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Research Article

Analysis of COL6A3, COL6A5 and COL6A6 Gene Expression in Breast and Prostate Tumors

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Abstract

Collagen VI is a ubiquitous extracellular matrix (ECM) protein that plays a structural role in the ECM and is found in close association with the basement membranes of a variety of cell types. Collagen VI that exists as a heterotrimer of three chains; 1(VI), 2(VI) and 3(VI) encoded by the COL6A1, COL6A2 and COL6A3 genes, respectively. Two new collagen VI genes have been described recently in humans; COL6A5 and COL6A6. The role of collagen VI in cancer is very poorly understood although it has recently been shown that the expression of the collagen VI genes has been shown to be altered in several different types of solid tumors. The aim of this study is to investigate the tissue distribution of COL6A3 and the two new collagen VI genes, COL6A5 and COL6A6 and to determine whether the expression of these genes is altered in breast and prostate tumors. We found that COL6A3 is expressed in a wide range of tissues with highest expression in skin and uterus. In contrast, COL6A5 and COL6A6 were expressed in a much smaller subset of tissues and, in general, not co-expressed in the same tissues. There were stage-specific differences in expression of COL6A3 in breast and prostate tumor panels. The expression of COL6A3 decreased with increasing tumor stage in both the breast and prostate models although the only the changes in prostate tumors were statistically significant (P=0.04). Our data suggest the exciting possibility that, since collagen VI gene expression decreases as the tumor becomes more malignant, collagen VI may be a clinically useful biomarker for tumor progression.

Keywords: collagen VI, gene expression, cancer

Introduction

Collagen VI is a ubiquitous protein that is expressed in the stroma of healthy connective tissues [1-3]. Classically, collagen VI exists as a heterotrimer composed of three genetically distinct protein chains; 1(VI), 2(VI) and 3(VI) [4-8] encoded by the COL6A1, COL6A2 and COL6A3 genes, respectively. The collagen VI assembly process is complex and incompletely understood. However, it is clear that individual collagen VI heterotrimers composed of the three chains associate into dimers and then following that, tetramers inside the cell. Tetramers are then secreted into the extracellular space where they associate end-to-end to form collagen VI microfibrils [9,10]. In normal tissues

these microfibrils surround fibroblasts, chondrocytes, and other mesenchymal cells and function in cell adhesion and attachment.

Recently, two additional collagen VI chains were identified in humans, 5(VI), 6(VI) encoded by the COL6A5 and COL6A6 genes [11,12]. A third new chain, 4(VI) is present in mice and other mammals but absent in humans. Domain architecture and phylogenetic analysis indicate that the new chains are most similar to the α3(VI) chain [11,12]. For example, the α3, α5 and α6 chains have large globular N-termini comprising multiple von Willebrand factor A-like (VWA) domains. α3(VI) has ten VWA modules (denoted N1 to N10), and α5(VI) and α6(VI) chain each have seven VWA domains (N1 to N7), compared to just a single VWA domain in the α1(VI) and α2(VI) chains. Each of the new chains has a 336 amino acid Gly-X-Y repeat triple helix and a globular C-terminus composed of several different types of protein modules. All collagen VI chains have a single Cys residue in the triple helical domain that is important for stabilizing higher-order assembly structures [10]. In the α3, α5 and α6 chains, this Cys is located 50 amino acids from the start of the triple helix. In contrast, the Cys residues in α1(VI) and α2(VI) are in a different relative position along the triple helix at amino acid These similarities between the α3, α5 and α6 chains suggests an assembly model for collagen VI where the α5 and α6 can substitute for α3 in assembling with α1 and α2 chains to provide for cell- or tissue-specific collagen VI assemblies.

