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Original Article

Characterization of laminin-332 gene expression in molecular subtypes of human bladder cancer

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Abstract: Human bladder cancer (BCa) exhibits morphological and molecular heterogeneity which can complicate treatment. Morphologically, more than 90% of BCa is classified as urothelial cell carcinoma (UCC). Among other histological variants, UCC with squamous differentiation (SqD) shows a worse prognosis than pure UCC. In addition, basal-squamous BCa is enriched for SqD, and these tumors have a poor prognosis. Therefore, it is critical to elucidate the mechanisms to drive the basal-squamous phenotype of human BCa. Laminin-332 is a major glycoprotein of the epithelial basement membrane. It is well known that laminin-332 is a favorable target for extracellular matrix proteases such as matrix metalloproteinases (MMPs) in various diseases. Accumulating evidence indicates the significant role of laminin-332 in tumorigenesis. Here, we analyzed the expression of laminin-332 genes (*LAMA3, LAMB3, LAMC2*) in molecular subtypes of human BCa using publicly available data from The Cancer Genome Atlas (TCGA). Additionally, we also used q-RT-PCR to characterize laminin-332 gene expression between distinct molecular subtypes of human BCa cell lines. Our analysis of publicly available data show that laminin-332 genes are highly expressed in the basal-squamous molecular subtype of human BCa. In addition, we show laminin-332 genes are highly expressed in basal-squamous human BCa cell lines. Moreover, the expression of both *LAMA3* and *LAMC2* are negatively correlated with expression of the luminal transcription factor (TF) *FOXA1* in the TCGA data. We also demonstrate that laminin-332 genes are downregulated by the overexpression of *FOXA1* in a human basal-squamous BCa cell line (5637). Taken together, these results suggest that laminin-332 gene expression may be a biomarker of BCa patients with basal-squamous disease.

Keywords: Bladder cancer, laminin-332, basal-squamous, FOXA1

Introduction

According to the American Cancer Society (ACS), it is estimated that 83,730 cases of bladder cancer (BCa) were diagnosed in 2021 [1]. BCa exhibits morphological and molecular heterogeneity which can influence systemic and targeted therapy. Morphologically, more than 90% of BCa is classified as urothelial cell carcinoma (UCC). Evidence suggests that UCC with squamous differentiation (SqD) confers a worse prognosis relative to conventional UCC. Several researchers demonstrated that the majority of early stage, non-muscle invasive BCa (NMIBCa), as well as a subset of muscle invasive BCa (MIBCa) exhibit a luminal gene expression signature enriched in luminal markers including Forkhead Box A1 (*FOXA1*), which is required for urothelial differentiation [2]. On the other hand, regions of SqD are often observed in late stage MIBCa, and these tumors exhibit a basal gene expression signature, including elevated expression of high molecular weight cytokeratins, cytokeratin 5 and 14 (*KRT5*, *KRT14*) [3-6]. Recent studies have classified human BCa into two major molecular subtypes termed “luminal” and “basal-squamous” based on the expression of select genes, including those described above. Currently, it is recognized that the basal molecular subtype of BCa with SqD is one of the most aggressive form of BCa [7]. Therefore, it is essential to identify molecular factors that contribute to the devel-

opment of the basal subtype with SqD of human BCa.

Laminins are large glycoproteins of the basement membrane consisting of α, β, and γ chains that form a cross-shaped heterotrimer (αβγ) [8]. To date, genes of five α chains, four β chains, and three γ chains have been identified, and a total of 16 laminin isoforms are defined by the combination with different α, β, γ chains. Laminin α, β, and γ chains have distinct tissue-specific expression patterns [9]. Therefore, individual laminin isoforms exhibit specific functions in regulating cell proliferation, adhesion, migration, survival and differentiation of tissue during mammalian development [8, 10, 11]. Among laminin isoforms, laminin-332 is a major glycoprotein of the basement membrane in the epithelium and consists of α3, β3 and γ2 chains encoded by the *LAMA3*, *LAMB3*, *LAMC2* genes, respectively [12]. Laminin-332 interacts with multiple cell surface receptors such as syndecan-1, integrin α3β1, and integrin α6β4 to regulate cell adhesion, migration, and tissue stability [13, 14].

