromised both proliferative activity and migration into lipid rafts [4]. In patient biopsy specimens, Notch-3 immunostaining correlated with expression of the carbonic anhydrase IX hypoxia marker [4].

This study expands this work. We initially examined the expression patterns of Notch ligand and receptors in prostate specimen prior to further evaluation of the Notch-3 receptor in prostate cancer models. We identify potential regulation of Notch-3 by androgens in prostate cancer xenografts. We report a role of hypoxia in the regulation of *NOTCH-3* mRNA levels *in vitro* and in patient specimens. We confirm modification of Notch-3 protein levels by hypoxia in additional prostate cancer cell lines and document reduction in activation and nuclear translocation of the Notch-3 intracellular domain under these conditions.

## 2. Materials and methods

### 2.1. *In vitro* models

Human prostate cancer cell lines (22Rv1, DU145, PC3, LnCaP) (ATCC, UK) were maintained in RPMI 1640 medium (Gibco, UK) supplemented with 10% foetal calf serum (Globepharm, UK) and 1% streptomycin-penicillin (Gibco, UK). Normal human prostate cell lines PWR-IE and RPWE-1 were routinely maintained in Keratinocyte SFM medium (Gibco, UK) supplemented with Bovine Pituitary Extract, recombinant Epidermal Growth Factor and 1% streptomycin-penicillin (Gibco, UK). Hypoxic conditions (0.5% oxygen, 48 hours) were achieved in a 1000 *in vivo* hypoxic chamber (BioTrace. UK).

### 2.2. *In vivo* model of castration resistance

Tissue specimens were obtained from CWR22 prostate carcinoma xenograft prepared as described in Roe et al. [41]. Briefly, 8 mm diameter ADT-naïve CWR22 tumor xenografts were allowed to grow to 12 mm prior to surgical


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

castration. Tumor diameter was monitored. Tissue samples were collected at baseline, when tumor diameter reached 8 mm (~one month post castration), 4 mm (~four month post castration) and in relapsing tumors, when the tumor diameter returned to 8 mm.

## 2.3. NOTCH-3 RT-PCR

RNA was extracted from 60–80% confluent aerobic and hypoxic cell culture flasks or pooled CWR22 tissue samples (N = 3/pool) using Qiagen RNA extraction kit (Qiagen, Crawley, UK). NOTCH-3 gene expression was examined using a pre-optimized single Taqman® assay (Applied Biosystem, UK). The comparative ΔΔCT method was used to analyze results which were normalized to β-actin housekeeping gene expression, in three biological replicates of each cell line. The benign PWR1E cell line was used as a reference sample to determine Notch-3 expression patterns associated with disease progression. Aerobic samples were used as reference to determine hypoxic induction of the gene in each cell line. Baseline tissue samples were used as reference to Notch-3 expression patterns in our CW22R castration resistance model.

## 2.4. Protein extraction

Prostate cells/CW22R tissue were trypsinised, and centrifuged (3 min, 1500 rpm, 4°C). To generate whole cell lysates, the pellet was resuspended in cold RIPA lysis buffer supplemented with a protease inhibitor cocktail (Santa Cruz, UK). Following 10 min incubation on ice, the lysates were centrifuged at 4°C for 20 min (15,000 rpm). Separation of nuclear and cytoplasmic proteins was achieved using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Fisher Scientific, UK) according to manufacturer's instruction.

## 2.5. Western blotting

Equal amounts of protein from the lysates were separated by electrophoresis on 6% polyacrylamide gels and then transferred onto Hybond-C Super nitrocellulose membrane (Amersham, UK). Western blots were probed with either rabbit monoclonal anti-Notch-3 antibody (#ab23426, 1:250 in milk, Abcam, UK), goat anti-NUMB (#ab4147, 1:1000 in milk, Abcam) or rabbit anti-HES1 (#ab41970, 1:1000 in BSA, Abcam). After washing, the membrane was incubated with Horseradish peroxidase conjugated goat secondary antibody (sc-2020, 1:1000 in BSA, Santa Cruz) or Anti-Rabbit IgG HRP Linked (#7074, 1:1000 in BSA, Cell signaling) antibodies. Pierce Luminal kit (Pierce, UK) was used for protein detection. Membranes were stripped prior to reprobing with either a mouse monoclonal anti-actin (1:10000 in milk, Sigma-Aldrich, UK), rabbit anti-tubulin (#2148S, 1:2000 in BSA, Cell Signalling), rabbit anti-Mek 1/2 (#8727, 1:1000 in


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

BSA, Cell Signalling, UK) or rabbit anti-histone H3 (#4499, 1:1000 in BSA, Cell signaling) antibodies.