Collagen VI is upregulated in various solid tumors and during tumor progression and has been shown to stimulate invasion and metastasis [13,14]. For example, the 3(VI) subunit was found to interact with the extracellular domain of TEM8 which is expressed predominantly in the endothelium of tumors [15]. COL6A3 expression was associated with colorectal cancer and may be a good biomarker for this tumor type [16,17]. One study reported that the 3(VI) chain was overexpressed 62-fold in a clonal ovarian tumor cell line which is known to be highly resistant to oxaliplatin [18,19]. In addition, the upregulation of 3(VI) collagen has been demonstrated in breast cancer [20,21] and tumor-specific alternative splicing of COL6A3 has been detected in breast, lung, prostate, and kidney cancer [22,23]. Furthermore, collagen VI expression was found to be reduced in experimental and spontaneous human fibrosarcomas; rhabdomyosarcomas, leiomyosarcomas, chondrosarcomas and liposarcomas [19]. These findings imply that the absence of collagen VI may contribute to the ability of a tumor to metastasize. While there are no reports associating COL6A5 with cancer, COL6A6 expression is altered in childhood leukemias [24] and breast cancer [25] and COL6A6 coding region variants reported in lung carcinoma [26]. Taken together, these studies clearly indicate that the regulation of the 3 and 6 chains of collagen VI are altered in tumorogenesis raising the exciting possibility that collagen VI may have utility as a surrogate marker for cancer.

The goal of this study was to examine the mRNA levels of COL6A3 and the two new collagen VI genes, COL6A5 and COL6A6, in two types of cancer; breast and prostate. This was achieved using commercially available cDNA expression panels derived from normal and select neoplastic adult human tissues using a quantitative PCR method.

Materials and Methods

Optimizing PCR conditions

The optimum reaction temperature and magnesium concentrations were determined using reverse-transcriptase PCR (RT-PCR) on a MJ Mini Personal Thermal Cycler (Bio-Rad) in temperature gradient mode. A master mix was created using the Expand High Fidelity PCR System of reagents (Roche). Primers were designed that were specific to COL6A3 and the novel collagen VI protein subunit genes, COL6A5 and COL6A6. These primers were then added to samples of human cDNA (Biochain Institute), creating a 50 l reaction volume containing 20pM primer concentration. This was performed according to the manufacturer recommendations (Expand High Fidelity PCR

System, Roche). The products were then electrophoresed on a 1% agarose gel with ethidium bromide. The gel was exposed to ultraviolet light and the fluorescence intensity of the bands analyzed (Quantity One Software ver.4.5.2 with GelDoc XR, Bio-Rad).

Distribution of collagen subunit gene expression in human tissues

The distribution of COL6A3, COL6A5 and COL6A6 mRNA in human tissues was determined using commercially available human expression panels (HMRT101, Origene). These panels, which are specifically designed for quantitative PCR (qPCR), contain cDNA made from mRNA purified from 48 different human tissues. The gene-specific primers (20pM final concentration) were added to the iQ SYBR Green Supermix (#170-882, Bio-Rad) which is also specifically designed for qPCR. This reagent is a pre-made master mix containing all the components for a qPCR analysis including thermostable DNA polymerase, dNTPs, SYBR Green DNA binding dye, Mg^{2+} ions and reaction buffer. The SYBR Green dye fluoresces upon binding to double-stranded DNA and this fluorescence is detected by the qPCR thermocycler (ICycler, Bio-Rad) over a preset number of cycles. Since the fluorescence is measured by the thermocycler at the end of every cycle, the PCR reaction is followed in real-time. Following assembly of the Master mix and primers the reaction solution was carefully pipetted into each well of the panel. The panels were set upon ice and then gently vortexed and centrifuged in a cell-culture centrifuge to remove any air bubbles. The reaction was run for 42 cycles. A 72°C extension step was utilized because our amplicon was greater than 120 base pairs. Each gene was amplified at the annealing temperature determined above. The expression profile of a known gene, actin, was analyzed using supplied primers (Origene). On each expression panel, two wells served as negative controls. The relative fluorescence units per doubling cycle were plotted for each gene.

Gene expression of novel collagen subunits in neoplastic human tissues

The mRNA levels for COL6A3, COL6A5 and COL6A6 were determined using commercially available human breast and prostate cancer expression panels (HCRT101 and HPRT101, Origene) optimized for qPCR. These expression panels contain 48 wells of cDNA from tumor samples that have been previously characterized and staged pathologically according to the American Joint Committee on Cancer (AJCC). The prostate expression panel contained normal tissue (stage 0) and tissue in benign prostatic hypertrophy (BPH). The qPCRs were conducted as described above for the tissue distribution panel including the inclusion of the positive control gene, β-actin. All the data was corrected for actin levels. For the breast cancer panel, multiple samples were present representing stage 0 (7 patients), 1 (10 patients), 2A (13 patients), 2B (7 patients), 3A (9 patients) and 3C (2 patients). The prostate cancer panel contained cDNA samples from 48 patients; stage 0 (7 patients), 1 (2 patients), 2 (18 patients), 3 (9 patients), 4 (1 patients) and BPH (11 patients).

