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Differential Expression of GDF-15, PSAP, and COL6A1 in Prostate Cancer Cells and TNMplot-Based Clinical Tissue Specimens of Prostate Cancer Adenocarcinoma

(Ekspresi Berbeza GDF-15, PSAP dan COL6A1 dalam Sel Kanser Prostat dan Spesimen Tisu Klinikal Berasaskan TNMplot bagi Adenokarsinoma Kanser Prostat)

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ABSTRACT

Distinguishing aggressive from indolent prostate cancer remains a key clinical challenge. Although prostate-specific antigen (PSA) is commonly used for diagnosis, its low sensitivity and specificity limit its ability to predict tumour aggressiveness. This study aimed to investigate the expression patterns of selected protein biomarkers, including growth/differentiation factor-15 (GDF-15), prosaposin (PSAP), and collagen-α1 (VI) chain (COL6A1), in prostate cancer cells (primary and metastatic) and tumour tissue samples. The immunocytochemical (ICC) analysis showed distinct expression patterns of these proteins across different prostate cancer cell lines, with GDF-15 being highly expressed in lymph node metastatic (LNCaP) cells. In contrast, PSAP and COL6A1 were primarily expressed in primary (22Rv1) and bone metastatic (PC-3) cells. The western blot analysis confirmed differential expression of GDF-15, PSAP, and COL6A1 across prostate cancer cell lines, with GDF-15 showing higher expression in LNCaP cells, whereas PSAP and COL6A1 were highly expressed in PC-3 cells. TNMplot analysis showed that the *GDF-15* gene was significantly upregulated in primary and metastatic prostate cancer tissues compared to non-tumour samples and was also markedly overexpressed in colon adenocarcinoma. Similarly, *PSAP* expression was elevated in primary prostate tumours relative to non-tumour tissues, with significantly higher levels observed in hepatocellular carcinoma. In contrast, *COL6A1* was consistently downregulated in prostate cancer tissues and across several other malignancies. These results suggest that GDF-15 may serve as a broad-spectrum cancer biomarker, whereas PSAP and COL6A1 may be associated with disease progression and metastatic behaviour. However, further studies are needed to validate their potential clinical applications as a biomarker.

Keywords: Prostate cancer; protein biomarkers; COL6A1; GDF-15; PSAP

ABSTRAK

Membezakan antara kanser prostat yang agresif dan tidak agresif kekal sebagai cabaran klinikal utama. Walaupun antigen khusus prostat (PSA) lazimnya digunakan untuk diagnosis, kepekaan dan pengkhususan yang rendah mengehadkan kemampuannya untuk meramalkan tahap agresif tumor. Penyelidikan ini bertujuan untuk mengkaji corak ekspresi biopenanda protein terpilih, termasuk pertumbuhan/faktor pembezaan 15 (GDF-15), prosaposin (PSAP) dan kolagen-α1 (VI) rantaian (COL6A1) dalam sel kanser prostat (primer dan metastatik) serta sampel tisu tumor. Analisis imunositokimia (ICC) menunjukkan corak ekspresi yang berbeza bagi biopenanda protein tersebut dalam pelbagai sel kanser prostat dengan GDF-15 didapati sangat tinggi dalam sel metastatik nodus limfa (LNCaP). Sebaliknya, PSAP dan COL6A1 terutamanya dijumpai dalam sel primer (22Rv1) dan sel metastatik tulang (PC-3) yang menunjukkan potensi peranan dalam perkembangan dan pengkhususan metastatik kanser prostat. Analisis pemendapan *Western* mengesahkan ekspresi yang berbeza bagi GDF-15, PSAP dan COL6A1 dalam pelbagai sel kanser prostat dengan GDF-15 menunjukkan ekspresi yang lebih tinggi dalam sel LNCaP, manakala PSAP dan COL6A1 pula menunjukkan ekspresi yang tinggi dalam sel PC-3. Analisis TNMplot menunjukkan bahawa gen *GDF-15* meningkat secara ketara dalam tisu kanser prostat primer dan metastatik berbanding sampel bukan tumor serta turut menunjukkan ekspresi berlebihan yang ketara dalam adenokarsinoma kolon. Begitu juga dengan ekspresi *PSAP* yang didapati meningkat dalam tumor prostat primer berbanding tisu bukan tumor dengan tahap yang jauh lebih tinggi diperhatikan dalam karsinoma hepatosel. Sebaliknya, *COL6A1* secara tekal didapati menurun dalam tisu kanser prostat serta pelbagai jenis kanser lain. Keputusan ini mencadangkan bahawa

GDF-15 berpotensi sebagai biopenanda kanser spektrum luas, manakala PSAP dan COL6A1 mungkin berkaitan dengan perkembangan penyakit dan tingkah laku metastatik. Walau bagaimanapun, kajian lanjut diperlukan untuk mengesahkan potensi aplikasi klinikal bagi biopenanda ini.

Kata kunci: Biopenanda protein; kanser prostat; COL6A1; GDF-15; PSAP

INTRODUCTION

Prostate cancer is the second most commonly occurring cancer, with a total of 1,414,259 new cases and 375,304 deaths reported in 2020. The highest incidence rate was reported in men aged 65 years and above in Northern and Western Europe and the Caribbean (Sung et al. 2021). In Malaysia, a recent report by the National Cancer Registry has shown a total of 6,441 new prostate cancer cases from 2017 to 2021, with approximately a 35.0% increase in cancer cases compared to cancer incidence between 2012 and 2016. From 6,441 cases reported from 2017 to 2021, 63.3% of prostate cancer patients were detected at stages III and IV (National Cancer Institute 2025).

The occurrence of prostate cancer at advanced stages (Stages III and IV) reflects a persistent issue of delayed diagnosis, as these stages are commonly linked to aggressive and high-risk disease. In Stage IV, the disease frequently involves metastasis to distant organs, particularly the lymph nodes and bones, reported in approximately 85-90% of advanced cases (Gandaglia et al. 2014), which is associated with limited treatment options and poorer clinical outcomes. In contrast, cases identified at earlier stages, typically confined to the prostate, are more responsive to treatment and demonstrate markedly lower cancer-specific mortality (Beksisa et al. 2020; McPhail et al. 2015). Therefore, there is a critical need to discover reliable biomarkers that enable early detection and accurate staging of prostate cancer to enhance patient prognosis.

Presently, prostate cancer is diagnosed based on digital rectal examination (DRE), prostate-specific antigen (PSA) level, and prostate biopsy (Omri et al. 2021; Washino et al. 2017). For the last decade, PSA, a serine protease kallikrein 3 proteins, has been the common protein biomarker used to detect prostate cancer occurrence before undergoing prostate biopsy. Despite being one of the most useful prostate cancer biomarkers, PSA-based screening is far from ideal due to some limitations, such as overdiagnosis and overtreatment, due to the low specificity and sensitivity in detecting prostate cancer (Khan et al. 2022). A previous study involving 1,986 subjects reported that 25% of patients had low PSA levels ranging from 2.5 to 4.0 ng/mL but were later diagnosed with prostate cancer following a biopsy (Chung et al. 2020). Therefore, a new protein biomarker with high sensitivity and specificity is critical to enhance prostate cancer diagnosis.

The limitations of PSA as a diagnostic marker have driven the development and validation of novel biomarkers to enhance the accuracy of prostate cancer detection. These potential protein biomarkers include collagen-α-1 (VI) chain (COL6A1), prosaposin (PSAP), and growth/differentiation factor-15 (GDF-15), which can serve as candidate biomarkers for detecting prostate cancer and predicting disease severity and patient outcomes. One of the three main collagen VI subunits is COL6A1, a major extracellular matrix (ECM) protein that has been reported to be highly expressed in castration-resistant prostate cancer (CRPC) tissues and contribute to tumour growth (Zhu et al. 2015). Our previous proteomic analysis also showed that COL6A1 is highly secreted by bone metastatic prostate cancer cells compared to normal prostate cells (Nurdin et al. 2016).