## 2.6. Human prostate tissue specimen profiling

This study was approved by the Adelaide and Meath Hospital (AMNCH) and St. James's Hospital Ethics committee, Dublin, Ireland. Prostate tumors and histologically normal adjacent formalin fixed paraffin embedded (FFPE) tissue from patients with primary disease treated by radical prostatectomy at AMNCH were obtained retrospectively in accordance to the approved guidelines, as described previously [42]. All patients provided written informed consent. To overcome the limited amount of RNA obtained from FFPE tissues, RNA samples were pooled. Four different pools were generated: prostate cancer, high grade prostatic intraepithelial neoplasia (HGPIN), histologically benign (HB) and benign prostatic hyperplasia (BPH). Prostate cancer pools were further subdivided into Gleason score $\geq 7$ and Gleason score $\leq 6$. Similarly, RNA was additionally extracted from Gleason score $\leq 6$ tumor specimens previously immunostained for HIF-1α and VEGF 39 to generate both "aerobic" (low HIF-1α, low VEGF staining intensity) and "hypoxic" (high HIF-1α, high VEGF staining intensity) pools. Each pool consisted of DNase treated total RNA (100 ng), isolated from microdissected tissue from four individual cases. cDNA (100 ng/sample) was prepared using a RNA-to-cDNA kit (Applied Biosystem, Warrington, UK) according to manufacturers' instructions and loaded on microfluidic cards. A low density array approach (Applied Biosystems, UK) was used to determine the expression of the Notch receptors (Notch 1–4) and ligands (Jagged-1, DLL1, DLL4). The comparative ΔΔCT method was used to analyze the results which were normalized to Phospho-Glucose-Kinase-1 (PGK-1) housekeeping gene expression or β-actin for experiments involving hypoxia. The BPH pool was used as a reference sample to examine disease progression. The aerobic pool was used as a reference sample to examine hypoxia.

## 2.7. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was calculated using SPSS software version 20.0. Differences in relative gene expression were compared using student t-tests. A p-value of $<0.05$ was considered statistically significant. Data are presented as Mean ± Standard Error of the Mean.


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

## 3. Results

### 3.1. NOTCH-3 expression is elevated in high grade (Gleason ≥7) prostate tumors

The gene expression levels of Notch receptors (1–4) and ligands (Jagged 1, DLL1, DLL4) were determined in a series of pooled human BPH, HB, HGPIN, low grade (Gleason ≤6) and high grade (Gleason ≥7) specimens (Fig. 1). The relative Ct quantification method was used to determine the expression levels of these genes with prostate tumorigenesis (BPH, HGPIN, low grade and higher grade tumors) relative to BPH specimens. All Notch ligands and the *NOTCH-2* receptor were expressed in BPH specimens. *JAGGED-1*, *DLL-1* and *NOTCH-2* expression levels were relatively stable across all pools. *DLL-4* expression was significantly reduced in HGPIN ($p < 0.001$) high grade tumors ($p < 0.001$). *NOTCH-1* expression was significantly increased in all pools ($p < 0.001$). *NOTCH-4* was detected in HB specimens only. *NOTCH-3* expression was significantly increased in HB specimen ($p < 0.001$), lost in HGPIN and detected at progressively higher levels in low ($p < 0.001$) and high grade tumors ($p < 0.001$).

### 3.2. Notch-3 expression patterns in prostate cancer cells

*NOTCH-3* mRNA levels were determined in a panel of prostate cancer cell lines (22Rv1, androgen-sensitive; LnCaP, metastatic androgen-sensitive; DU145 and PC3, metastatic, androgen-refractory) and compared to basal levels measured in a benign prostate line (PWR1E) (Fig. 2A). In androgen-sensitive cell lines, *NOTCH-3* expression was significantly upregulated in 22Rv1 ($p = 0.004$) but

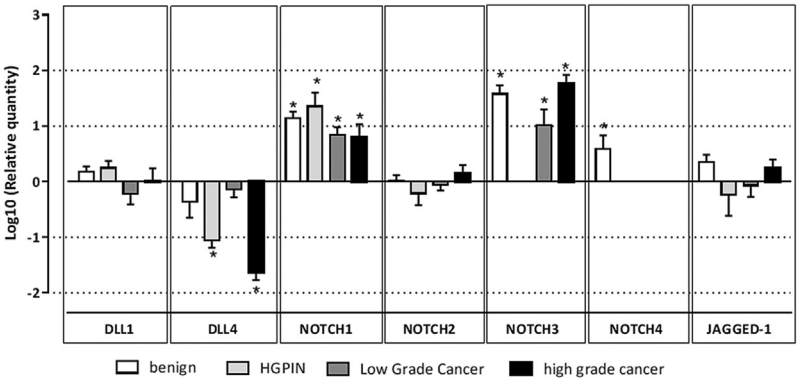


**Fig. 1.** Expression pattern of Notch ligands and receptors in human prostate tissue specimens. A low density gene array approach was used to compare expression of genes in the Notch signalling pathway between histologically benign, BPH, HGPIN and malignant prostate specimens (low grade (Gleason score < = 6), high grade (Gleason score > = 7)) in pooled mRNA (N = 4 patients/pool). The $2^{-\Delta\Delta CT}$ method was used for quantification of each sample relative to BPH control. The Log10 Rq values are presented in each pool. N = 2, mean ± SEM. *, $p < 0.05$.