Data analysis

For each qPCR experiment, the threshold cycle data generated from the quantitative PCR amplification was normalized to the relative fluorescence unit at which the most number of sample curves were linear (ICycler Software, Bio-Rad). This provided a normalization of the threshold cycle across all samples. Each value was then subtracted from the threshold cycle of the control gene, β-actin yielding a delta cycle threshold (ΔC_t). This value was then subtracted from the tissue with the lowest detectable expression, obtaining a threshold cycle relative to the tissue with the lowest detectable expression, a so-called delta delta cycle threshold value ($\Delta\Delta C_t$). The relative fold expression was determined by calculating the doubling value using $2^{\Delta\Delta Ct}$. Stages having one or two samples were excluded from analysis. Data from the breast and prostate cancer arrays were analyzed in spreadsheet using Microsoft Excel (Microsoft Corp). Statistical analyses were performed using SigmaStat (Version 3.1, Systat Software).

Results

Collagen VI subunit gene expression in human tissues

The COL6A3 gene was expressed in all human tissues with the lowest relative expression seen in optic nerve and the highest expression in ovary, uterus, and skin (Figure 1A). The expression of COL6A3 in uterus, which is predominantly smooth muscle, was 2778-fold greater than the lowest tissue, optic nerve. The next highest expressing tissues, ovary and skin, were 1031 and 1176-fold higher than optic nerve, respectively (see Figure 1A, inset). The expression of COL6A3 in mammary gland and fat was approximately 50 and 90-fold that of optic nerve, respectively. Its expression in prostate was 135-fold more than optic nerve.