An earlier study by Kishimoto, Hiraiwa and O'Brien (1992) reported that PSAP is a highly conserved glycoprotein ranging from 68 to 73 kDa and serves as a precursor to saposins, which are primarily involved in sphingolipid degradation. It undergoes glycosylation, is secreted extracellularly, and is processed intracellularly to maintain lipid homeostasis and proper lysosomal function. A subsequent study by Hu et al. (2010) demonstrated that PSAP knockdown significantly reduced the migratory and invasive capabilities of metastatic prostate cancer cells. Clinically, PSAP has also been shown to differentiate between primary and advanced stages of prostate cancer, as reported by Koochekpour et al. (2012).

GDF-15, also referred to as macrophage inhibitory cytokine 1 (MIC-1), belongs to the transforming growth factor-β (TGF-β) superfamily that is involved in the regulation of inflammatory response in atrophic inflammatory lesions of the prostate (Lambert et al. 2015). This finding was further supported by Tsui et al. (2012), who reported higher GDF-15 expression in metastatic prostate cancer cells (PC-3) and associated it with upregulation of interleukin-6 (IL-6). Moreover, subsequent research by Li et al. (2015) showed that elevated levels of serum MIC/GDF-15 in prostate cancer patients compared to non-tumour patients were positively associated with PSA levels.

However, despite all these studies, the comparison of the levels of these protein biomarkers (GDF-15, PSAP, and COL6A1) in different prostate cancer cell lines, such as primary prostate (22Rv1), bone metastatic (PC-3), and lymph node metastatic (LNCaP) prostate cancer cells, has not been reported previously. Therefore, this study aims to compare the expression profiles of these selected protein biomarkers in primary versus metastatic prostate cancer cells to determine which may serve as robust biomarkers for prostate cancer. Assessing the protein expression throughout these stages is critical to further validate its potential as a biomarker for prostate cancer diagnosis and prognosis. By utilising bioinformatics tools such as

the TNMplot database, this study will also systematically evaluate targeted gene expression patterns, validate the relevance of biomarkers, and assess their potential clinical utility.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Eagle's Minimum Essential Medium (EMEM) powder, Kaighn's modification of Ham's F-12 Ham Medium (F-12K), and Roswell Park Memorial Institute (RPMI)-1640 medium were purchased from Sigma Aldrich® (St Louis, USA); fetal bovine serum (FBS) was purchased from Tico Europe (Amstelveen, Netherlands); 100x penicillin-streptomycin solution and radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (10x) (Cat. no.: 08714-04) were purchased Nacalai Tesque (Kyoto, Japan); 2-step plus poly-HRP Anti-Mouse/Rabbit IgG detection system kit was purchased from Elabscience® (Texas, USA); mouse monoclonal antibody to human GDF-15 (Cat. No. sc-377195) was purchased from Santa Cruz (California, USA); rabbit polyclonal antibodies to PSAP (Cat. No. AB180751), and COL6A1 (Cat. No. AB151422) were purchased from Abcam (Cambridge, United Kingdom); and BCA working reagent was purchased from Thermo Fisher Scientific (Loughborough, UK).

CELL LINES

Human primary/non-aggressive prostate cancer cells (22Rv1) (Cat No: CRL-2505™), lymph nodes-metastasised LNCaP (Cat No: CRL-1740™), and bone-metastasised PC-3 (Cat No: 1435™) were purchased from the American Type Culture Collection (ATCC®) (Virginia, USA). 22Rv1 and LNCaP cells were cultured in RPMI 1640 medium, while PC-3 cells were cultured in F-12K medium. The complete medium for all cells was supplemented with $NaHCO_3$, 10% FBS, and a 1% antibiotic mixture (penicillin/streptomycin). All cells were incubated at 37 °C with 5% carbon dioxide (CO_2), and the growth medium was refreshed every 3 days to maintain optimal growth conditions.

IMMUNOCYTOCHEMISTRY (ICC) STAINING OF THE TARGETED PROTEIN BIOMARKERS

22Rv1 and metastatic prostate cancer cells (PC-3 and LNCaP) were seeded on cleaned coverslips (22.1 cm^2) in 60 mm culture dishes at seeding densities of 2×10^6. After 24 h of incubation, the existing media from the seeded cells were removed, and the cells were rinsed three times with 1 mL of PBS. The ICC analysis was carried out using the 2-step plus poly-HRP anti-mouse/rabbit IgG detection system (with DAB solution) kit, as previously described (Roslan et al. 2023). The cells were incubated with primary antibodies (such as anti-GDF-15, PSAP, and COL6A1) at a 1:200 dilution overnight at 4 °C in the chiller. Positive staining was indicated by yellow-brownish staining, while the negative control appeared blue-purple.

PROTEIN ISOLATION AND CELL DISRUPTION OF CELL LYSATES

The LNCaP, 22Rv1, and PC3 cell lines were seeded at a density of 1×10^6 cells per well in 6-well plates, with 2 mL of the appropriate culture medium per well. The cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. After incubation, the cells were washed twice with 500 µL of ice-cold PBS to remove residual media and other contaminants. Lysis was initiated by adding 300 µL of RIPA buffer directly to each well, followed by a brief 1-minute incubation. The lysed cells were scraped thoroughly, and the resulting lysate was transferred to a fresh 1.5 mL microcentrifuge tube. The lysates were incubated on ice for 20 min and centrifuged at 13,000 × *g* for 15 min at 4 °C to separate the soluble protein fraction. The clear supernatant was carefully collected into a new microcentrifuge tube, making it ready for subsequent protein quantification and western blot analysis.

AUTOMATED WESTERN BLOTTING USING THE JESS SYSTEM

Prior to protein quantification, the protein concentration of cell lysates from each sample was quantified using the BCA assay in a 96-well plate. Automated Western blotting was conducted as previously described by Roslan et al. (2023). Mouse monoclonal antibodies specific to human GDF-15, PSAP, and COL6A1 were utilised as primary antibodies for detection. Protein quantification was normalised to the total protein content in each capillary using a protein normalisation reagent (Protein Simple, California, USA).

EXPRESSION OF THE TARGET GENE IN PROSTATE CANCER TISSUES USING THE TNMpLOT DATABASE

To further evaluate the expression of the targeted protein biomarkers in the tissue of prostate cancer patients, an open-access bioinformatics tool such as the TNMplot database (https://www.tnmplot.com) (Bartha & Gyorffy 2021) was used. The expression of the targeted genes in primary tumour and metastatic prostate cancer tissues was compared with that in non-tumour tissues. In this study, 'non-tumour' refers to non-cancerous (benign or normal) tissue that does not exhibit malignant characteristics. The expression of the selected markers was also compared in different types of cancer, including lung, prostate, colon, liver, and stomach adenocarcinomas.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS (version 27.0, Chicago, USA) and GraphPad Prism 9 (La Jolla, CA, USA). Protein expression levels across different cell lines were compared using one-way ANOVA, followed by the Tukey post-hoc test. Data are presented as the mean ± Standard Error of the Mean (SEM). For gene expression data from the TNMplot database, the Mann-Whitney U test was used to compare the expression of the selected gene between non-tumour and primary tumour prostate cancer

tissues. In addition, the Kruskal-Wallis test was used to compare the expression of the targeted gene in non-tumour, primary tumour, and metastatic prostate cancer patients. A *p*-value of ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

EXPRESSION OF THE TARGETED PROTEIN BIOMARKERS IN PRIMARY AND METASTATIC PROSTATE CANCER CELLS

In the present study, to address the aggressiveness of prostate cancer, the expression of selected protein biomarkers was evaluated in prostate cancer cells with three metastatic potentials: primary (22Rv1), bone metastatic (PC3), and lymph node metastatic. ICC staining showed a yellow-brownish staining pattern against anti-human GDF-15 protein expression in 22Rv1 and LNCaP prostate cancer cells. However, a distinct yellow-brownish staining of GDF-15 was observed in the cytoplasm of LNCaP cells as compared to the 22Rv1 cells. No positive staining was observed in PC-3. Positive staining for PSAP and COL6A1 protein expression was observed in primary prostate cancer cells (22Rv1) and bone metastases (PC3) prostate cancer cells, whereby distinct yellow-brownish staining of COL6A1 was observed in the cytoplasm and membrane of PC-3 cells as compared to the 22Rv1 cells. For PSAP, the expression was observed in the cytoplasm of PC-3 cells and in both the cytoplasm and membrane of 22Rv1 cells. No yellow-brownish staining was observed in the lymph node metastases (LNCaP) prostate cancer cells for both targeted proteins (Figure 1). In the present study, no detectable staining signal was observed in the negative control cells for all anti-human antibodies (GDF-15, PSAP, and COL6A1), which were processed without the primary antibody (Figure 1). This finding confirmed the specificity of the staining protocol for all antibodies used in the present study.