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

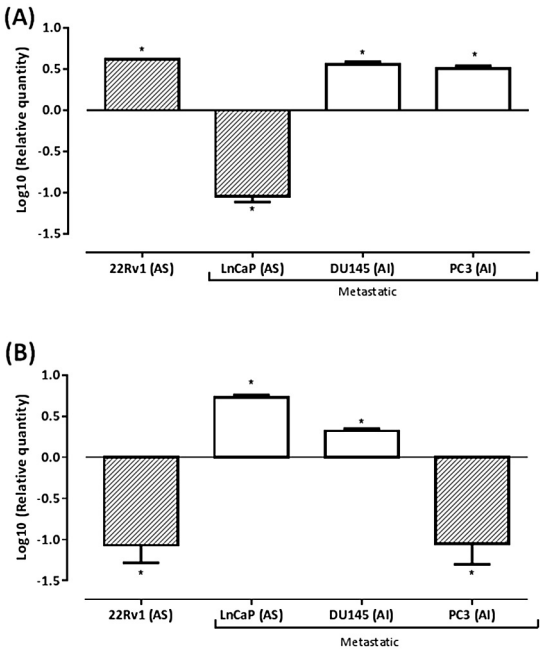


**Fig. 2.** *NOTCH-3* expression in prostate cancer cell lines. *NOTCH-3* mRNA levels were measured in a panel of prostate cell lines. (A) *NOTCH-3* Log10 Rq values in *in vitro* models of primary (22Rv1, androgen-sensitive (AS)) and metastatic androgen-sensitive (LnCaP), and androgen-independent (AI) (DU145, PC3) cell lines relative to PWRE1 benign prostate cells. (B) *NOTCH-3* Log10 Rq values in hypoxic (0.5% $O_2$, 48 hrs) relative to aerobic prostate cancer cell lines. N = 3, mean ± SEM. *, $p < 0.05$.

strongly downregulated (12-fold) in the LnCaP metastatic line ($p = 0.007$). *NOTCH-3* expression was significantly upregulated in the two other androgen-independent metastatic lines tested (DU145 ($p = 0.04$), PC3 ($p = 0.04$)). This pattern was confirmed by western blot analysis for the full-length Notch-3 receptor in whole cell lysates (Fig. 3A). Notch-3 activation was evidenced through the detection of the nuclear intracellular domain (N3ICD). Notch-3 expressing 22Rv1, PC3 and DU145 cells also expressed the notch regulatory NUMB protein and the Notch-target HES-1 (Fig. 3B).

## 3.3. Notch-3 expression patterns in androgen independent prostate cancer cells

Notch-3 expression was examined in an *in vivo* xenograft model of castration resistance. Notch-3 gene and protein expression levels were measured at baseline, one month and four month following castration and in relapsing tumors. A trend towards a reduction in *NOTCH-3* mRNA levels following castration was observed. Castration-induced decrease in Notch-3 levels was confirmed by Western blot. Detection of the receptor was reduced in long-term castrated xenografts and restored to baseline levels at relapse (Fig. 4).


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

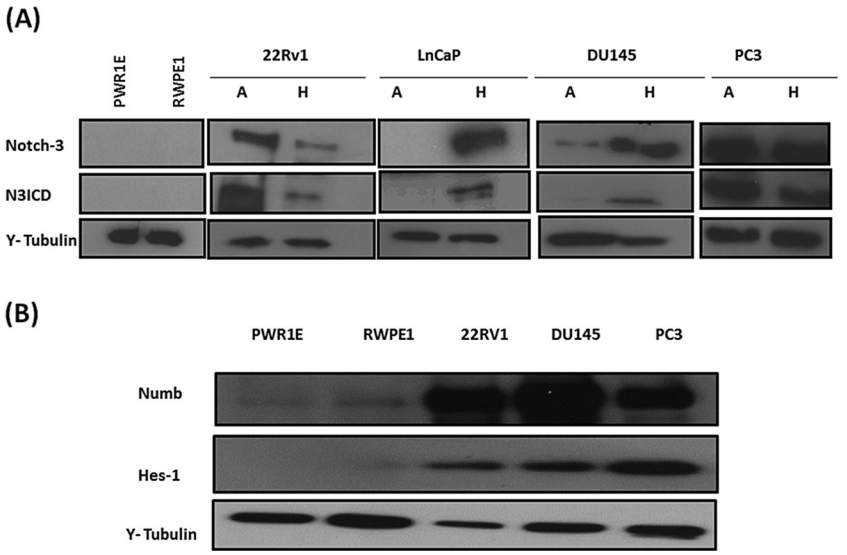


**Fig. 3.** Notch-3, NUMB and HES-1 protein expression in prostate cancer cell lines. (A) Representative immunoblot for the full length Notch-3 protein (244 kDa) and its truncated form (N3ICD (120KDa)) in PWR1E and RWPE1 normal prostate cells and a panel of aerobic and hypoxic prostate cancer cells (22Rv1, DU145, PC3). (B) Representative immunoblot for the NUMB and HES-1 proteins in PWR1E and RWPE1 normal prostate cells and a panel of prostate cancer cells. (22Rv1, DU145, PC3). Tubulin immunostaining was used as a loading control. A: aerobic, H: hypoxic (0.5% $O_2$, 48 hrs) The full western blots are presented in the supplementary material.

## 3.4. Hypoxia prevents nuclear translocation of the Notch-3 nuclear intracellular domain

We next determined whether Notch-3 was responsive to hypoxic shock (48 hrs, 0.5% $O_2$). *NOTCH-3* expression in hypoxic cells was measured in our panel of prostate cell lines and compared to that of aerobic controls (Fig. 2B). *NOTCH-3* was increased in response to hypoxic exposure in DU145 (2-fold) ($p = 0.04$) and LnCaP (2-fold) ($p = 0.04$) but downregulated in 22Rv1 (15-fold) ($p = 0.02$) and PC3 (20-fold) ($p = 0.02$) cells. These changes in gene expression correlated with up- or downregulation of the full length Notch-3 receptor protein, as evidenced by western blotting in aerobic and hypoxic whole cell protein lysates (Fig. 3A). Hypoxia appeared to downregulate Notch-3 protein levels in 22Rv1 and PC3 cells, while the protein was detected as higher levels in both LnCaP and DU145 cells. Notch-3 activation was evidenced through the detection of the nuclear intracellular domain (N3ICD). To further examine the regulation of Notch-3 by hypoxia, expression of N3ICD was measured in nuclear and cytoplasmic protein lysates in 22Rv1, DU145 and PC3 cells (Fig. 5A). The N3ICD was detected in the nucleus of aerobic cells whereas in samples collected from hypoxic cells, amounts were below detection levels. To begin to evaluate the potential consequences for this reduction, the expression of NUMB, RBPSUH and HES-1 was determined. However no modifications in expression patterns in hypoxic, compared to aerobic samples were detected (Fig. 5B).