Different from other laminin isoforms, it is well known that laminin-332 is the target of proteases such as matrix metalloproteinases (MMPs) [15]. The short arm at the N terminus of laminin γ2 chain is cleaved by MMP-2 and MT1-MMP (MMP-14). In particular, MMP-14 cleaves off domain III (DIII, known as EGF-like domain) of the laminin γ2 chain and the released DIII fragment binds to epidermal growth factor receptor (EGFR), leading to the acceleration of cell migration [15-19]. Other MMPs (MMP-3, 12, 13, 19, 20) are also reported to cleave the laminin γ2 chain [20, 21]. In addition, cleavage of the laminin β3 chain by matrilysin 1 (MMP-7) promotes cell migration in colon cancer cells [22]. Other non-MMP proteases are implicated in the processing of laminin-332, including neutrophil elastase, BMP-1, mTLD, and cathepsin S [23-26]. Thus, although abundant evidence suggests that laminin-332 is a substrate for various proteases, and this substrate function contributes to tumor aggressiveness, the mechanisms of laminin-332 gene regulation in distinct molecular subtypes of human BCa remains unclear.

Our findings show that laminin-332 genes are highly expressed in the basal-squamous molecular subtype of human BCa, as well as in representative basal-squamous BCa cell lines. Additionally, we demonstrate that laminin-332 genes are downregulated by overexpression of *FOXA1* in a basal-squamous human BCa cell line 5637. These results suggest that laminin-332 genes are associated with basal-squamous BCa and may serve as a marker to identify this aggressive molecular subtype.

Materials and methods

Cell culture

All human BCa cell lines were purchased as described previously [27] and authenticity was confirmed by short tandem repeat (STR) analysis. The BCa cell lines RT4 and T24 were cultured in McCoy's Modified Medium with 10% FBS. UMUC1 and UMUC3 cells were cultured in Minimal Essential Medium (MEM) supplemented with 10% FBS. SCaBER, HT1197, HT1376 and TCCSUP cell lines were cultured in MEM following the addition of Non-Essential Amino Acids (NEAA) and 10% FBS. 5637 and SW780 cell lines were cultured in Roswell Park memorial Institute (RPMI) 1640 following the addition of 10% FBS.

RNA extraction and quantitative real-time PCR (q-RT-PCR)

Total RNA was extracted using the RNeasy approach (Qiagen, Hilden, Germany) according to manufacturer's protocol. For cDNA synthesis, reverse transcription was performed using M-MLV reverse transcriptase (Thermo Fisher) via manufacturer instructions. q-RT-PCR was performed using QuantaStudio7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Taqman probes used in this study were as follows. *LAMA3* (Hs00165042_m1), *LAMB3* (Hs00165078_m1), *LAMC2* (Hs01043717_m1). Relative gene expression differences were calculated by the ΔΔCt method. 18S ribosomal RNA was used as an endogenous reference.

Overexpression of FOXA1 in basal-squamous subtype of human BCa cell line

The day before transfection, 2×10^5 cells of 5637 BCa cells were plated in 6 well plates (Corning Inc, Corning, NY). On the following day, attached cells were transfected with 2.5 mg of the following plasmid constructs: pCMV6-Entry (CMV empty vector; Origene, Rock-

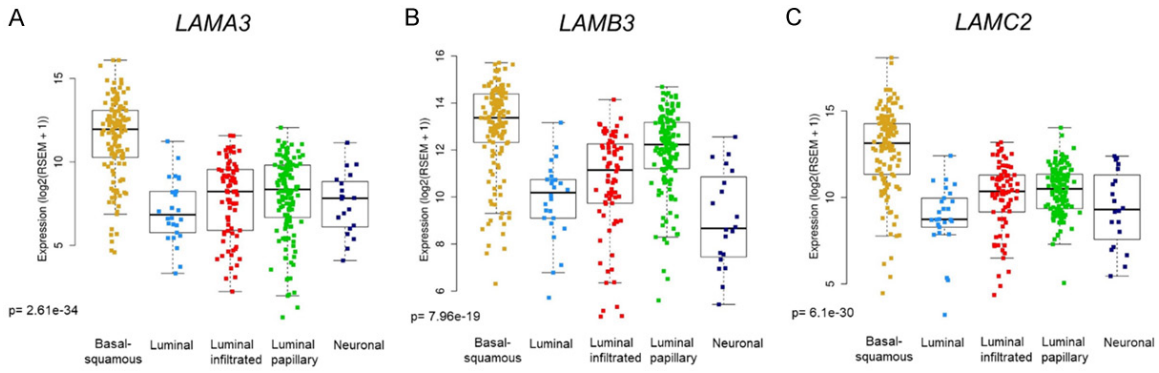

Figure 1. Laminin-332 (*LAMA3*, *LAMB3*, *LAMC2*) gene expression is associated with basal-squamous BCa. (A) *LAMA3*, (B) *LAMB3*, (C) *LAMC2* are enriched in basal-squamous subtype (basal-squamous vs. luminal, luminal infiltrated, luminal papillary, and neuronal, *P*<0.0001, Mann-Whitney U test).

