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Original Research

Expression profiles of proto-oncogene TWIST1 and tumor metastasis suppressor gene LASS2 in bladder cancer

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Abstract: Transcription factor proto-oncogene TWIST1 and tumor metastasis suppressor gene LASS2 have been reported to be involved in various carcinomas but their expression profiles and prognostic significances in bladder cancer are largely unknown. We aimed to determine these genes' expression levels both at mRNA and protein level in bladder cancer. mRNA expression levels of TWIST1 and LASS2 genes were examined using real-time quantitative PCR (qPCR) in human bladder tumors and paired normal adjacent tissues obtained from 44 patients. Protein expression profiles of both genes were detected by immunohistochemical staining in formalin-fixed and paraffin-embedded tissues from the same patients. The expression profiles of TWIST1 mRNA in bladder tumors were significantly lower than the normal adjacent tissues and linked to both the stage and the grade. The expression profiles of LASS2 mRNA in bladder tumors were also significantly lower than the normal adjacent tissues reflecting the potential tumor suppressor profile of the gene, independently from stage or grade. By immunohistochemistry, TWIST1 and LASS2 positive expression rates were found as 14.3% (6/42) and 38.1% (16/42), respectively. As potential molecular markers for bladder carcinoma, both TWIST1 and LASS2 transcripts seem to play role during the tumorigenesis and development of bladder cancer. Lack of a functional link and/or weak inverse link between TWIST1 and LASS2 transcripts and immunohistochemical findings may reflect the potential associations of transcription regulation mechanisms and merit further investigations. To the best of our knowledge, this is the first report investigating the combined expression profile of TWIST1 and LASS2 in bladder cancer both at mRNA and protein level.

Key words: Bladder cancer; TWIST1; LASS2; Gene expression; Immunohistochemistry.

Introduction

Urinary bladder cancer ranks ninth in worldwide cancer incidence and approximately 145.000 patients die annually (1). Bladder cancer (BC) is the second leading cause of cancer death in patients with urinary tract malignancies. Although 70%–80% of these tumors are noninvasive (no muscle invasion; <T2), approximately 70% of them recur and among these recurrent ones, 10%–15% proceed to muscle invasion and metastasis (2). Bladder cancer like other cancer types stems from the accumulation of many genetic and epigenetic changes that lead to the activation of proto-oncogenes and/or inactivation of tumor suppressor genes.

Sphingolipids (SLs) are one of the three major classes of membrane lipids in eukaryotic cells. The building block of all SLs is ceramide which is an important intracellular signaling molecule involved in regulating differentiation, proliferation and apoptosis (3). Six members (CerS1-6; Lass 1-6) of the ceramide synthase gene family have been identified in mammals (4-5). These six known mammalian CerS build up a family of structurally related proteins that differ in terms of their substrate specificity and tissue specific expression pattern (4). *Homo sapiens* longevity assurance homolog 2 of yeast LAG1 (LASS2), also recognized as tumor metastasis suppressor gene 1 (TMSG1, GenBank accession number AF189062), is a gene isolated from a human liver cDNA library by the laboratory of Shanghai Medical College, Fudan University (Shanghai, China), and is a human homolog of the yeast (*Saccharomyces cerevisiae*) longevity assurance gene, LAG1 (6-7). The ability of LASS2 in inhibiting the colony formation of human hepatoma cells provided a clue to link this gene with the regulation of cell growth (8). CerS2 (LASS2) has a truncated form which lacks 150 residues at the N-terminus and since mRNA levels of the truncated form were extremely low in a metastatic cell line compared to a non-metastatic cell line, this protein was named as Tumor Metastasis Suppressor Gene-1 (TMSG-1) (3). Ceramide may play a certain role in tumor invasion and metastasis by affecting the length of telomere via telomerase activity and thus influencing cell life span (9). Silencing of LASS2 gene can promote growth, proliferation, invasion, and metastasis of human prostate carcinoma cell line in vitro and in vivo through the increase of the V-ATPase activity and extracellular hydrogen ion concentration, and this results with the activation of secreted matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), and degradation of extracellular matrix (ECM) (10). In addition, LASS2 promotes cell apoptosis by significantly inhibiting bcl-2

expression (9).