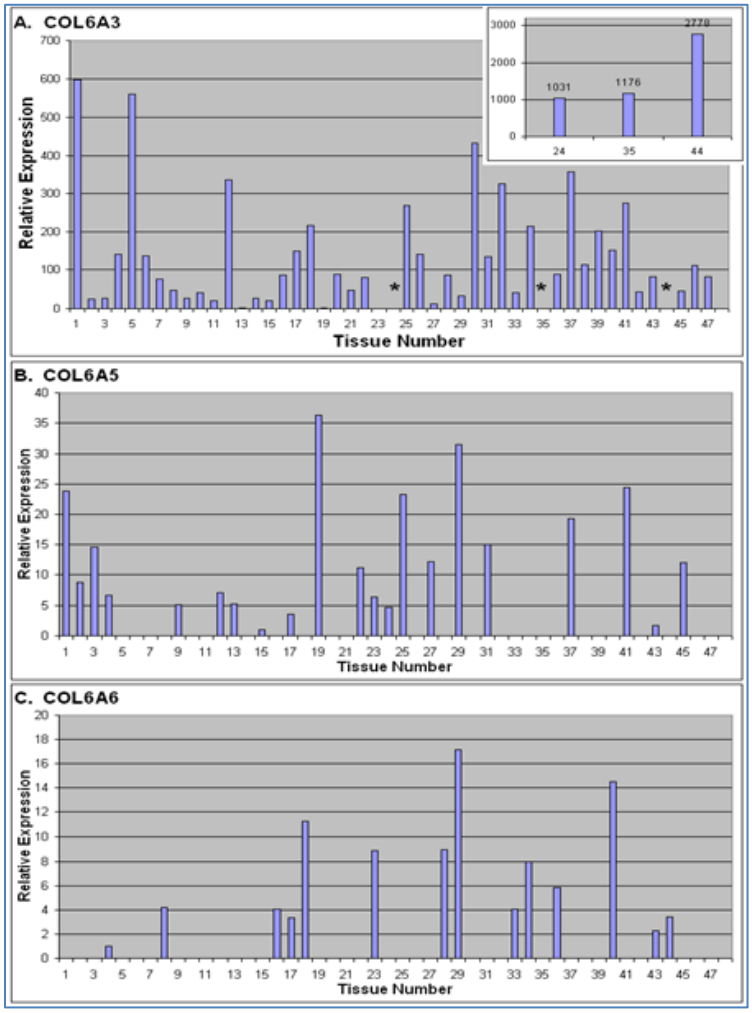

Figure 1: Distribution of gene expression in adult human normal tissues of COL6A3 (A), COL6A5 (B), COL6A6 (C) using human expression panels. Collagen subunit gene specific primers were used to amplify the genes with levels assessed through quantitative PCR. The threshold cycle numbers were normalized to a supplied control gene, β-actin and then normalized to the threshold cycle of the tissue expressing the lowest amount of collagen VI subunit mRNA. The data for COL6A3 was normalized to expression in optic nerve. The data for COL6A5 was normalized to larynx. COL6A6 data was normalized to bronchus. Tissue 1: Adrenal Gland, 2: Bone Marrow, 3: Brain, 4: Bronchus, 5: Cervix, 6: Colon, 7: Duodenum, 8: Epididymis, 9: Esophagus, 10: Fat, 11: Heart, 12: Small Intestine, 13: Intracranial artery, 14: Kidney, 15: Larynx, 16: Liver, 17: Lung, 18: Lymph Node, 19: Plasma blood leukocyte, 20: Mammary Gland, 21: Muscle, 22: Nasal Mucosa, 23: Optic Nerve, 24: Ovary, 25: Oviduct, 26: Pancreas, 27: Parathyroid, 28: Pericardium, 29: Pituitary, 30: Placenta, 31: Prostate, 32: Rectum, 33: Salivary Gland, 34: Seminal Vesicle, 35: Skin, 36: Spleen, 37: Stomach, 38: Testis, 39: Thymus, 40: Thyroid, 41: Tonsil, 42: Urethra, 43: Urinary Bladder, 44: Uterus, 45: Uvula, 46: Vagina, 47: Vena Cava, Blank. Tissues 24, 35, and 44 are labeled with an asterisk (*) in panel A and graphed in the inset. In these tissues, COL6A3 was expressed between 400-2100 fold greater than the next largest tissue.

The expression of COL6A5 and COL6A6 was significantly less than COL6A3 in the same tissues (Figure 1B & 1C). In general, these two genes were expressed in fewer tissues. For COL6A5, the expression was greatest in plasma blood leukocyte and lowest detectable expression in larynx and several tissues recorded no COL6A5 expression at all. COL6A5 was expressed in prostate but not mammary gland or fat.

Pituitary was the tissue with the greatest expression of COL6A6 and lowest in bronchus although, as with COL6A5, many tissues failed to express COL6A6 (Figure 1C). COL6A6 mRNA was not present in mammary gland, prostate or fat. Four tissues; urinary bladder, lung, pituitary, and optic nerve expressed both COL6A5 and COL6A6. We also noted that the C_t values for COL6A3 were much higher than the other two genes suggesting that COL6A3 is expressed at higher levels overall.

Expression of COL6A3, COL6A5 and COL6A6 in breast cancer

The COL6A3 expression data in breast cancer tumors was not normally distributed according to a normality test. It was noted that although there was variability between samples within any particular disease stage, a trend was apparent (Figure 2A). In general, expression of COL6A3 decreased with advancing tumor stage from stage 0 to stage 2B. The exception was stage 3A where COL6A3 mRNA expression was increased. This was supported by the Kruskal-Wallis One Way ANOVA on Ranks test. This test revealed a statistically significant decrease in the expression of COL6A3 with advancing stages of breast cancer (p=0.04) (Figure 2B). However, the Dunn's post-test analysis revealed that there were no significant expression differences between tumor stages when compared pairwise.