ville, MD), pCMV6-FOXA1 (Origene; RC206045) using Lipofectamine 3000 (Life Technologies, Carlsbad, CA). After 48 hours incubation, RNA was extracted for analysis.

Statistical analysis

GraphPad Prism6 (GraphPad Software, San Diego, CA) and R 3.5.0 [36] were used for statistical analysis. P<0.05 was considered as a statistically significant. Mann-Whitney U test was used to compare *LAMA3*, *LAMB3*, *LAMC2* expression between Luminal/Non-type and Basal-squamous subtypes of human BCa cell lines. Student's t test was used to compare *FOXA1*, *LAMA3*, *LAMB3*, *LAMC2* expression between parental 5637 and *FOXA1*-overexpressing 5637 cells. RNAseq-based gene expression data for the TCGA BLCA cohort (n= 408) was downloaded from the Broad Firehose GDAC (https://gdac.broadinstitute.org/). Gene expression subtypes, histological subtypes, and squamous pathology classifications for the TCGA BLCA cohort were obtained from the Supplementary Data of Robertson et al. (2018) [7]. The Kruskal-Wallis test was applied to compare *LAMA3*, *LAMB3*, *LAMC2* expression levels in the TCGA cohort based on groups defined by the molecular categorization of conventional histomorphometric examination, and gene expression subtype, respectively. Mann-Whitney U test was applied to compare *LAMA3*, *LAMB3*, *LAMC2* expression levels between basal-squamous and all other molecular subtypes combined (luminal, luminal infiltrated, luminal papillary, and neuronal) in the TCGA BLCA cohort.

Results

Elevated expression of laminin-332 genes (LAMA3, LAMB3, LAMC2) was observed in basal-squamous BCa

First, we investigated publicly available data from TCGA in order to determine the association of molecular subtype of human BCa with laminin-332 genes (*LAMA3*, *LAMB3*, *LAMC2*). Our analysis revealed the differential expression of *LAMA3*, *LAMB3*, and *LAMC2* across molecular subtypes of human clinical samples classified as basal-squamous, luminal, luminal infiltrated, luminal papillary, and neuronal (**Figure 1A-C**; *LAMA3*, *P*=3.78e-32; *LAMB3*, *P*=2.65e-25; and *LAMC2*, *P*=3.07e-29, Kruskal-Wallis test). Intriguingly, expression of *LAMA3*, *LAMB*3, and *LAMC2* is markedly different in the basal-squamous subtype relative to all other molecular subtypes combined (basal-squamous vs. luminal, luminal infiltrated, luminal papillary, and neuronal combined, *LAMA3*, *P*= 2.61e-34; *LAMB3*, *P*=7.96e-19; and *LAMC2*, *P*=6.1e-30, Mann-Whitney U test). These results and the expression data illustrated in **Figure 1** suggest that laminin-332 gene expression is elevated in basal subtype of human BCa specimens.

Laminin-332 genes (LAMA3, LAMB3, LAMC2) are highly expressed in basal-squamous BCa cell lines

In order to confirm the elevated expression of laminin-332 genes observed in the basal-squamous subtype of human BCa cancer tissues,