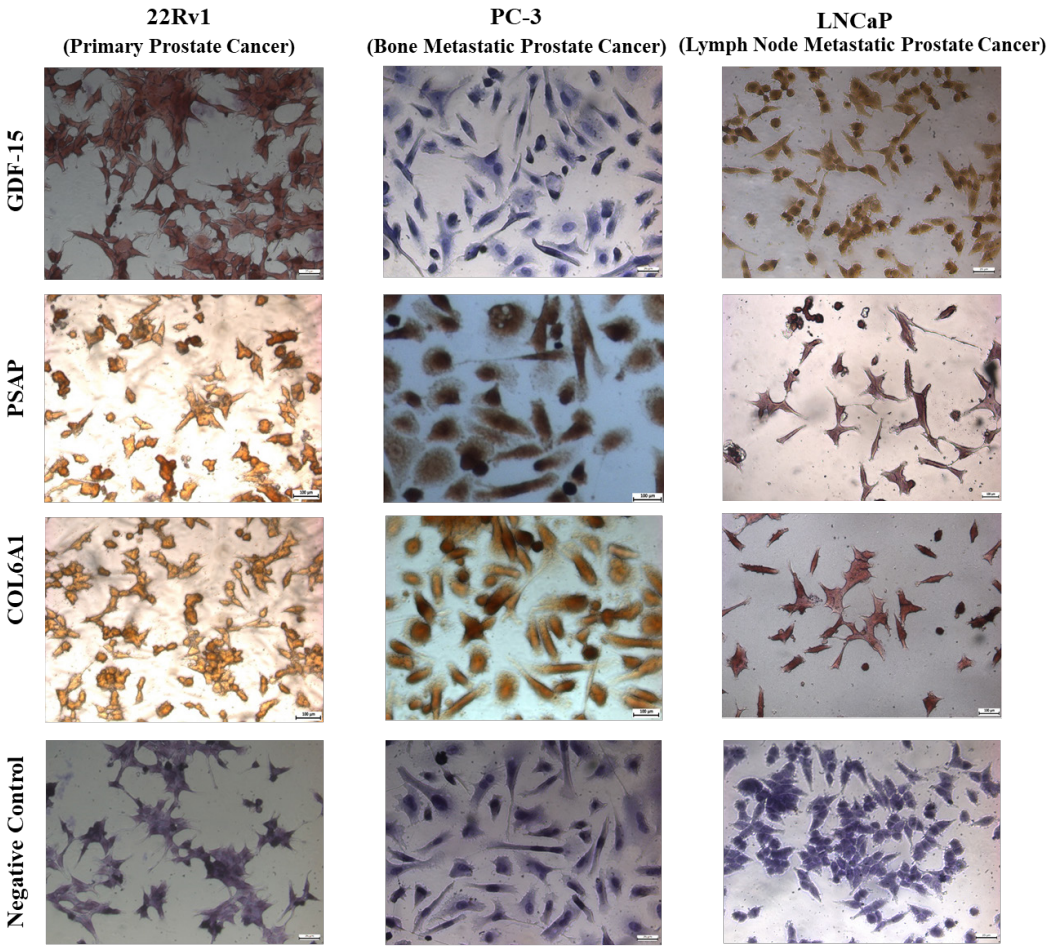

FIGURE 1. Immunocytochemistry of GDF-15, PSAP, and COL6A1 protein expressions in primary (22Rv1), bone metastases (PC-3), and lymph node metastases (LNCaP) prostate cancer cells with an optimised primary antibody dilution of 1:200 ratio under a light microscope at 400x magnification. Positive expression of GDF-15 was observed in primary prostate cancer cells (22Rv1) and lymph node metastases (LNCaP) prostate cancer cells. Positive expression of PSAP and COL6A1 was observed in primary prostate cancer cells (22Rv1) and bone metastatic prostate cancer cells (PC3)

Based on the findings obtained from the ICC assay, the Automated Jess Simple Western blot assay was utilised to quantify the expression of the selected protein biomarkers, including GDF-15, PSAP, and COL6A1, in various prostate cancer cell lines (22Rv1, PC-3, and LNCaP). Prior to quantifying the targeted protein biomarkers, the total protein content of whole-cell lysates from different prostate cancer cell lines was measured using the BCA assay. Figure 2 illustrates the protein amount of the cell lysates, whereby PC-3 cells (294±20.78) exhibited at least 1.7-fold and 2-fold higher protein lysate compared to LNCaP (167±27.22) and 22Rv1 (147±27.00) cells, respectively. The results confirm that all samples fall within an acceptable concentration range for automated Western blot analysis. Proper protein quantification with the BCA assay ensures that equal amounts of protein are loaded onto the gel, minimising variability and enabling accurate interpretation of the western blot results.

Our western blot findings showed that GDF-15 was successfully detected in all prostate cancer cell lines, although the expected molecular weight of GDF-15 at 30-40 kDa was slightly shifted to 45-47 kDa in all detected samples (Figure 3). This shift may be attributed to post-translational modifications, such as glycosylation or retention of the precursor. The results were also consistent across multiple replicates (Supplementary Figure 1), confirming the reproducibility of these findings. At least a 1.5-fold (p<0.05) higher expression of GDF-15 was noted in LNCaP cells as compared to 22Rv1 and PC-3 cells (Figure 3), which confirmed the results from ICC staining. This finding aligned with the study by Siddiqui et al. (2022), who reported that GDF-15 expression was significantly five to six times lower in PC-3 cells compared to the LNCaP prostate cells. Their findings demonstrated that overexpression of GDF-15 in prostate cancer cells significantly enhanced migration and invasion, whereas silencing GDF-15 reduced cell invasiveness and prevented bone destruction, suggesting that GDF-15 modulates the bone microenvironment in prostate cancer cells and enhances bone metastasis. A recent study by Yamamichi et al. (2024) showed that secreted GDF15-derived propeptide accelerated bone metastasis by increasing the osteogenic potential of human osteoblasts and osteoclasts in prostate cancer cells.

Similarly, PSAP was detected in all prostate cancer cell lines, but instead of a single band at its expected molecular weight (58 kDa), multiple bands were observed, indicating possible proteolytic cleavage, alternative splicing, or post-translational modifications. Three clear bands at the ~90 kDa, ~60 kDa, and ~40 kDa were observed in cell lysates from primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells, with higher intensity observed in the PC-3 cells as compared to 22Rv1 and LNCaP cells (Supplementary Figure 2). The presence of higher-molecular-weight bands (~90 kDa) suggests glycosylation or dimerisation, whereas the lower-molecular-weight bands (~40 kDa) may result from proteolytic cleavage. PSAP exhibited a significantly higher expression (2.5-fold; p<0.05) in PC-3 cells compared to 22Rv1 and LNCaP cells at ~85-90 kDa (Figure 4). However, PSAP expression was not significantly different among 22Rv1, PC-3, and LNCaP cells, as observed at ~60-62 kDa, which is the nearest predicted band for PSAP. In contrast, PSAP expression was significantly lower in PC-3 cells than in 22Rv1 and LNCaP cells at ~37-39 kDa. This finding is consistent with a previous study demonstrating that both intracellular and extracellular expression of PSAP is significantly elevated in androgen-independent prostate cancer cells (PC-3) compared to androgen-sensitive (LNCaP) and normal prostate cells (Koochekpour et al. 2005). Furthermore, silencing PSAP expression in prostate cancer cells has been reported to reduce their migratory and invasive capabilities (Hu et al. 2010).