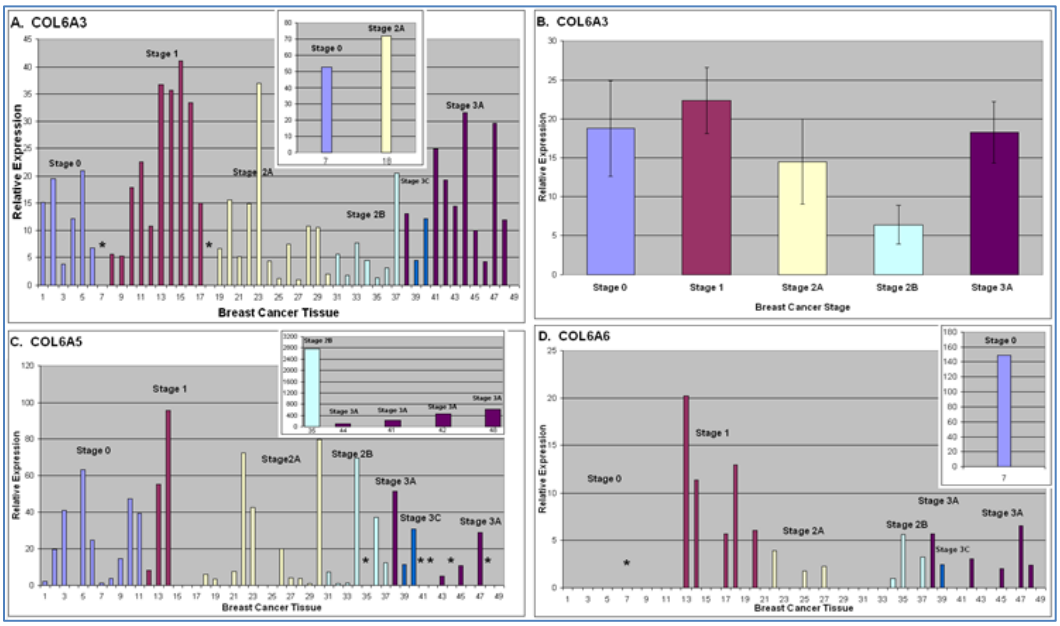

Figure 2 : Expression of COL6A3 (A), COL6A5 (C), and COL6A6 (D) in human breast cancer tissues using human cancer expression panels. Panel B plots the mean value for each stage of COL6A3 expression with standard deviations. Collagen subunit gene specific primers were used to amplify the genes with levels assessed through quantitative PCR. The relative fluorescence unit per cycle was generated. The threshold cycle numbers were normalized to a supplied control gene, β-actin and then normalized to the threshold cycle of the tissue expressing the lowest amount of collagen VI subunit mRNA. In panel A., the COL6A3 data was normalized to Well 27, a tissue in disease Stage 2A. The COL6A5 data was normalized to Well 29, a tissue in disease Stage 2A (panel C). COL6A6 was normalized to Well 34, Stage 2B (panel D). Each panel consisted of 48 wells containing cDNA generated from mRNA from breast cancer tissue staged according to the American Joint Committee on Cancer staging system for breast cancer. Wells 1-7 represent disease stage Wells 8-17 are tissue in stage I. Wells 18-30 represent tissue in stage IIA. Wells 31-37 represent tissue in stage IIB. Wells 38, 41-43, 45-48 represent tissue in stage IIIA. Wells 39-40, 44 represent tissue in stage IIIC. Several data had large values (asterisk) and these were graphed separately (inset). In COL6A3 (panel A), the data contained two tissues (wells 7, Stage 0, and 18, Stage 2A) in which gene expression was 10-30 fold greater than the next largest value. These two tissues were plotted in the inlay graph depicted above. The data for COL6A5 (panel C) also contained fold expression values for 5 tissues (Wells 35 Stage 2B, and Stage 3A tissues: 41, 42, 44, 48) which were significantly greater than the rest of the data (panel D). Likewise, the COL6A6 expression of well 7 (asterisk) was 100 fold greater than the next largest value (inset).

As shown in Figures 2C and 2D, the expression of COL6A5 and COL6A6 amongst the stages was highly variable and found to not be statistically significant using the Kruskal-Wallis One Way ANOVA on Ranks test (p=0.642 for COL6A5 and p=0.473 and COL6A6). One of the stage 2B samples expressed COL6A5 at a very high level (2800-fold more than the lowest expressed cDNA). In addition, stage 3A exhibited extreme variability in expression of COL6A5. Several tissues were 40-500 fold more than other samples within the stage. The expression of COL6A6 was less consistent with just 18 samples yielding data. Expression of COL6A6 was notably absent in all but one of the stage 0 tissue samples.

Expression of the COL6A3, COL6A5, and COL6A6 genes in prostate tumor panel

Expression of COL6A3 was observed in all samples (Figure 3A) and the data passed a normality test. The expression of COL6A3 amongst stages with >7 samples (stage 0, 2, 3 and BPH) was found to be statistically significant using a one-way ANOVA test (p=0.016, alpha 0.638). In pairwise comparisons, normal or stage 0 disease compared to stage 2 or 3 was found to be statistically significant using the Holm-Sidak method with an overall p value of 0.05. The expression of COL6A3 in stage 0 was found to be greater than stage 2 (p=0.002) and stage 3 (p=0.023) (see Figure 3B and Table 1). The BPH and Stage 3 comparison approached statistical significance (p=0.064).