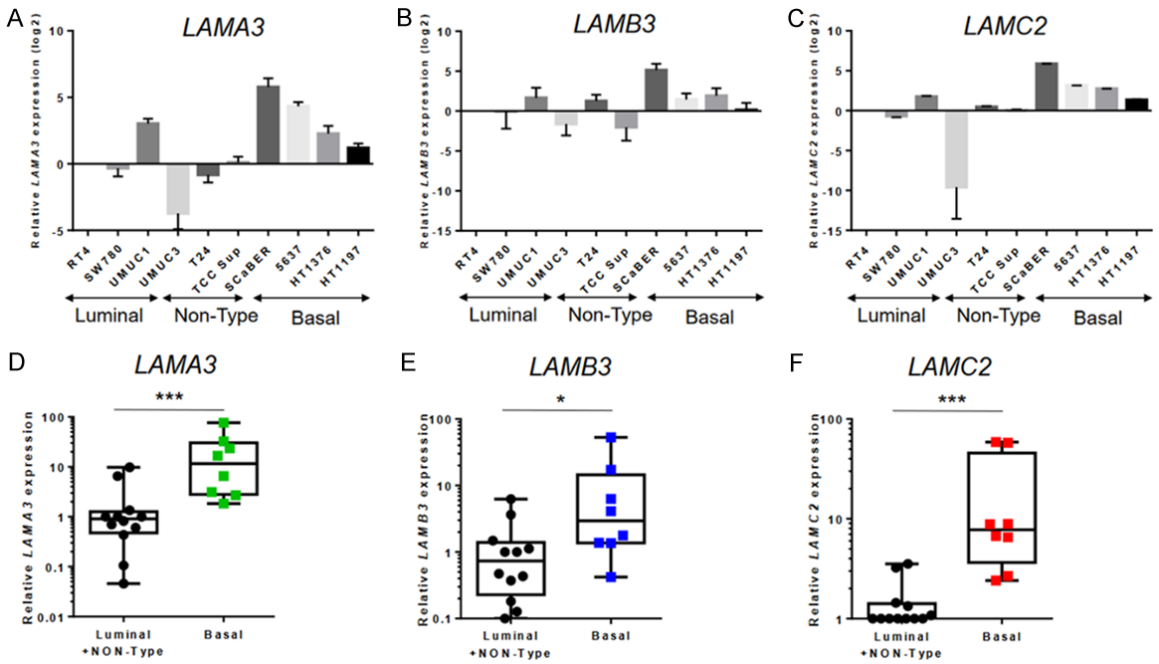

Figure 2. Laminin-332 genes are highly expressed in basal-squamous BCa cell lines. (A-C) q-RT-PCR analysis of mRNA expression of *LAMA3* (A), *LAMB3* (B), and *LAM2C* (C) in 10 human BCa cell lines. Data are expressed as the mean ± S.D. from two independent experiments. (D-F) Data of Luminal/Non-Type vs. Basal are expressed as the medians ± S.D. **P*<0.05, ***P<0.001, Mann-Whitney U test.

we next utilized q-RT-PCR to examine the expression of laminin-332 genes in a panel of human BCa cell lines that are representative of luminal and basal-squamous BCa, as well as a group of cell lines which does not fit under either gene expression subtype ("non-type") [27]. q-RT-PCR results showed that *LAMA3* (**Figure 2A**), *LAMB3* (**Figure 2B**) and *LAMC2* (**Figure 2C**) expression is significantly higher in cell line models of basal-squamous disease when compared with luminal and "non-type" cell lines. (*LAMA3*, *P*=0.0007; *LAMB3*, *P*= 0.0149; *LAMC2*, *P*=0.0002, Mann-Whitney U test) (**Figure 2D-F**). These results are in agreement with our observations that high expression of these genes is observed in human BCa basal-squamous subtype samples.

FOXA1 expression is negatively associated with Laminin-332 genes (LAMA3, LAMC2) in human BCa

The above observations led us to raise the question about what factors result in the differential expression of laminin-332 between distinct molecular subtypes of human BCa. It is known that FOXA*1* plays a significant role in the development of normal urothelium and it is expressed in UCC, while FOXA1 is lost in UCC with SqD [28-30]. Moreover, FOXA1 is also known as a pioneer factor that regulates gene regulation by changing chromatin structure [31]. In the TCGA BCa data set, distinct expression patterns of *FOXA1* and laminin-332 genes are observed in the basal-squamous and luminal subtypes (**Figure 3A**). A subsequent analysis shows *FOXA1* expression is negatively correlated with expression of *LAMA3* (Spearman's rank correlation *r*=-0.35, *P*=4.13e-13) and *LAMC2* (Spearman's rank correlation *r*=-0.32, *P*=2.14e-11) (**Figure 3B** and **3C**). These results suggest that expression of *FOXA1* and laminin-332 genes (*LAMA3* and *LAMC2*) are negatively associated in human BCa tissues.