TWIST1 belongs to the basic-helix-loop-helix (bHLH) family of transcription factors and is implicated in lineage-specific cellular differentiation and survival. TWIST1 governs early mesodermal patterning and osteogenesis in vertebrates (11). Twist was originally identified in Drosophila as a protein involved in establishing dorso–ventral polarity and shares extensive homology with another bHLH transcription factor Dermo-1 (Twist2) (12). Both Twist1 and Twist2 can promote anchorage-independent growth which is a common property of oncogene products (13). Germline haploinsufficiency of the human TWIST1 gene is responsible for the Saethre–Chotzen syndrome (14). TWIST1 induces epithelial-mesenchymal transition (EMT) which is the process whereby cells lose cell-cell and cell-basal membrane contacts during gastrulation movements and neural crest formation in early embryonic development. EMT is a key step during carcinoma progression and metastasis development and is characterized by the the loss of epithelial markers expression (E-cadherin, catenin) and neoexpression of mesenchymal markers (N-cadherin, vimentin, SMS-actin) (15). TWIST is the up-stream of E-cadherin and can enhance beta-catenin expression through reduction of E-cadherin and thus TWIST, E-cadherin, and beta-catenin may be used as pathologic markers for a stage progression prediction (16). Cancer cells gain stem cell-like characteristics via EMT (17). TWIST is a potential oncogene promoting the proliferation and inhibiting the apoptosis. TWIST also promotes the synthesis of vascular endothelial growth factor (VEGF) involved in tumor progression and metastasis (15). Expression of TWIST1 protein counteracts the proapoptotic effects of N-MYC by repression of p19ARF and thereby hampers Tp53 function. Overexpression of TWIST1 also induces angiogenesis and chromosomal instability (11). Besides, the fact that down-regulation of TWIST in DU145 and PC3 cells with nonfunctional p53 pathway also promotes the apoptosis may imply the antiapoptotic role of TWIST through both p53-dependent and p53-independent pathways (18). Twist may be a direct positive or negative transcriptional regulator for many genes (19).

The expression of anti-apoptotic protein Bcl-2 which assists Twist-1 to transport into the nucleus (20) is inhibited by LASS2 and apoptosis is promoted (9). These two distinct important scientific findings triggered us to discover the possibility of an indirect correlation between LASS2 and TWIST1 and thus we aimed to provide a different perspective by evaluating the expression levels of these two genes.

Materials and Methods

Patient population

The present study was based on a consecutive series of patients with bladder cancer who underwent transurethral resection or cystectomy at the Department of Urology, Samsun, Turkey between 2014 and 2016. A total of 44 fresh bladder carcinoma specimens and paired normal adjacent tissues (NAT) were taken into eppendorf tubes including 0.9% filter-sterilized NaCl to preserve tissue integrity. All specimens were obtained freshly immediately after surgery and stored at -80°C until processing. Histological grading and disease staging were based on the criteria of the 2004 WHO/ISUP and 2009 TNM system. Written informed consent was obtained from all the subjects and the study was approved by the Institutional Review Board of Ondokuz Mayis University, Turkey (Decision no: OMU KAEK 2013/390).

RNA extraction and RT-PCR

Total RNA was prepared from frozen tissues using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's protocol. RNA concentrations were quantified with UV-VIS spectrophotometer (Shimadzu, UV-2600) in 10 mM Tris-HCl pH 7.5 and the integrity of the specimens was evaluated with the gel image of 28S/18S rRNA bands. For cDNA synthesis, RNA samples were treated with RNase-free Dnase I to remove all potential genomic DNA molecules (1 U DNase or sometimes 2 U DNase when required). First-strand cDNA was synthesized from 100 ng total RNA with oligo dT18 primer using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) in a total volume of 20 µl.

Real-time PCR

At the end of the optimization tries with both the housekeeping and target genes, 10 ng cDNA use was found sufficient for a successful real-time PCR experiment. cDNAs encoding target genes (TWIST1 and LASS2) and endogenous control (GAPDH) were amplified with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Lithuania) using either Light Cycler 480 analyzer (Roche, Switzerland) or BIO-RAD CFX96 system (Bio-Rad Company, USA), with the primer sequences previously reported: LASS2 (21), TWIST1 and GAPDH (22). PCR conditions: 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. The initial denaturation step was performed at 95°C for 10 min and at the end of the PCR cycles, melting curve analyses were performed to confirm the generation of the specific PCR products (95°C for 10 sec and 60°C for 1 min). The levels of TWIST1 and LASS2 expressions were analyzed by $2^{-\Delta\Delta Ct}$ method (23). At the end of the reactions, PCR products were electrophoresed on 2.5% agarose gels to further identify the specificity of the amplicons (LASS2:134 bp; TWIST1: 201 bp; GAPDH: 226 bp). To eliminate possible genomic DNA and reagent contaminations, RT- (reverse transcriptase minus) and NTC (no template control) were included in all the reactions performed.