Similar to PSAP, the analysis of COL6A1 also showed multiple molecular weight forms across different cell lines, deviating from its expected 109 kDa size, suggesting post-translational modifications and dimerization. A total of two clear band intensities for COL6A1 protein were detected, which are at ~ 150-164 kDa and ~93-100 kDa (Supplementary Figure 3). The higher-molecular-weight bands (150-164 kDa) suggest glycosylation or cross-linking, while the lower-molecular-weight bands (~93-100 kDa) may indicate proteolytic cleavage. This identified band has slightly deviated from the predicted COL6A1 band size (~109 kDa). A significantly 44.5-fold and 30-fold higher expression of COL6A1 was noted in PC-3 cells as compared to 22Rv1 and LNCaP cells, respectively, at ~ 150-164 kDa (Figure 5). However, COL6A1 expression was not detected in PC-3 at ~93-100 kDa, which is the nearest predicted band for COL6A1. No significant difference in the COL6A1 expression in 22Rv1 cells as compared to LNCaP cells (Figure 5). The role of COL6A1 in prostate cancer proliferation has been highlighted in an earlier study, where COL6A1 knockdown led to a significant reduction in the proliferative capacity of prostate cancer cells. Further silencing COL6A1 resulted in cell-cycle arrest at the G1/S phase and inhibition of the JAK2-STAT pathway, leading to suppressed tumour growth (Zhu et al. 2015).

COMPARATIVE EXPRESSION OF SELECTED GENE BIOMARKERS IN TISSUES OF PROSTATE CANCER PATIENTS

To confirm the clinical relevance of the selected biomarkers, a TCGA database from TNMplot, a bioinformatics tool, was utilised to examine their expression patterns across an extensive dataset comprising adjacent normal, primary tumour, and metastatic prostate cancer tissues. TNMplot was chosen over transcriptomic techniques such as qRT-PCR and RNA-Seq due to its extensive pre-analysed patient dataset, which enabled more efficient, large-scale validation of biomarker expression patterns (Bartha & Győrffy 2021). The results indicate significant alterations in gene expression, with varying degrees of up- and downregulation across genes.

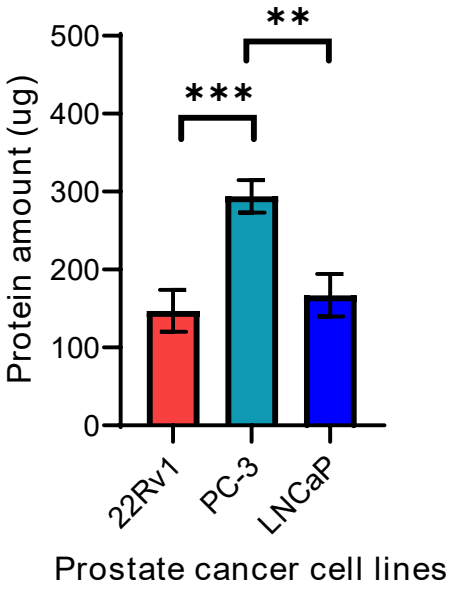

FIGURE 2. Protein amount (µg) of cell lysates in primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells, quantified based on BCA assay. A significantly higher amount of protein was isolated from PC-3 cells than from 22Rv1 and LNCaP cells. Data were analysed using One-way ANOVA, and Tukey's post hoc test was used to assess significant differences among the groups. The values are presented as mean ± SD with *, **, and *** as $p<0.05$, $p<0.01$, and $p<0.001$, respectively

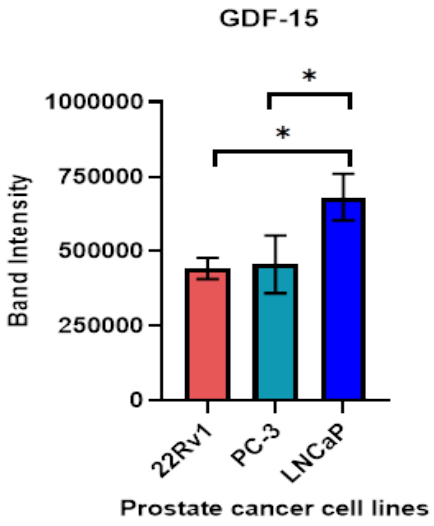

FIGURE 3. Expression of GDF-15 in primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells. A significantly higher expression of GDF-15 was noted in LNCaP cells as compared to 22Rv1 and PC-3 cells. Data were analysed using One-way ANOVA, and Tukey's post hoc test was used to assess significant differences among the groups. The values are presented as mean ± SD with *, **, and *** as $p<0.05$, $p<0.01$, and $p<0.001$, respectively

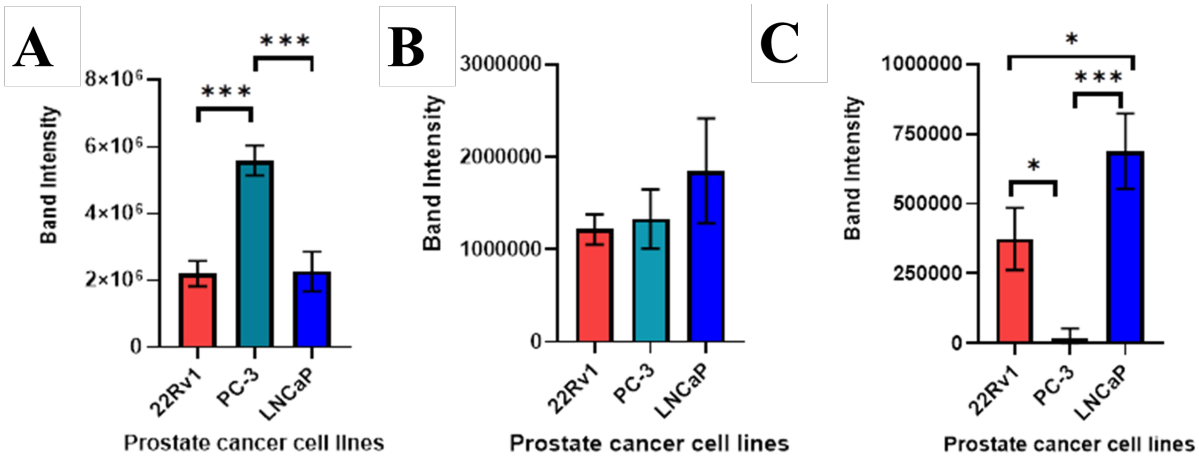

FIGURE 4. Expression of PSAP in primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells. The predicted band size for PSAP is ~58 kDa; however, in the present study, three PSAP bands were detected at (A) ~85-90 kDa, (B) ~60-62 kDa, and (C) ~37-39 kDa. Significantly higher PSAP expression was noted in PC-3 cells compared to 22Rv1 cells at ~85-90 kDa. In contrast, no significant difference in PSAP expression was observed at ~ 60-62 kDa, which is the nearest predicted band for PSAP. Significantly lower PSAP expression was observed in PC-3 cells compared with 22Rv1 and LNCaP cells at ~37-39 kDa. Data were analysed using One-way ANOVA, and Tukey's post hoc test was used to assess significant differences among the groups. The values are presented as mean ± SD with *, **, and *** as $p<0.05$, $p<0.01$, and $p<0.001$, respectively