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

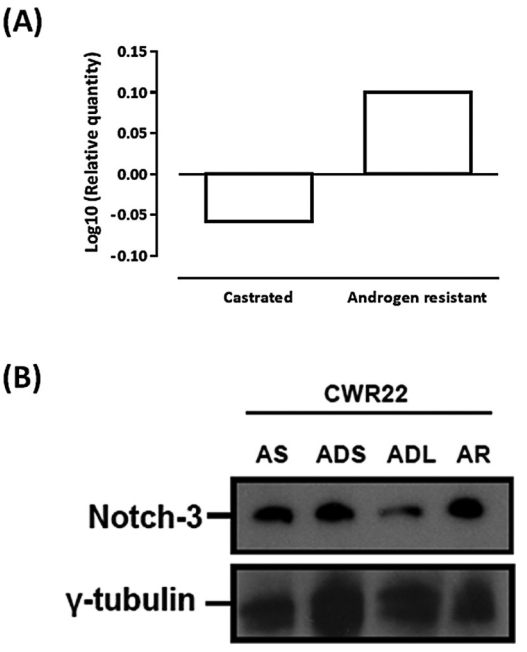


**Fig. 4.** Notch-3 expression in CWR22 xenografts. (A) *NOTCH-3* Log10 Rq values measured in castrated and castration resistant relative to androgen sensitive CWR22 pooled tissue specimens. (B) Representative immunoblot of full length Notch-3 receptor in androgen sensitive (AS), one month post castration (ADS), 7 month post castration (ADL) and in androgen resistant relapsed tumors (CR). Tubulin immunostaining was used as a loading control. The full western blots are presented in the supplementary material.

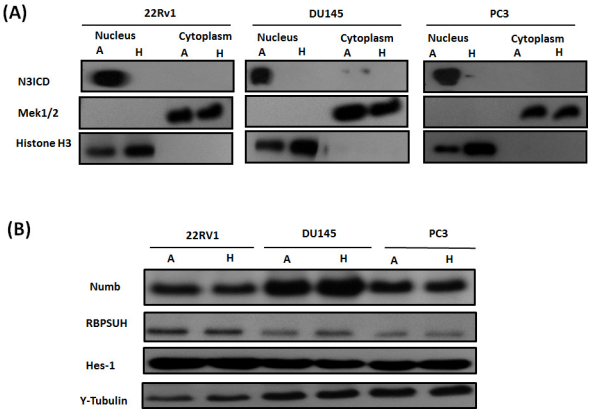


**Fig. 5.** Loss of notch-3 intracellular domain nuclear translocation under hypoxic conditions. (A) Representative immunoblot for the truncated Notch-3 protein (120 kDa) in the cytoplasmic and nuclear protein fraction of 22Rv1, DU145 and PC3 cells. (B) Representative immunoblot for the NUMB, RBPSUH and HES-1 protein in aerobic and hypoxic 22Rv1, DU145 and PC3 cells. A: aerobic, H: hypoxia (5% $O_2$, 48 hrs). The full western blots are presented in the supplementary material.


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

### 3.5. NOTCH-3 mRNA levels are elevated in hypoxic tumor specimen

To further elucidate potential role of hypoxia on *NOTCH-3*, expression was determined in low grade (Gleason score ≤6) human tumor specimens, whose HIF-1α and VEGF immunostaining intensities were previously characterized [43]."Hypoxia" was defined as combined high HIF-1α and high VEGF staining intensities, whereas samples staining weakly for both markers were considered "aerobic". Relative to benign specimens, *NOTCH-3* expression was significantly reduced in the aerobic pool ($p = 0.049$) and non-significantly elevated in the "hypoxic" pool (Fig. 6). *NOTCH-3* expression was significantly higher in the hypoxic, when compared to the aerobic pools ($p = 0.005$).

## 4. Discussion

The intrinsic role of the Notch pathway in prostate tumorigenesis is increasingly documented [35]. The tumor- and metastatic-specific expression patterns of Notch family members have predominantly identified the Notch-1 receptor as a key marker of Notch signaling deregulation in prostate cancer. Despite evidence for its elevated expression in tumors [6, 19, 20], diminished expression of its cleaved form and its downstream effector HEY-1 was reported in adenocarcinoma foci when compared with benign tissues [44]. The mechanistic basis for this loss remains unexplained, but it correlates with loss of phosphatase and tensin homolog gene (PTEN) expression [44] and calcium/calmodulin-dependent kinase II overexpression [45]. Highly involved in tumor vasculogenesis [46], DLL-4 expression is low in prostate tissue [47].