Overexpression of FOXA1 downregulated laminin-332 genes (LAMA3, LAMB3, LAMC2) in a human BCa cell line with basal-squamous subtype

Based on the inverse correlation between expression of *FOXA1* and laminin-332 genes noted above, we hypothesized that *FOXA1* may repress expression of laminin-332 genes in the basal-squamous BCa cells. To test this hypothesis, we transiently overexpressed *FOXA1* gene

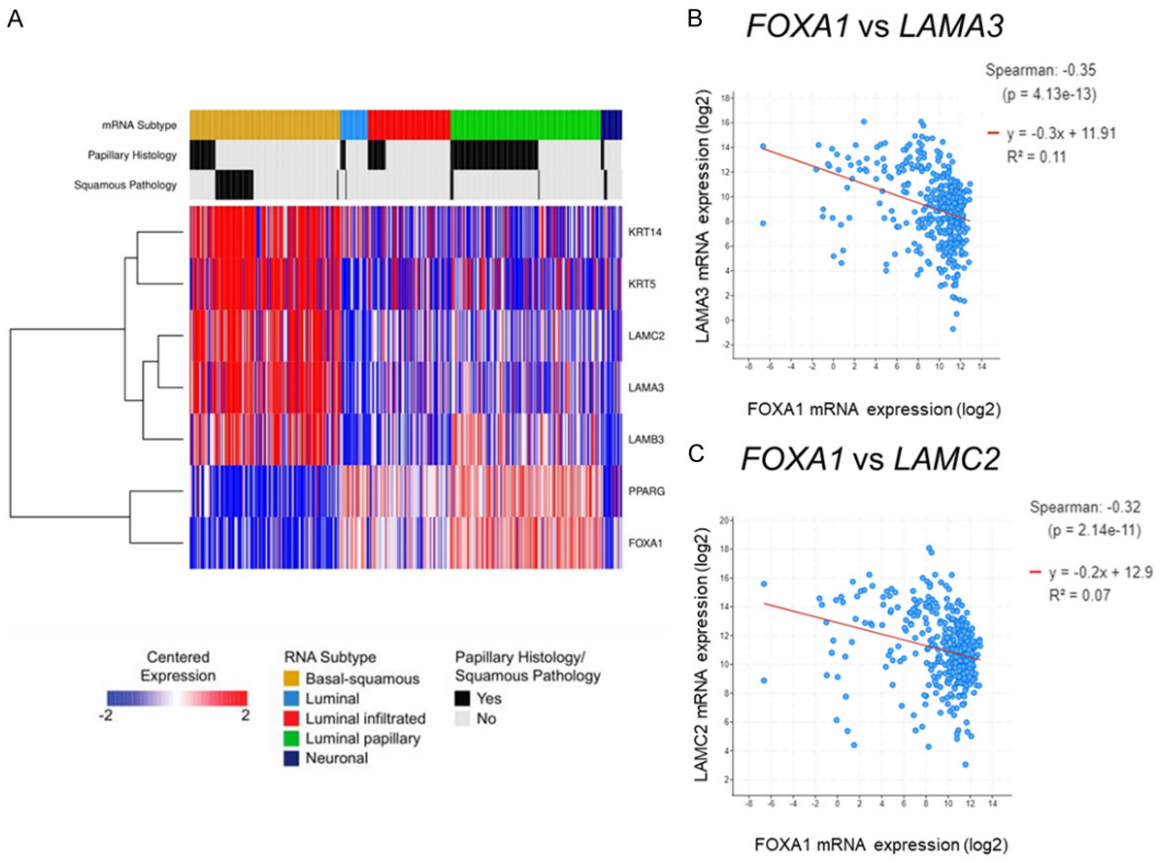

Figure 3. *FOXA1* is negatively associated with Laminin-332 genes (*LAMA3*, *LAMC2*) in human BCa. (A) *LAMA3*, *LAMB3*, *LAMC2* expression were co-clustered with luminal markers (*FOXA1*, *PPARG*) and basal markers (*KRT5*, *KRT14*) and displayed by heatmap as expression values (log2 (normalized RSEM + 1)) from the Cancer Genome Atlas (TCGA) BCa cohort (*n*=408). Annotation tracks show gene expression subtype, histological subtype, and squamous pathology classification. Spearman's rank correlation analysis between *FOXA1* and *LAMA3* (B), *FOXA1* and *LAMC2* (C) mRNA in human BCa.