Table 1: COL6A3 expression in prostate cancer stages: comparison between stages using the Holm-Sidak pairwise multiple comparison test with overall p = 0.05. COL6A5 was expressed in only 10 of the 48 patient tumor samples and not at all in stages 1, 3, and 4. Similarly, COL6A6 was expressed in 12 tissues. Overall, the expression of COL6A5 and COL6A6 was extremely small and inconsistent in prostate cancer with only sample #6 (stage 0) expressing all three collagen VI genes.

Comparison	Δ of Means	P value	Significant?
Stage 1 vs. Stage 3	3.14	0.0020	Yes
Stage 1 vs. Stage 2	1.98	0.0227	Yes
BHP vs. Stage 3	1.74	0.0636	No
Stage 2 vs. Stage 3	1.16	0.1380	No
Stage 1 vs. BHP	1.4	0.1560	No
BHP vs. Stage 2	0.58	0.4710	No

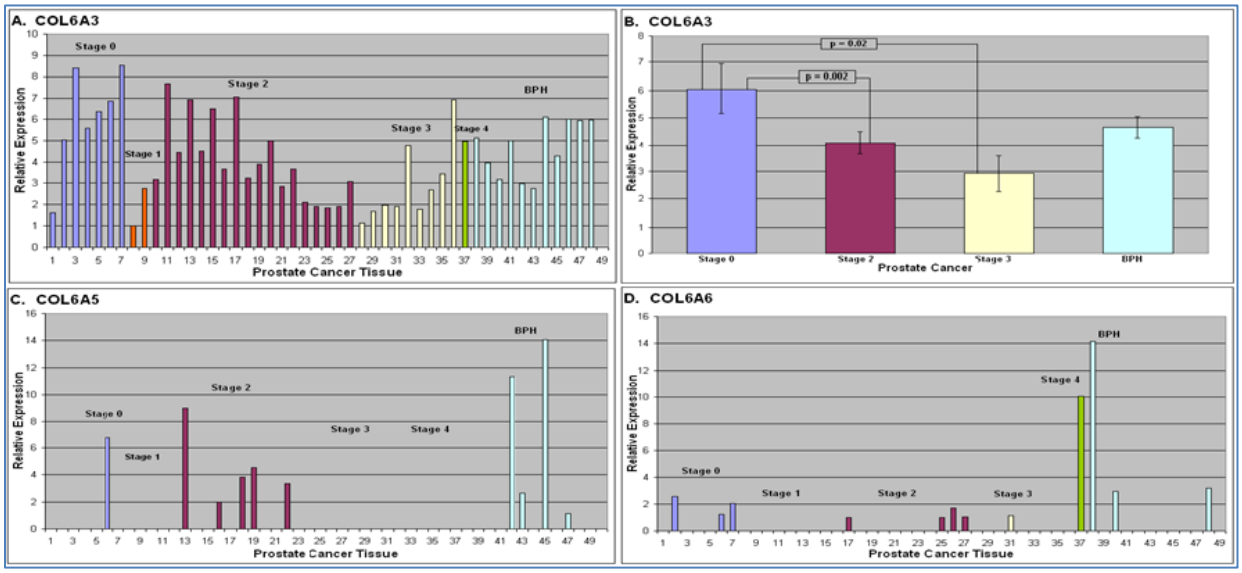

Figure 3: Expression of COL6A3 (A), COL6A5 (C), and COL6A6 (D) in human prostate cancer tissues using human cancer expression panels. Panel B plots the median value for each stage of COL6A3 expression with standard deviations.The COL6A3 data was normalized to Well 8, Stage 1 disease (shown in panel A). The COL6A5 data was normalized to Well 47, a tissue sample consistent with benign prostatic hypertrophy (panel B). COL6A6 was normalized to Well 25, Stage 2 (panel D). Each expression panel consisted of 48 wells containing cDNA from prostate cancer tissue staged according to the American Joint Committee on Cancer staging system for prostate cancer. Wells 1-7 represent disease stage 0. Wells 8-9 are tissue in stage I. Wells 10-26 represent tissue in stage II. Wells 29-36 represent tissue in stage III. Well 37 represents tissue in stage IV. Wells 38-48 represent benign prostatic hypertrophy (BPH).