Along with Notch 2 and Notch 4, the Notch 3 receptor has been less extensively studied than Notch 1. Despite previous report of undetectable expression of Notch 3 and Notch 4 in PC3 cells, [19] our examination of the mRNA levels of Notch receptors and ligand identified significant upregulation of Notch-1 and Notch-3

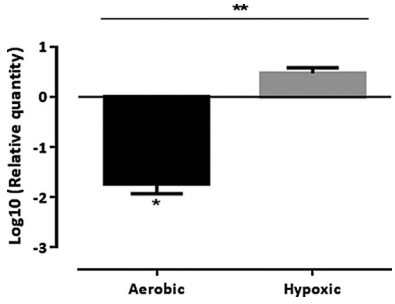


**Fig. 6.** Notch-3 expression in hypoxic prostate cancer. *NOTCH-3* Log10 Rq values in pooled mRNA samples from prostate cancer biopsies associated with low ("aerobic") and high ("hypoxic") HIF and VEGF immunostaining. N = 2, mean ± SEM. *, $p < 0.05$.


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

and downregulation of DLL4 expression in prostate cancer, when compared to BPH patient specimens. Similarly. Danza et al. identified a significant positive correlation between Notch-3 immunostaining intensity and Gleason grade, with a higher frequency of Notch-3 nuclear staining in high grade tumours [4].

Our study identifies that Notch-3 mRNA levels and protein expression are elevated in a panel of prostate cancer cell lines (22Rv1, DU145 and PC3 cells), when compared to the PWR1E normal prostate cell lines. Notch-3 was downregulated at both mRNA and protein levels in the metastatic LnCaP cell line, consistent with the reported increased Notch-3 expression in DU145 and PC3 cells compared to LnCaP [4]. The same tumor specific pattern of expression was present when the Numb protein was examined. The intricate relationship between Notch and Numb is poorly reported in prostate cancer, but is likely a key factor to prostate tumorigenesis [48]. The presence of Notch-3 and Numb did not correlate with detection or absence of the Notch-target protein HES-1. While this suggests that HES-1 may not be regulated by Notch-3 in these cells, examination of the Notch pathway in the TRAMP mouse model identified a significant functional role of the Jagged1-2/Notch3/Hey1 axis in prostate tumorigenesis [47].

Comparison of the levels of Notch-3 expression in these three metastatic cell lines additionally suggests potential involvement of Notch-3 in the transition from androgen-sensitive (LnCaP) to androgen-resistant state (DU145 and PC3 cells). This hypothesis was supported by the observed trend toward initial reduction in Notch-3 mRNA and protein levels following castration of CWR22 xenografts and restoration of expression with the emergence of a castration resistant relapsing tumor. A cross-talk between Notch and androgen signaling has been proposed though Jagged1 overexpression [49], sequestration of Hey-1 in the cytoplasm of prostate cancer cells [50, 51] and numb upregulation [52]. Further evaluation of the inter-relationship between androgen signaling and Notch-3 is warranted.

A number of parallels exist between the cellular behavior of notch-activated and hypoxic prostate cancer cells [35], but direct evidence of this cross talk is scarce. Reports of the hypoxia responsiveness of the Notch pathway consistently highlight the Notch-3 receptor as a key member of this response [4, 53, 54]. The regulation of the Notch-3 receptor was reported in hypoxic LnCaP cells and correlated to carbonic anhydrase IX staining intensity in prostate cancer specimen [4]. Notch-3 expression was preserved and regulated cell proliferation in LnCaP cells chronically exposed to 2% oxygenation levels [4]. Our results strengthen evidence for a role of Notch-3 in hypoxic prostate cancer. We confirm modification of Notch-3 expression and activity under 0.5% oxygenation in 22Rv1, DU145, PC3 in addition to LnCaP cells. This induction did not correlate with a modification in the level of Numb or RBPSUH protein expression levels. At this low level of oxygen, examination of the cytoplasmic and nuclear protein fraction identified loss of the


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

Notch-3 intracellular domain in the nuclear and cytoplasmic fraction of hypoxic 22Rv1, DU145 and PC3 cells. Notch-3 nuclear activity was increased in LnCaP cells chronically exposed to 2% oxygenation levels [4]. Our results suggest possible dependence of Notch-3 regulation with oxygen levels. At very low oxygen levels, hypoxia appears to either inhibit cleavage of the receptor in these cells or prevent ligand-receptor interaction. Gamma-secretase activity seems increased by (1%) hypoxia [3] in a possibly HIF-1 α dependent process [3], whereas at 2% oxygen, mRNA and protein expression levels of Notch ligands (Jagged-1, Jagged-2, DLL1 and DLL-4) were significantly reduced in PC3, DU145 and LnCaP cells [54].

Analysis of *NOTCH-3* mRNA levels in patient specimens confirmed previous reports of a tumour-specific pattern that correlates with the degree of malignancy and hypoxia [4, 55]. The identification of elevated *NOTCH-3* in specimen defined as hypoxic through analysis of alternate hypoxia markers (HIF-1α and VEGF) than previously used (carbonic anhydrase IX) further support the need to evaluate Notch-3 in hypoxic prostate tumours. In light of the reported increased nuclear localization of Notch-3 in high grade tumours [4], evaluation of the clinical relevance of the cytoplasmic and nuclear localization of this receptor is particularly required.

In conclusion, this study strengthens previous evidence for a role for Notch-3 in the regulation of prostate cancer cells under hypoxic conditions with potential implications for Notch-targeted therapies. Further evaluation of activation mechanisms of the Notch-3 receptor and characterization of its target genes are warranted to define the clinical relevance of the Notch-hypoxia interaction in prostate tumors.