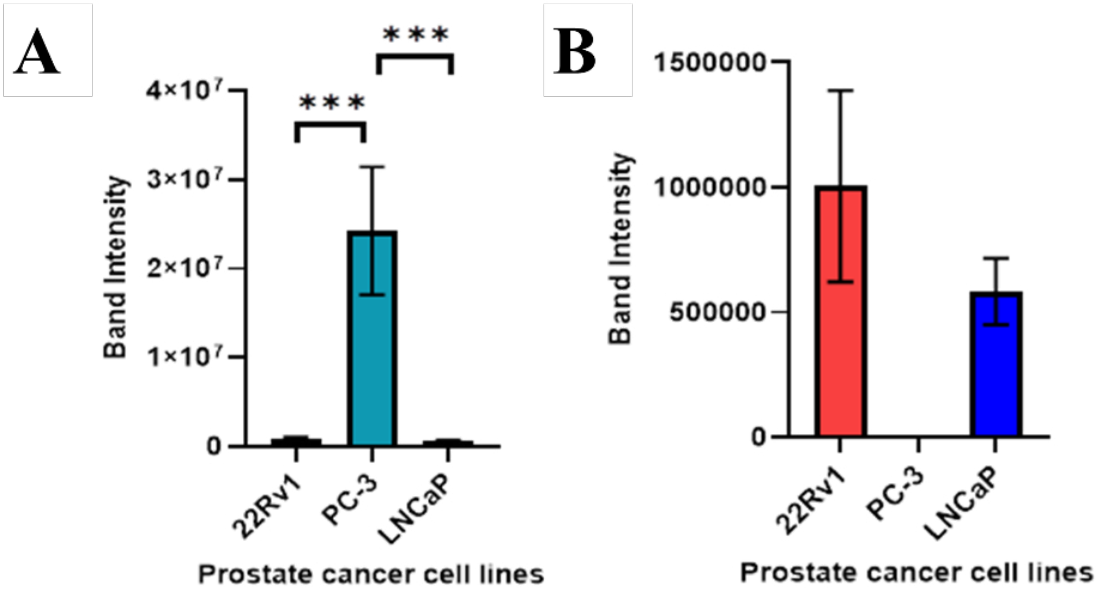

FIGURE 5. Expression of COL6A1 in primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells. The predicted band size for COL6A1 is ~109 kDa; however, in the present study, two COL6A1 bands were detected at (A) ~150-164 kDa and (B) ~93-100 kDa. A significantly higher expression of COL6A1 was noted in PC-3 cells as compared to 22Rv1 and LNCaP cells at ~ 150-164 kDa. However, COL6A1 expression was not detected in PC-3 at ~93-100 kDa, which is the nearest predicted band for COL6A1. Data were analysed using One-way ANOVA, and Tukey's post hoc test was used to assess significant differences among the groups. The values are presented as mean ± SD with *, **, and *** as $p<0.05$, $p<0.01$, and $p<0.001$, respectively

A significant increase in the expression of *GDF-15* and *PSAP* genes was observed in the primary tumour of prostate cancer patients compared to the non-tumour tissue samples (Table 1; Supplementary Figure 4(A) and 4(B)). Notably, among the targeted genes, *GDF-15* exhibited a six-fold upregulation in the primary tumour as compared to the non-tumour tissue samples, suggesting that these targeted genes could be the potential markers for prostate cancer detection. In contrast, *COL6A1* was found to be significantly downregulated 6-fold in the primary tumour of prostate cancer patients as compared to the non-tumour tissue samples (Table 1; Supplementary Figure 4(C)).

To further evaluate the expression of the selected genes in metastatic prostate cancer patients, the expression data from the TNMplot were further analysed. Table 2 and Supplementary Figure 5 present comparative expression levels of the targeted genes (*GDF-15, PSAP*, and *COL6A1*) in primary tumours and metastatic prostate cancer patients relative to non-tumour tissue samples. Similarly, the analysis also shows significant variations in gene expression patterns among these three groups, with distinct trends of upregulation and downregulation. In the present study, *GDF-15* displayed a remarkable upregulation in primary tumours as compared to non-tumour tissue (Table 2; Supplementary Figure 5(A)), with at least a 5-fold increment. A similar trend was also observed when comparing non-tumour tissue to metastatic tissues (fold change: 5.53, p=0.011). In contrast, no significant differences in the level of expression of *GDF-15* between primary and metastatic prostate cancer patients, suggesting the potential role of *GDF-15* in cancer progression.

For *PSAP*, a significantly higher expression of the targeted genes was observed in the primary tumour compared to non-tumour tissue samples (Table 2; Supplementary Figure 5 (B)). However, expression of both targeted genes did not differ significantly between metastatic and primary tumour tissues (Table 2), suggesting that their roles may be more relevant to early tumour development than to metastasis. The findings also demonstrate that *COL6A1* expression was downregulated in primary tumour and metastatic prostate cancer samples compared with non-tumour tissue samples. However, the differences do not show any significant changes across any tissue types (Table 2; Supplementary Figure 5(C)).

The analysis highlights potential biomarkers, such as GDF-15, for distinguishing malignant tissues (primary and metastatic) from non-tumour tissues. The significant increase of GDF-15 and PSAP in prostate cancer patients compared to non-tumour tissues has been shown previously (Stopka-Farooqui et al. 2024). Clinical studies have also shown that an elevated increase in serum GDF-15 is significantly higher in Gleason score prostate cancer compared to the low-risk (with low Gleason score) and non-malignant (Bansal et al. 2017; Song et al. 2021). A significant increase in circulating GDF-15 is associated with poor response to docetaxel and shorter overall survival in metastatic CRPC (Mahon et al. 2024). A study by Koochekpour et al. (2012) reported that serum PSAP levels were significantly elevated in patients with metastatic CRPC compared to non-tumour controls. Conversely, serum PSAP levels were significantly reduced in patients with localised prostate cancer relative to the normal group. To date, limited studies have reported on the expression of COL6A1 in prostate cancer patients.

COMPARATIVE EXPRESSION OF SELECTED GENE BIOMARKERS IN DIFFERENT TYPES OF CANCER

To further evaluate the potential of the selected biomarkers, the TNMplot database was utilised to investigate the expression patterns of the chosen gene biomarkers across various cancer types. Based on the latest global cancer statistics, lung, prostate, colorectal, stomach, and liver cancers are among the top five cancers with the highest incidence rates in men (Bray et al. 2024). Consequently, these five cancer types were selected for further analysis. The present findings showed significant upregulation of *GDF-15* in prostate and colon adenocarcinomas, with

TABLE 1. Summary of the fold changes of the expression of the selected genes in non-tumour as compared to the primary tumour of prostate cancer patients

Selected genes	Non-tumour		Primary tumour		Fold changes	*P* value
	Median	N	Median	N		
GDF-15	1021.00	198	6302.00	478	6.17	0.001
PSAP	19513.00	201	29838.5	494	1.53	0.001
*COL6A*1	20598.00	201	3369	491	- 6.11 *	0.001

Data wre obtained from the TNMplot database and analysed using the Mann-Whitney U test. The outlier's data were removed prior to statistical analysis in non-tumour and primary tumours

GDF-15, growth/differentiation factor-15; PSAP, prosaposin; COL6A1, collagen-α-1 (VI) chain

Fold changes were calculated using the following formula: (Median Primary Tumour / Median Non-Tumour). The gene is upregulated in the primary tumour compared with the non-tumour tissue

*Fold changes were calculated using the following formula: (Median Non-tumour / Primary Tumour). The gene is downregulated in the primary tumour compared with the non-tumour tissue

TABLE 2. Summary of the fold changes of the expression of the selected genes in non-tumour as compared to the primary tumour and metastatic prostate cancer patients

Selected genes	Tissue Samples	Median	N	Normal vs. Primary		Normal vs. Metastatic		Primary vs. Metastatic	
				Fold changes *	*P* value	Fold changes **	*P* value	Fold changes ***	*P* value
GDF-15	Non-Tumour	456	98	5.06	0.013	5.53	0.001	1.09	0.904
	Primary Tumour	2307	278						
	Metastatic	2523	6						
PSAP	Non-Tumour	11603	106	1.16	0.004	0.97	0.510	0.83	0.145
	Primary Tumour	13460	283						
	Metastatic	11231	6						
COL6A1	Non-Tumour	3265.95	106	- 1.73	0.248	- 2.68	0.400	- 1.55	0.64
	Primary Tumour	1887	282						
	Metastatic	1217	6						

Data were obtained from the TNMplot database and analysed using Kruskal-Wallis's test. The outlier's data were removed prior to statistical analysis in the non-tumour, primary tumour, and metastatic groups

Fold changes were calculated using the following formula: * (Median Primary Tumour / Median Non-tumour) or **(Median Metastatic / Median Non-tumour) or ***(Median Metastatic / Median Primary Tumour)

GDF-15, growth/differentiation factor-15; PSAP, prosaposin; COL6A1, collagen-α-1 (VI) chain

The gene is downregulated in the primary tumour or metastatic prostate cancer compared to the non-tumour.

the most pronounced increases in *GDF-15* expression, at least 6-fold and 12-fold higher levels, respectively, in primary tumour tissues compared to non-tumour tissues (Supplementary Table 1). In contrast, *GDF-15* expression was downregulated in lung squamous cell carcinoma, with a minimum 2-fold decrease in primary tumour tissues relative to non-tumour tissues (Supplementary Table 1). These results suggest that *GDF-15* may play a dual role in cancer biology, acting as a potential oncogenic marker in certain cancer types while being suppressed in others, such as lung squamous cell carcinoma. This tissue-specific expression pattern highlights the role of GDF-15 in tumour progression and warrants further investigation into its functional mechanisms across different cancers.