Discussion

Collagen VI is produced by every cell type that synthesizes an extracellular matrix (ECM) and is considered to be a fundamental component of basement membranes. Quantitative PCR analysis demonstrated that COL6A3 is expressed in a wide range of tissues with highest expression in skin and uterus consistent with other studies [12,27,28]. In contrast, the expression of COL6A5 and COL6A6 is more restricted and these two chains are generally not co-expressed in the same tissues. Detailed COL6A5 and COL6A6 localization studies by immunohistochemistry has been conducted in two tissues whether the two genes are co-expressed; skin [29] and skeletal muscle [28,30]. In these tissues, the two chains have partially non-overlapping expression suggesting they may assemble into different collagen VI isoforms that impart subtle differences on the heterotrimeric collagen VI structure that provide for different functional properties.

Little is known about the role of extracellular matrix components or collagen VI in tumor biology. Several papers have reported changes in collagen VI expression levels in breast cancer [20,21], and other tumor types [13,14,16,21,23,31-33] and alternative splicing in prostate cancer [22,34]. While nothing is known about the expression of COL6A5 and COL6A3 in cancer there are several studies on the expression of COL6A3. For example, high COL6A3 expression is associated with worse gastric [35-37] and colorectal [16,17,38], pancreatic [39], ovarian [18,31] cancer and decreased COL6A3 methylation in glioblastomas [40], This study represents the first step in the analysis of COL6A3 and two novel subunits of collagen VI and their roles in normal and neoplastic human prostrate and breast tissue.

We detected stage-specific differences in expression of COL6A3 in the breast and prostate tumor panels. In the breast cancer samples, the levels of COL6A3 declined from stage 0 to stage 2B but increased in the most advanced stage 3A tumors. This is in contrast to Iyenger et al., who reported the expression of COL6A3 by qPCR in late stage or larger tumors to be to be 4 times greater than that seen in smaller or early disease stages [20]. This study was conducted in mice and does not report the staging system utilized. Also, tumor progression in mice may not parallel tumor progression in humans. The importance of tumor size relative to gene expression is unknown.

The extracellular matrix components in prostate cancer are known to promote cancer cell differentiation and alter gene-expression profiles [41]. However, there is little information on COL6A3 expression in prostate cancer. We show a statistically significant decline in COL6A3 mRNA expression as the stage of prostate cancer increased suggesting that COL6A3 is downregulated as prostate cancer advances. This is in contrast to the increased gene expression of COL6A3 in other tumor types. While our findings need to be independently confirmed, COL6A3 mRNA levels may demonstrate clinical utility as an adjunct in prostate cancer staging. Specific COL6A3 splicing variants are reported to be associated with prostate tumors [22,23], colon cancer [42] and pancreatic ductal adenocarcinoma [43]. Our PCR approach is based on a single COL6A3 amplicon and does not take into account different splice variants. However, cancer-associated alternative splicing is emerging as critical for chemo-resistance as the tumor evades cell death [44-46] and COL6A3 is a potential candidate gene for tumor detection or as a target for new therapies.

Our study is limited in two ways. First, larger tissue sample numbers will increase the power of the statistical analyses. For example, in the breast cancer panel a trend in COL6A3 expression that approached statistical significance was noted. Second, little is known about the history of these tumor samples. For example, patients undergoing treatment may have different gene expression patterns compared to non-treated patients. Since the relevant history is not available it is difficult to assess whether this if a factor in the present analysis.

Our data suggest that reduced collagen VI levels are associated with a less differentiated cell phenotype raising the exciting possibility that collagen VI could be a clinically useful biomarker for breast and prostate tumor

progression. Finally, collagen VI has been implicated in other pathologies besides cancer. For example, collagen VI levels are altered in hepatic fibrosis [47], endometriotic cysts [48], arthrofibrosis [49], and in the vasculature of the subependymal germinal layer of the premature infant creating a predisposition towards intraventricular hemorrhage [50]. Therefore, a collagen VI biomarker assay may be useful as a clinical tool in the diagnosis of these pathologies as well as cancer.

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