## Declarations

## Author contribution statement

Armelle Meunier, Angela N. Flores, Niamh McDermott, Karla Rivera-Figueroa, Antoinette Perry, Thomas Lynch, Kathrine Røe Redalen, Laure Marignol: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

## Funding statement

This work was supported by the Irish Cancer Society (PCA12MAR) and the National Center on Minority Health and Health Disparities of the National Institutes of Health (MD001452), MHIRT at Mount Sinai School of Medicine.


http://dx.doi.org/10.1016/j.heliyon.2016.e00104

## Competing interest statement

The authors declare no conflict of interest.

## Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2016.e00104

## References

[1] S. Chiba, Notch signaling in stem cell systems, Stem Cells 24 (2006) 2437–2447.

[2] S. Artavanis-Tsakonas, M.D. Rand, R.J. Lake, Notch signaling: cell fate control and signal integration in development, Science 284 (1999) 770–776.

[3] J.C. Villa, D. Chiu, A.H. Brandes, F.E. Escorcia, C.H. Villa, W.F. Maguire, C.J. Hu, E. de Stanchina, M.C. Simon, S.S. Sisodia, D.A. Scheinberg, Y.M. Li, Nontranscriptional role of Hif-1alpha in activation of gamma-secretase and notch signaling in breast cancer, Cell Rep. 8 (2014) 1077–1092.

[4] G. Danza, C. Di Serio, M.R. Ambrosio, N. Sturli, G. Lonetto, F. Rosati, B.J. Rocca, G. Ventimiglia, M.T. del Vecchio, I. Prudovsky, N. Marchionni, F. Tarantini, Notch3 is activated by chronic hypoxia and contributes to the progression of human prostate cancer, Int. J. Cancer 133 (2013) 2577–2586.

[5] J. Webber, T.C. Stone, E. Katilius, B.C. Smith, B. Gordon, M.D. Mason, Z. Tabi, I.A. Brewis, A. Clayton, Proteomics analysis of cancer exosomes using a novel modified aptamer-based array (SOMAscan) platform, Mol. Cell Proteomics 13 (2014) 1050–1064.

[6] J. Shou, S. Ross, H. Koeppen, F.J. de Sauvage, W.Q. Gao, Dynamics of notch expression during murine prostate development and tumorigenesis, Cancer Res. 61 (2001) 7291–7297.

[7] A.E. Ross, L. Marchionni, M. Vuica-Ross, C. Cheadle, J. Fan, D.M. Berman, E.M. Schaeffer, Gene expression pathways of high grade localized prostate cancer, Prostate (2011).

[8] M.A. Villaronga, C.L. Bevan, B. Belandia, Notch signaling: a potential therapeutic target in prostate cancer, Curr. Cancer Drug Targets 8 (2008) 566–580.

[9] D. Kong, E. Heath, W. Chen, M. Cher, I. Powell, L. Heilbrun, Y. Li, S. Ali, S. Sethi, O. Hassan, C. Hwang, N. Gupta, D. Chitale, W.A. Sakr, M. Menon, F. H. Sarkar, Epigenetic silencing of miR-34a in human prostate cancer cells

and tumor tissue specimens can be reversed by BR-DIM treatment, Am. J. Transl. Res. 4 (2012) 14–23.

[10] J. Domingo-Domenech, S.J. Vidal, V. Rodriguez-Bravo, M. Castillo-Martin, S.A. Quinn, R. Rodriguez-Barrueco, D.M. Bonal, E. Charytonowicz, N. Gladoun, J. de la Iglesia-Vicente, D.P. Petrylak, M.C. Benson, J.M. Silva, C. Cordon-Cardo, Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells, Cancer Cell 22 (2012) 373–388.

[11] S. Sharad, A. Srivastava, S. Ravulapalli, P. Parker, Y. Chen, H. Li, G. Petrovics, A. Dobi, Prostate cancer gene expression signature of patients with high body mass index, Prostate Cancer Prostatic Dis. 14 (2011) 22–29.

[12] L. Marignol, Targeting notch in prostate cancer–combination is the key, Nat. Rev. Urol. 11 (2014) 419.

[13] J.M. Barth, K. Kohler, How to take autophagy and endocytosis up a notch, BioMed research international 2014 (2014) 960803.

[14] S. Yamamoto, W.L. Charng, H.J. Bellen, Endocytosis and intracellular trafficking of Notch and its ligands, Curr. Top Dev. Biol. 92 (2010) 165–200.

[15] A. Gulino, L. Di Marcotullio, I. Screpanti, The multiple functions of Numb, Exp. Cell Res. 316 (2010) 900–906.

[16] S. Pece, S. Confalonieri, R.R. P, P.P. Di Fiore, NUMB-ing down cancer by more than just a NOTCH, Biochim Biophys Acta 1815 (2011) 26–43.

[17] R. Kopan, M.X. Ilagan, The canonical Notch signaling pathway: unfolding the activation mechanism, Cell 137 (2009) 216–233.

[18] E.C. Lai, Notch signaling: control of cell communication and cell fate, Development 131 (2004) 965–973.

[19] N. Scorey, S.P. Fraser, P. Patel, C. Pridgeon, M.J. Dallman, M.B. Djamgoz, Notch signalling and voltage-gated Na+ channel activity in human prostate cancer cells: independent modulation of in vitro motility, Prostate Cancer Prostatic Dis. 9 (2006) 399–406.

[20] S. Sethi, J. Macoska, W. Chen, F.H. Sarkar, Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis, Am. J. Transl. Res. 3 (2011) 90–99.