The literature on GDF-15 is often characterised by a paradoxical dual role, whereby it functions as both a tumour promoter and, under specific conditions, a tumour suppressor or a mediator of drug sensitivity. For instance, GDF-15 has been shown to promote the progression of colon cancer cells in an inflammatory microenvironment by activation of c-FOS and EMT pathways (Ding et al. 2020), and elevated serum concentrations are significantly correlated with increased adenoma recurrence and shorter survival rates in colorectal cancer (Brown et al. 2012; Wallin et al. 2011). High serum/plasma GDF-15 levels are also associated with more advanced stages of liver metastasis (Shen et al. 2018; Wang et al. 2017) and are also significantly elevated in gastric cancer, where GDF-15 shows promise for early-stage detection (Ge et al. 2020). In contrast, Wang et al. (2017) support the protective role of GDF-15. Reduced GDF-15 expression in 5-fluorouracil (5-FU)-resistant colon cancer cells was associated with enhanced migration and resistance to apoptosis, and restoring GDF-15 expression resensitized these cells to 5-FU. Furthermore, lower plasma GDF-15 levels were significantly associated with improved immunotherapy efficacy and better prognosis in patients with advanced non-small cell lung cancer (NSCLC) (Hong et al. 2023). This clear duality highlights the importance of GDF-15 expression, which must be interpreted cautiously and in a tissue-specific context.

PSAP expression was significantly upregulated across all cancer types in primary tumour tissues compared with non-tumour tissues (Supplementary Table 2). The fold changes in *PSAP* expression ranged from 1.15-fold to 1.77-fold higher in primary tumour tissues, with liver hepatocellular carcinoma exhibiting the highest increase (1.77-fold) among the cancer types examined. These findings suggest that elevated *PSAP* expression in tumour tissues is not specific to a particular cancer type, indicating its potential role as a general marker of tumour progression rather than a cancer-specific biomarker. Apart from prostate cancer, reports on PSAP upregulation in other malignancies are limited, with current evidence primarily observed in gastric cancer (Wen et al. 2022; Zhang et al. 2025), head and neck squamous cell carcinoma (Datta et al. 2024), breast cancer (Ali et al. 2015), and gallbladder cancer (Sahasrabuddhe et al. 2014). A recent study by Zhang et al. (2025) showed that PSAP knockdown significantly inhibited the invasion and migration of gastric cancer cells.

Significant downregulation of *COL6A1* was observed not only in prostate adenocarcinoma but also in other cancer types (Supplementary Table 3). For instance, lung adenocarcinoma and lung squamous cell carcinoma exhibited at least a 2-fold decrease in *COL6A1* expression, while colon adenocarcinoma showed a 4-fold decrease compared to non-tumour tissues. Notably, prostate adenocarcinoma demonstrated the most pronounced downregulation of *COL6A1* in primary tumour tissues relative to non-tumour tissues. These findings suggest that *COL6A1* downregulation is a common feature across multiple cancer types, with prostate adenocarcinoma showing the most significant reduction, potentially implicating its role in tumour progression or tissue-specific cancer biology.

Consistent with our findings, recent evidence indicates that COL6A1 is downregulated in bladder cancer, and its suppression promotes tumour growth by regulating fibrillin-1 (FBN1) (Chen et al. 2024). However, previous studies have reported contrasting findings. Huang et al. (2022) identified COL6A1 as an epithelial–mesenchymal transition (EMT)-related protein involved in colorectal cancer metastasis, while Zhang et al. (2021) demonstrated that elevated COL6A1 expression in osteosarcoma tissues promotes cell invasion and migration via the TGF-β/COL6A1 signalling pathway. Similarly, COL6A1 overexpression has been reported in non-small cell lung cancer, particularly in cases with bone metastases (Li et al. 2021), as well as in pancreatic cancer (Owusu Ansah et al. 2019), glioblastoma (Chen et al. 2024), and clear cell renal cell carcinoma (Wan et al. 2015), where it is frequently associated with poor prognosis. In prostate cancer, significantly elevated levels of urinary COL6A1 have been reported compared to non-cancerous controls (Davalieva et al. 2017). Zhu et al. (2015) also demonstrated that COL6A1 levels were higher in androgen-independent LNCaP prostate cancer cells and in castration-resistant prostate cancer tissues than in androgen-dependent LNCaP cells and hormone-sensitive tumour tissues, respectively. In addition, suppression of COL6A1 expression was associated with decreased cell proliferation, likely involving the JAK2–STAT signalling pathway. In our current study, the use of publicly available platforms such as TNM plot, which integrate large-scale transcriptomic datasets from multiple cohorts, may partially account for the observed discrepancies, as the analyses are based on bulk gene expression profiles that are inherently influenced by tumour heterogeneity, cellular composition, and variations in stromal content. However, these contrasting findings regarding the role of COL6A1 in cancer progression highlight the need for further investigation to validate and clarify its functional significance across different cancer types.

CONCLUSION

The present study provides valuable insights into the expression patterns of key protein biomarkers, including GDF-15, PSAP, and COL6A1, across different prostate cancer cell lines and their potential roles in prostate cancer metastasis. GDF-15 showed prominent expression in LNCaP cells, while PSAP and COL6A1 were more abundant in PC-3 cells. Analysis using TNMplot further demonstrated increased expression of *GDF-15* and *PSAP* in primary prostate tumours, supporting their potential utility as diagnostic indicators. In contrast, the widespread downregulation of COL6A1 across multiple cancer types highlights the complexity of its roles as a biomarker in cancer biology. These findings also suggest that GDF-15 and PSAP could function as general cancer markers. However, further research is warranted to validate these biomarkers and elucidate their functional mechanisms in prostate cancer and other malignancies. Further understanding of the expression patterns of these biomarkers in larger sample sizes, especially for metastatic tumours, is crucial and can aid in biomarker selection for prostate cancer diagnostics and targeted therapies.