in a basal human BCa cell line (5637) and utilized q-RT-PCR to investigate the difference of laminin-332 gene expression at 48 hours after transfection between empty vector (control) and *FOXA1*-overexpressing cells. q-RT-PCR result shows that overexpression of *FOXA1* was successfully performed (**Figure 4A**) and all laminin-332 genes (*LAMA3*, *LAMB3*, and *LAMC2*) are significantly decreased in *FOXA1*-overexpressed 5637 cells (**Figure 4B-D**), suggesting that *FOXA1* plays a role in suppressing the expression of laminin-332 genes in the basal subtype.

Discussion

In this study, we characterized the expression of laminin-332 genes (*LAMA3, LAMB3*, *LAMC2*) using publicly available data from TCGA as well as in 10 commonly used human BCa cell lines. Our results show that these genes are highly expressed in human BCa specimens with the basal-squamous subtype, as are the basal markers *KRT6A*, *KRT14*. These results were confirmed by data showing high expression of *LAMA3*, *LAMB3*, and *LAMC2* in a basal-squamous BCa cell line. Collectively, these results demonstrated that laminin-332 genes are expressed in a subtype-specific manner in human BCa. Limitations of this current study include the retrospective analysis of publicly available human data.

Laminin-332 is a component of the basement membrane and contributes to normal epithelial cell adhesion, migration via integrin α3β1 and cell stability via integrin α6β4. Moreover, it has been reported that laminin-332 stimulates BCa cell motility and invasion [32]. Laminin-332 is also known to be favorable target for MMPs. Among MMPs, MMP-2 is overexpressed in basal-squamous subtypes when compared to lumi-