[21] S. Reichrath, C.S. Muller, B. Gleissner, M. Pfreundschuh, T. Vogt, J. Reichrath, Notch- and vitamin D signaling in 1. glioblastoma multiforme (GBM) cell lines, J. Steroid Biochem. Mol. Biol. 121 (2010) 420–424.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00104

[22] L.C. Ding, L. She, D.L. Zheng, Q.L. Huang, J.F. Wang, F.F. Zheng, Y.G. Lu, Notch-4 contributes to the metastasis of salivary adenoid cystic carcinoma, Oncol. Rep. 24 (2010) 363–368.

[23] P. Sansone, G. Storci, C. Giovannini, S. Pandolfi, S. Pianetti, M. Taffurelli, D. Santini, C. Ceccarelli, P. Chieco, M. Bonafe, p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland expanded in vitro as mammospheres, Stem Cells 25 (2007) 807–815.

[24] A.W. Studebaker, G. Storci, J.L. Werbeck, P. Sansone, A.K. Sasser, S. Tavolari, T. Huang, M.W. Chan, F.C. Marini, T.J. Rosol, M. Bonafe, B.M. Hall, Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner, Cancer Res. 68 (2008) 9087–9095.

[25] S. Yeasmin, K. Nakayama, M.T. Rahman, M. Rahman, M. Ishikawa, K. Iida, Y. Otsuki, H. Kobayashi, S. Nakayama, K. Miyazaki, Expression of nuclear Notch3 in cervical squamous cell carcinomas and its association with adverse clinical outcomes, Gynecol. Oncol. 117 (2010) 409–416.

[26] X. Chen, A. Stoeck, S.J. Lee, I.M. Shih, M.M. Wang, T.L. Wang, Jagged1 expression regulated by Notch3 and Wnt/beta-catenin signaling pathways in ovarian cancer, Oncotarget 1 (2010) 210–218.

[27] S.G. Jung, Y.D. Kwon, J.A. Song, M.J. Back, S.Y. Lee, C. Lee, Y.Y. Hwang, H.J. An, Prognostic significance of Notch 3 gene expression in ovarian serous carcinoma, Cancer Sci. 101 (2010) 1977–1983.

[28] L. Marignol, M. Coffey, M. Lawler, D. Hollywood, Hypoxia in prostate cancer: A powerful shield against tumour destruction? Cancer Treat Rev. (34) (2008) 313–327.

[29] T. Yong, A. Sun, M.D. Henry, S. Meyers, J.N. Davis, Down regulation of CSL activity inhibits cell proliferation in prostate and breast cancer cells, J. Cell Biochem. 112 (2011) 2340–2351.

[30] A.O. Rehman, C.Y. Wang, Notch signaling in the regulation of tumor angiogenesis, Trends Cell Biol. 16 (2006) 293–300.

[31] H. Zhong, A.M. De Marzo, E. Laughner, M. Lim, D.A. Hilton, D. Zagzag, P. Buechler, W.B. Isaacs, G.L. Semenza, J.W. Simons, Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases, Cancer Res. 59 (1999) 5830–5835.

[32] A. Lekas, A.C. Lazaris, C. Deliveliotis, M. Chrisofos, C. Zoubouli, D. Lapas, T. Papathomas, I. Fokitis, L. Nakopoulou, The expression of hypoxia-inducible

 http://dx.doi.org/10.1016/j.heliyon.2016.e00104

factor-1alpha (HIF-1alpha) and angiogenesis markers in hyperplastic and malignant prostate tissue, Anticancer Res. 26 (2006) 2989–2993.

[33] M.V. Gustafsson, X. Zheng, T. Pereira, K. Gradin, S. Jin, J. Lundkvist, J.L. Ruas, L. Poellinger, U. Lendahl, M. Bondesson, Hypoxia requires notch signaling to maintain the undifferentiated cell state, Dev. Cell 9 (2005) 617–628.

[34] K. Horii, Y. Suzuki, Y. Kondo, M. Akimoto, T. Nishimura, Y. Yamabe, M. Sakaue, T. Sano, T. Kitagawa, S. Himeno, N. Imura, S. Hara, Androgen-dependent gene expression of prostate-specific antigen is enhanced synergistically by hypoxia in human prostate cancer cells, Mol. Cancer Res. 5 (2007) 383–391.

[35] L. Marignol, K. Rivera-Figueroa, T. Lynch, D. Hollywood, Hypoxia, notch signalling, and prostate cancer, Nat. Rev. Urol. 10 (2013) 405–413.

[36] L.M. Anderson, G.H. Gibbons, Notch: a mastermind of vascular morphogenesis, J. Clin. Invest. 117 (2007) 299–302.

[37] T. Gridley, Notch signaling in vascular development and physiology, Development 134 (2007) 2709–2718.

[38] J. Pallares, F. Rojo, J. Iriarte, J. Morote, L.I. Armadans, I. de Torres, Study of microvessel density and the expression of the angiogenic factors VEGF bFGF and the receptors Flt-1 and FLK-1 in benign, premalignant and malignant prostate tissues, Histol. Histopathol. 21 (2006) 857–865.

[39] D.G. Bostwick, K.A. Iczkowski, Microvessel density in prostate cancer: prognostic and therapeutic utility, Semin. Urol. Oncol. 16 (1998) 118–123.