Immunohistochemistry

Paraffin sections (4 µm thick) were used for immunohistochemical study with anti Lass2 (1:200, ab85567 Abcam, Cambridge UK) rabbit polyclonal antibody using Ventana Benchmark® XT autostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). Human kidney tissues (ductus epithelial cells) were used as positive controls. Negative control was obtained by replacement of primary antibody with PBS. Semiquantitative scores were used to analyze the immunostaining of each case and only nuclear staining was evaluated. THA extent of immunostaining was scored according to the percentage of positive cells as 1 (1-25%), 2 (26-50%), 3 (51-75%),

or 4 (>75%). Staining intensity was scored on a scale of 1-3 as follows: 1, weak yellow staining; 2, dark yellow staining; 3, dark brown staining. The expressions of Lass2 in each slide were scored as the sum of intensity and extent of positive-staining cells. The slide with a final staining score of ≥3 was defined as positive expression.

Twist1 immunohistochemical analysis was carried out using streptavidin biotin peroxidase method with monoclonal mouse anti twist (anti twist 2c 1a, ab50887 Abcam) as a primer antibody. Paraffin sections (4 µm thick) were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution for 10 min incubation. The sections were submitted to citrate buffer solution at 90°C for 35 min in a microwave oven and cooled for 20 min. After the application of protein blocking agent, the slides were then incubated for 3 hours at room temperature with Twist1 primer antibody. The sections were then incubated with biotinylated anti-immunoglobulin and streptavidin peroxidase conjugate for 10 min. Freshly made 3.3'-diaminobenzidine (DAB) (Abcam) was used as a monitoring agent. The sections were then counterstained with Mayer's hematoxylin for 60 second. Since no staining was obtained when cervix carcinoma and colon adenocarcinoma were used as positive controls, the evaluation of the positive staining was carried out with mesenchymal cells in the stroma of urothelial carcinoma and vascular endothelial cells. Expression of Twist1 was assessed semiquantitatively based on only nuclear staining. The extent of immunostaining and staining intensity were as in the case of Lass2. Negative Twist1 expression was defined as a final score of <3 and ≥3 as positive Twist1 expression.

Statistical analysis

All statistical analyses were performed using a SPSS 22.0 software and p values <0.05 were considered statistically significant. mRNA expressions were evaluated with $2^{-\Delta\Delta Ct}$ method (23). Normal distribution was first tested using Shapiro-Wilk test. For real-time PCR data, Mann-Whitney U test and sign test were used. The Pearson's X^2 test and Fisher's Exact test were used for IHC data. The correlations between the mRNA and protein levels of the investigated genes were evaluated with Spearman's Rho test.

Results

Cohort

The characteristics of the patients are summarized in Table I.

Expression levels of TWIST1 and LASS2 genes at mRNA level

To evaluate the transcript levels of TWIST1 and LASS2, cut-off reference value ≥2 was considered as upregulation and <2 as downregulation. The expression profiles of TWIST1 mRNA in bladder tumors were significantly lower than the normal adjacent tissues (Median: 0.11; P<0.05) and this was linked to both the stage (P=0.0037) and the grade (P=0.001) (Fig. 1A and Fig. 1B). The expression profiles of LASS2 mRNA in blad-

Table I. Clinicopathological features of the patients with bladder cancer.

Variables	n	%
Age,yr	28-87(median 72.5)	
Sex		
Male	40	90.9
Female	4	9.1
Grade		
Low	16	36.4
High	28	63.6
Tumor stage		
pTa- pT1	38	86.4
pT2-pT4	6	13.6
Metastasis		
Absent	39	88.6
Present	5	11.4