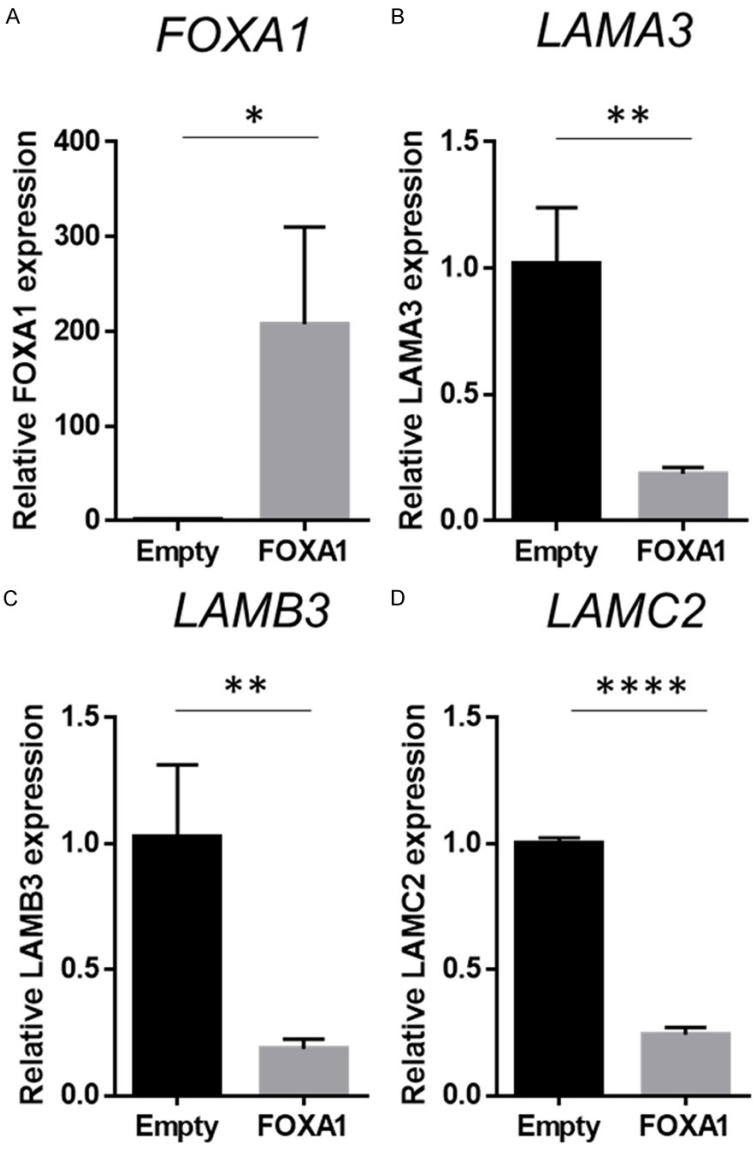

Figure 4. Overexpression of *FOXA1* downregulated laminin-332 genes (*LAMA3*, *LAMB3*, *LAMC2*) in a human BCa cell line with basal-squamous subtype. The day before transfection, 2×10^5 cells of 5637 bladder cancer cells were plated in 6 well plates. Next day, attached cells were transfected with 2.5 mg of the following plasmid constructs: pCMV6-Entry (CMV empty vector), pCMV6-FOXA1 using Lipofectamine 3000. After 48 hrs incubation, RNA was extracted for q-RT-PCR analysis. mRNA expression of *FOXA1*, *LAMA3*, *LAMB3*, and *LAMC2* were compared between empty vector - overexpressed and *FOXA1*-overexpressed 5637 cell line. Data are expressed as the mean ± S.D. from three independent experiments. **P*<0.05, **P<0.01, ****P<0.0001 (Student's *t* test).

nal-papillary subtypes of human BCa [33]. MMP-7 expression is also significantly elevated in basal-squamous subtypes when compared to luminal-papillary subtypes of human BCa [34].

Considering our results showing the expression of laminin-332 in basal-squamous subtypes of human BCa, laminin-332 may play a critical role in promoting cell adhesion and migration, as well as invasion in basal-squamous BCa. This would most likely occur through the cleavage of laminin-332 by these MMP-2 and MMP-7. If so, this may explain the reason why UCC with SqD exhibits a worse prognosis relative to pure UCC. More detailed studies are required to elucidate the mechanisms of how laminin-332 contributes to poor prognosis in human basal-squamous BCa.

Furthermore, the observation of elevated laminin-332 expression in the basal-squamous subtype led us to raise the question of what factors contribute to the differential expression of laminin-332 between distinct molecular subtypes of human BCa. Our previous study demonstrated that three luminal subtype transcription factors (TF)s *FOXA1*, *GATA3*, and *PPARG* cooperate to shift the basal-squamous subtype to a luminal phenotype in human BCa cell lines [27]. *FOXA1* is known as a pioneer factor that opens chromatin to regulate gene expression as well as a TF, and it plays a critical role in regulating gene expression that drives urothelial differentiation [30]. Additionally, loss of *FOXA1* is associated with high grade, late stage and enhanced BCa proliferation [28].

Therefore, we specifically addressed the role of *FOXA1* in laminin-332 gene expression by transiently overexpressing *FOXA1* in a human BCa cell line exhibiting a basal-squamous subtype (5637). Our results showed that overexpression of *FOXA1* in 5637 cells resulted in the downregulation of all three laminin-332 genes, suggesting that FOXA1 plays a role in repressing laminin-332 gene expression in the basal-squam-

ous subtype. However, one limitation of this study is the fact that the exact mechanism by which FOXA1 represses laminin-332 remains unknown. Intriguingly, FOXA1 repressed the expression of transforming growth factor beta3 (TGFB3) by binding to the upstream enhancer of TGFB3 gene in prostate cancer (PCa) cells [35]. Therefore, laminin-332 gene suppression by FOXA1 may be also induced by a similar mechanism. As future work, chromatin immunoprecipitation sequencing (Chip-seq) analysis will be required to identify FOXA1 binding regions to regulate laminin-332 genes (*LAMA3*, *LAMB3*, *LAMC2*) in human BCa tissue as well as in *in vitro* models.

In summary, we show that laminin-332 genes are highly expressed in basal-squamous molecular subtypes of human BCa cohort using TCGA data as well as in human BCa cell lines.

Moreover, these three genes are downregulated by overexpressing FOXA1 in 5637 cells.

Collectively, these results suggest that laminin-332 genes are associated with basal-squamous BCa and may be a biomarker of BCa patients with basal subtype.

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Disclosure of conflict of interest

None.

Abbreviations

BCa, Bladder Cancer; UCC, Urothelial Cell Carcinoma; SqD, Squamous Differentiation; NMIBCa, Non-Muscle Invasive Bladder Cancer; MIBCa, Muscle Invasive Bladder Cancer; TCGA, The Cancer Genome Atlas; Chip-seq, Chromatin immunoprecipitation sequencing.

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