[40] A.D. Lassaletta, N.Y. Elmadhun, T.A. Burgess, C. Bianchi, A.A. Sabe, M.P. Robich, L.M. Chu, F.W. Sellke, Microvascular notch signaling is upregulated in response to vascular endothelial growth factor and chronic myocardial ischemia, Circ. J. 78 (2014) 743–751.

[41] K. Roe, A. Bratland, L. Vlatkovic, H.B. Ragnum, M.G. Saelen, D.R. Olsen, L. Marignol, A.H. Ree, Hypoxic tumor kinase signaling mediated by STAT5A in development of castration-resistant prostate cancer, PLoS One 8 (2013) e63723.

[42] A.S. Perry, G. O'Hurley, O.A. Raheem, K. Brennan, S. Wong, A. O'Grady, A. M. Kennedy, L. Marignol, T.M. Murphy, L. Sullivan, C. Barrett, B. Loftus, J. Thornhill, S.M. Hewitt, M. Lawler, E. Kay, T. Lynch, D. Hollywood, Gene expression and epigenetic discovery screen reveal methylation of SFRP2 in prostate cancer, Int. J. Cancer 132 (2013) 1771–1780.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00104

[43] R. Foley, L. Marignol, A.Z. Thomas, I.M. Cullen, A.S. Perry, P. Tewari, A. O'Grady, E. Kay, B. Dunne, B. Loftus, R.W. Watson, J.M. Fitzpatrick, K. Woodson, T. Lehman, D. Hollywood, T.H. Lynch, M. Lawler, The HIF-1alpha C1772T polymorphism may be associated with susceptibility to clinically localised prostate cancer but not with elevated expression of hypoxic biomarkers, Cancer Biol. Ther. 8 (2009).

[44] J.T. Whelan, A. Kellogg, B.M. Shewchuk, K. Hewan-Lowe, F.E. Bertrand, Notch-1 signaling is lost in prostate adenocarcinoma and promotes PTEN gene expression, J. Cell Biochem. 107 (2009) 992–1001.

[45] O.A. Mamaeva, J. Kim, G. Feng, J.M. McDonald, Calcium/calmodulin-dependent kinase II regulates notch-1 signaling in prostate cancer cells, J. Cell Biochem. 106 (2009) 25–32.

[46] N.M. Kofler, C.J. Shawber, T. Kangsamaksin, H.O. Reed, J. Galatioto, J. Kitajewski, Notch signaling in developmental and tumor angiogenesis, Genes Cancer 2 (2011) 1106–1116.

[47] A.R. Pedrosa, J.L. Graca, S. Carvalho, M.C. Peleteiro, A. Duarte, A. Trindade, Notch signaling dynamics in the adult healthy prostate and in prostatic tumor development, Prostate 76 (2016) 80–96.

[48] A.N. Flores, N. McDermott, A. Meunier, L. Marignol, NUMB inhibition of NOTCH signalling as a therapeutic target in prostate cancer, Nat. Rev. Urol. 11 (2014) 499–507.

[49] Y. Yu, Y. Zhang, W. Guan, T. Huang, J. Kang, X. Sheng, J. Qi, Androgen receptor promotes the oncogenic function of overexpressed Jagged1 in prostate cancer by enhancing cyclin B1 expression via Akt phosphorylation, Mol. Cancer Res. 12 (2014) 830–842.

[50] R.E. Reiter, I. Sato, G. Thomas, J. Qian, Z. Gu, T. Watabe, M. Loda, R.B. Jenkins, Coamplification of prostate stem cell antigen (PSCA) and MYC in locally advanced prostate cancer, Genes Chromosomes Cancer 27 (2000) 95–103.

[51] B. Belandia, S.M. Powell, J.M. Garcia-Pedrero, M.M. Walker, C.L. Bevan, M.G. Parker, Hey1 a mediator of notch signaling, is an androgen receptor corepressor, Mol. Cell Biol. 25 (2005) 1425–1436.

[52] X.H. Liu, Y. Wu, S. Yao, A.C. Levine, A. Kirschenbaum, L. Collier, W.A. Bauman, C.P. Cardozo, Androgens up-regulate transcription of the Notch inhibitor Numb in C2C12 myoblasts via Wnt/beta-catenin signaling to T cell factor elements in the Numb promoter, J. Biol. Chem. 288 (2013) 17990–17998.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00104

[53] A. Pietras, K. von Stedingk, D. Lindgren, S. Pahlman, H. Axelson, JAG2 induction in hypoxic tumor cells alters Notch signaling and enhances endothelial cell tube formation, Mol. Cancer Res. 9 (2011) 626–636.

[54] G. Danza, C. Di Serio, F. Rosati, G. Lonetto, N. Sturli, D. Kacer, A. Pennella, G. Ventimiglia, R. Barucci, A. Piscazzi, I. Prudovsky, M. Landriscina, N. Marchionni, F. Tarantini, Notch signaling modulates hypoxia-induced neuroendocrine differentiation of human prostate cancer cells, Mol. Cancer Res. 10 (2012) 230–238.

[55] Q. Long, B.A. Johnson, A.O. Osunkoya, Y.H. Lai, W. Zhou, M. Abramovitz, M. Xia, M.B. Bouzyk, R.K. Nam, L. Sugar, A. Stanimirovic, D.J. Williams, B.R. Leyland-Jones, A.K. Seth, J.A. Petros, C.S. Moreno, Protein-coding and microRNA biomarkers of recurrence of prostate cancer following radical prostatectomy, Am. J. Pathol. 179 (2011) 46–54.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00104