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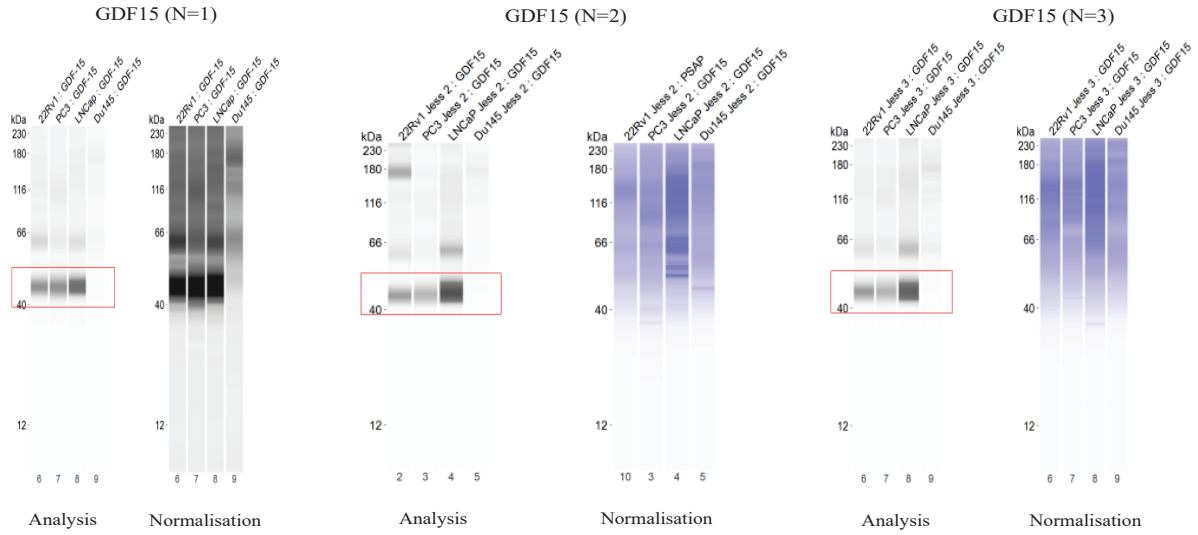
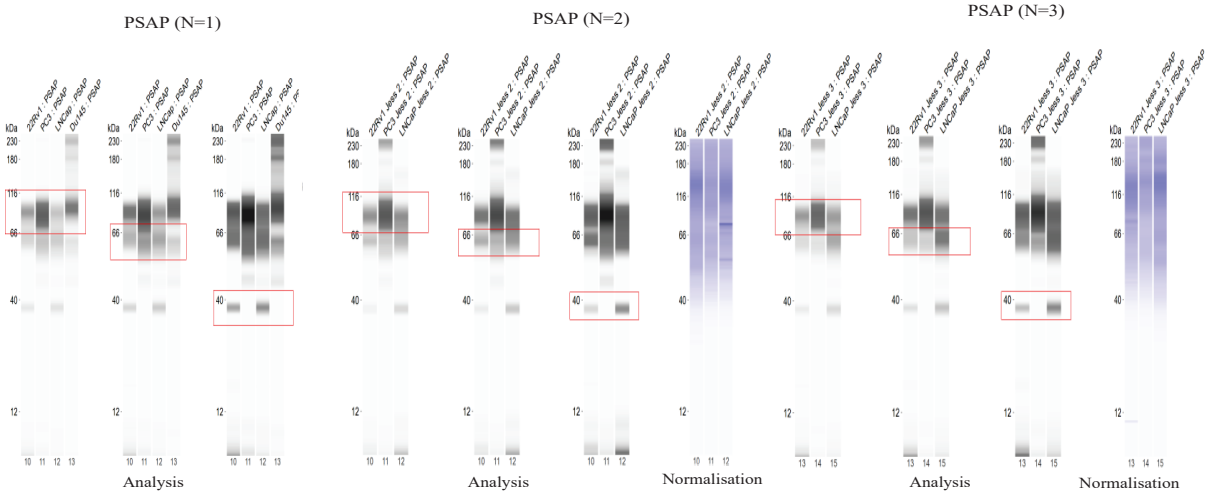
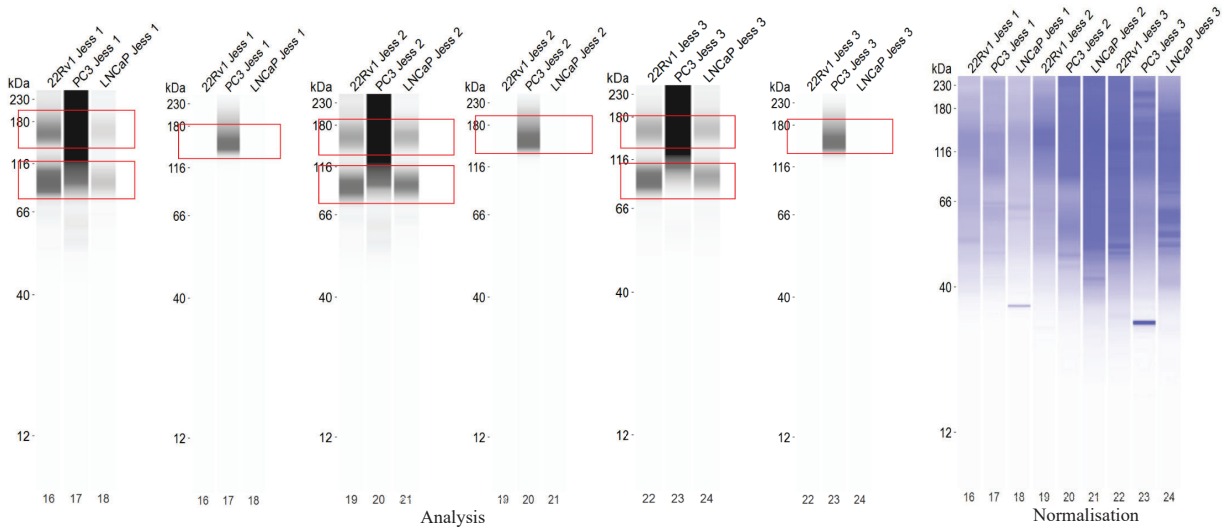


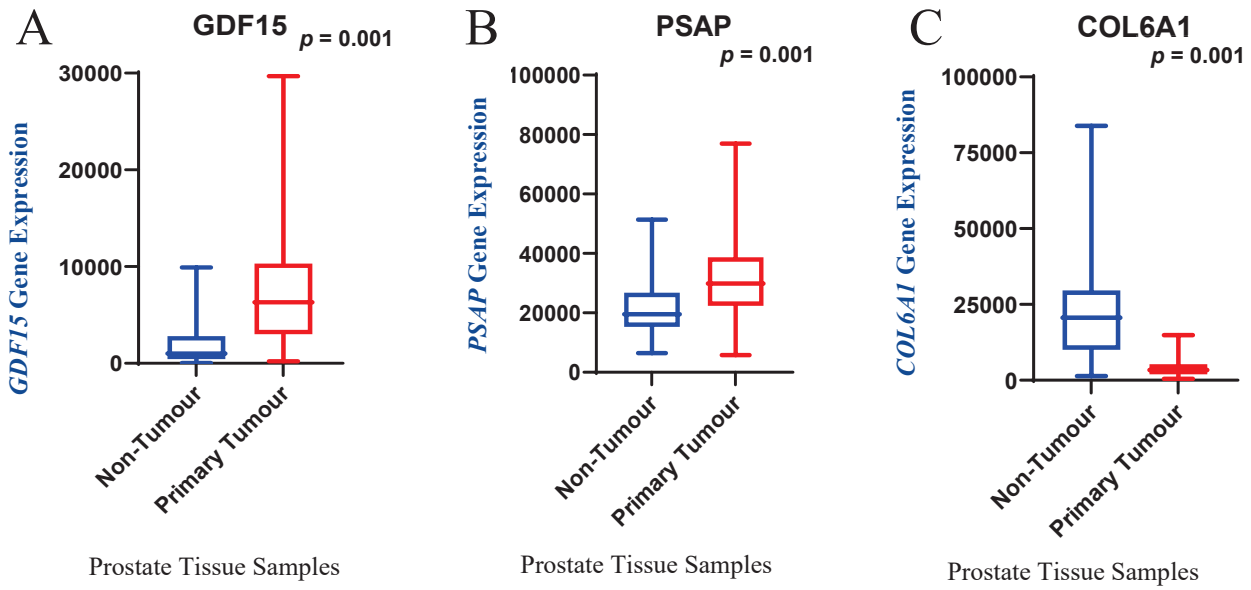
SUPPLEMENTARY FIGURE 1. Band intensity of GDF-15 based on the automated JESS Western blot analysis. One clear band at the ~ 40 kDa was observed in all cell lysates from primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells, with higher intensity observed in the LNCaP cells as compared to 22Rv1 and PC-3 cells. The isolated proteins were prepared, and the quantified proteins were analysed for protein expression levels using an automated Western blot (Jess system). An antibody against GDF-15 was used. Protein normalisation and separation (Protein Simple, USA) were used to normalise and separate the proteins. N=1 to N=3; triplicate



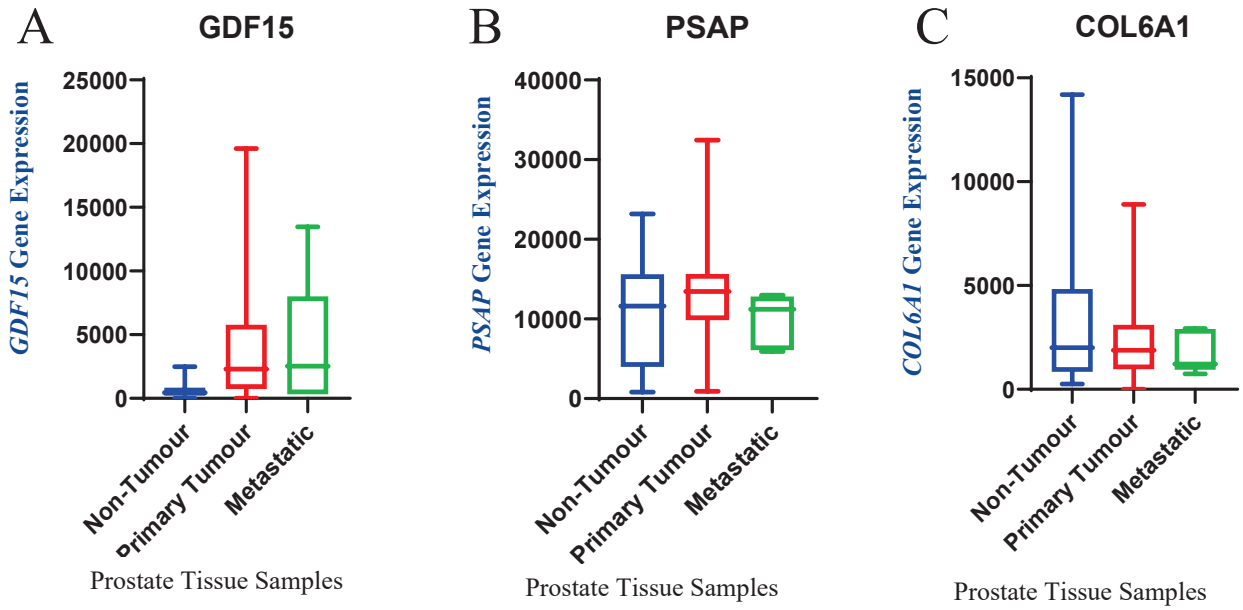
SUPPLEMENTARY FIGURE 2. Band intensity of PSAP based on the automated JESS Western blot analysis. Three clear bands at the ~90 kDa, ~60 kDa, and ~40 kDa were observed in cell lysates from primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells, with higher intensity observed in the PC-3 cells as compared to 22Rv1 and LNCaP cells. The isolated proteins were prepared, and the quantified proteins were analysed for protein expression levels using an automated Western blot (Jess system). An antibody against GDF-15 was used. Protein normalisation and separation (Protein Simple, USA) were used to normalise and separate the proteins. N=1 to N=3; triplicate



SUPPLEMENTARY FIGURE 3. Band intensity of COL6A1 based on the automated JESS Western blot analysis. Three clear bands at the ~100 kDa and ~150 kDa were observed in cell lysates from primary (22Rv1), bone metastatic (PC-3), and lymph nodes metastatic (LNCaP) prostate cancer cells, with higher intensity observed in the PC-3 cells as compared to 22Rv1 and LNCaP cells. The isolated proteins were prepared, and the quantified proteins were analysed for protein expression levels using an automated Western blot (Jess system). An antibody against GDF-15 was used. Protein normalisation and separation (Protein Simple, USA) were used to normalise and separate the proteins. N=1 to N=3; triplicate



SUPPLEMENTARY FIGURE 4. Comparison of the expression of (A) *GDF-15,* (B) *PSAP,* and (C) *COL6A1* genes in normal and primary tumour prostate cancer tissue based on the TNMplot database. The outliers in the data were removed based on the boxplot analysis in SPSS. Data were analysed using the Mann-Whitney U test. A significant increase in the *GDF-15 and PSAP* genes was observed in the primary tumour of prostate cancer patients compared to the non-tumour tissue samples. Meanwhile, *COL6A1* was found to be downregulated in the primary tumour of prostate cancer patients as compared to the non-tumour tissue samples. NGDF-15, growth/differentiation factor-15; PSAP, prosaposin; AMACR, COL6A1, collagen-α-1 (VI) chain



SUPPLEMENTARY FIGURE 5. Comparison of the expression of (A) *GDF-15,* (B) *COL6A1,* and (C) *PSAP* genes in normal, primary tumour, and metastatic prostate cancer tissue based on the TNMplot database. The outliers in the data were removed based on the boxplot analysis in SPSS. Data were analysed using a Kruskal-Wallis's test. GDF-15, growth/differentiation factor-15; PSAP, prosaposin; COL6A1

SUPPLEMENTARY TABLE 1. Summary of the fold changes of the expression of the *GDF-15* genes in non-tumour as compared to primary tumour in different types of cancer

Types of cancer	Non-tumour							Primary tumour							Fold changes	*P* value
	Median	Mean	Q1	Q3	Min	Max	N	Median	Mean	Q1	Q3	Min	Max	N		
Lung AC	453	607.41	251.75	830.25	47	2549	460	928.5	1390.53	385.75	1886.25	14	6373	490	2.05	0.001
Lung SC	461.50	625.74	261.75	834.25	44	2454	450	218.50	343.26	99.25	462	6	1539	458	- 2.11	0.001
Prostate	1021.00	1936.74	428.25	2814.25	40.00	9910.00	198	6302.00	7774.24	3012.00	10321.25	200.00	29692.00	478	6.17	0.001
Colon	199.50	266.35	104.75	1353	15	1202	298	2472	3572.38	345	4804	67	14632	449	12.39	0.001
Stomach	520.5	732.07	134.25	1025.25	3	3643	278	1181.5	1629.47	534	2371.5	62	7323	362	2.27	0.001
Liver	215	478.53	88.5	655.25	2	23226	206	826	1346.64	409.25	1821.75	19	5984	344	3.84	0.001

Data were obtained from the TNMplot database and analysed using the Mann-Whitney U test. The outlier's data were removed prior to statistical analysis in non-tumour and primary tumours

Fold changes were calculated using the following formula: (Median Primary Tumour / Median Non-tumour)

GDF-15, growth/differentiation factor-15

The gene is downregulated in the primary tumour compared to the non-tumour

SUPPLEMENTARY TABLE 2. Summary of the fold changes of the expression of the *PSAP* genes in non-tumour as compared to primary tumour in different types of cancer

Types of cancer	Non-tumour							Primary tumour							Fold changes	*P* value
	Median	Mean	Q1	Q3	Min	Max	N	Median	Mean	Q1	Q3	Min	Max	N		
Lung AC	29036	33662.25	20898	41824	5101	100890	481	36518	42323.24	25760.25	55204.5	4974	130873	518	1.26	0.001
Lung SC	29485	33096.44	22789.5	40864.5	6473	94284	469	34013	38793.51	23380.75	50324	5833	127651	498	1.15	0.001
Prostate	19513.00	21795.99	15303.00	26810.50	6421	51382	201	29838.5	31810.03	22390.50	38678.5	5813.00	76954.00	494	1.53	0.001
Colon	13471.5	16818.65	10236	20976.75	4185	49593	304	17523.5	21847.47	12169.5	28290.75	1601	74792	454	1.30	0.001
Stomach	18322	21167.81	13277.75	26365.75	7113	59558	294	21521	24817.3	14932	31768	2831	81488	369	1.17	0.001
Liver	12889	14355.75	9696	16076.75	4105	40666	225	22833	25581.35	17701.5	31339.25	6123	75815	362	1.77	0.001

Data were obtained from the TNMplot database and analysed using the Mann-Whitney U test. The outlier's data were removed prior to statistical analysis in non-tumour and primary tumours

Fold changes were calculated using the following formula: (Median Primary Tumour / Median Non-tumour)

PSAP, prosaposin

SUPPLEMENTARY TABLE 3. Summary of the fold changes of the expression of the *COL6A1* genes in non-tumour as compared to primary tumour in different types of cancer

Types of cancer	Non-tumour							Primary tumour							Fold changes	*P* value
	Median	Mean	Q1	Q3	Min	Max	N	Median	Mean	Q1	Q3	Min	Max	N		
Lung AC	12616.5	14524.66	7557.5	19267	1045	51555	482	4996	6471.08	2610.5	8592	410	25821	513	-2.53	0.001
Lung SC	13378	14982.81	9077	19573	1198	49120	471	5661	7190.93	3009	9345	228	28104	477	-2.36	0.001
Prostate	20598.00	22550.17	10039.50	29629	1415	83860	201	3369	4070.74	1976	5333	426	14855	491	- 6.16	0.001
Colon	16660	28367.12	6421	43595	1563	145806	311	4022	5386.02	2148	7551	86	24373	449	- 4.14	0.001
Stomach	8946	15313.61	4699	3971	718	67043	279	7876.5	10347.45	20883	13721	426	41983	370	-1.14	0.003
Liver	2603	2985.94	1841.5	3701.5	495	8742	213	1974.5	2541.27	921.75	3460.5	66	10591	360	- 1.32	0.001

Data were obtained from the TNMplot database and analysed using the Mann-Whitney U test. The outlier's data were removed prior to statistical analysis in non-tumour and primary tumours

Fold changes were calculated using the following formula: (Median Primary Tumour / Median Non-tumour)

COL6A1, collagen-α-1 (VI) chain

The gene is downregulated in the primary tumour compared to the non-tumour