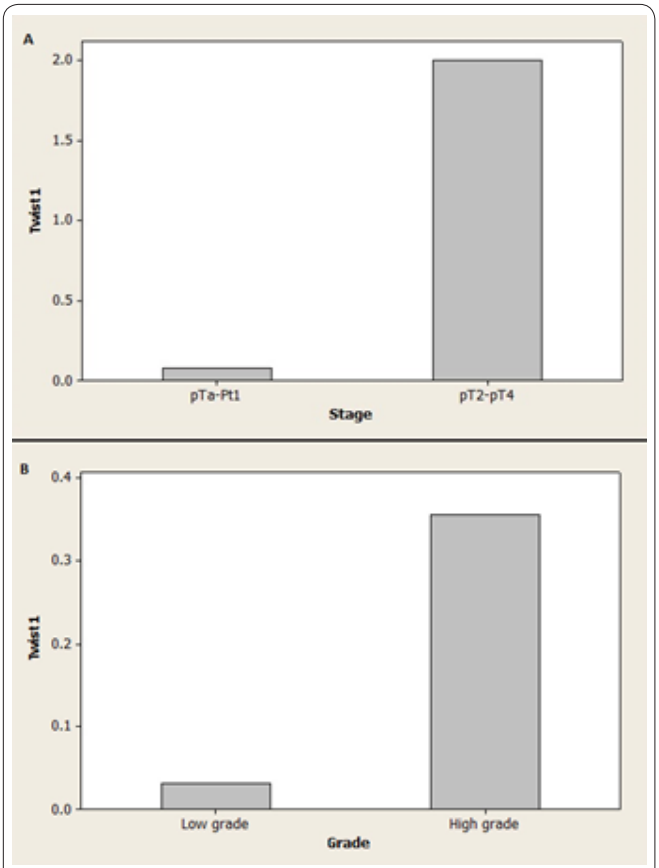

Figure 1. (**A**) TWIST1 transcript levels correlate with tumor stage and (**B**) tumor grade.

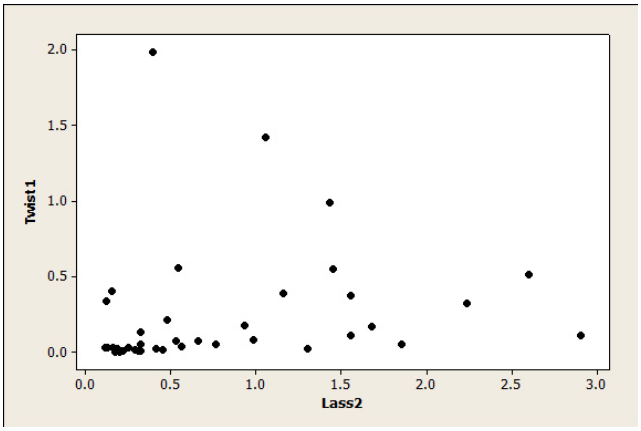

Figure 2. TWIST1 and LASS2 transcripts correlate with each other. Spearman-Rho coefficient of correlation: 57.5%, p <0.05.

der tumors were also significantly lower than the normal adjacent tissues (Median: 0.54; P<0.05) reflecting the potential tumor suppressor profile of the gene. However, this mRNA profile was not linked to stage or grade (P>0.05). TWIST1 transcript levels were positively correlated with LASS2 transcript levels (coefficient of correlation=0.57, P<0.05) (Fig. 2).

TWIST1 and LASS2 immunohistochemical expressions

By immunohistochemistry, LASS2 and TWIST1 positive expression rates were found as 38.1% (16/42) and 14.3% (6/42) respectively (Fig. 3 and Fig. 4A). Neither of them correlated with the investigated clinicopathological parameters (P>0.05). TWIST1 protein was mainly expressed in the stromal cells of specimens (Fig. 4B.)

mRNA/protein correlation of TWIST1 and LASS2 genes

No correlation was found between TWIST1 mRNA expression levels and immunohistochemical findings (Spearman's Rho, P>0.05). A weak and reverse correlation was found between LASS2 expression levels and immunohistochemical findings in terms of the total score and the extent of staining (Spearman's Rho, P<0.05).

Discussion

Carcinogenesis is a multistep process characterized by the gradual accumulation of mutations in cancer cells which also modify their stromal surroundings to create a supportive environment to permit tumor progression. The tumor stroma which is the connective-tissue framework of the tumor includes fibroblasts, immune and inflammatory cells, fat cells and blood-vessels. Cancer associated fibroblasts are important promoters of tumor growth and progression. Jouppila-Mättö et al. evaluated TWIST expression in pharyngeal squamous cell carcinoma and reported that the stromal expression of TWIST was clearly more common than the expression in the epithelial tumor cell compartment. Though there are no published data about the half-life of TWIST or its stability in cell nucleus, the case probable for trancription factors may also be valid for TWIST and explain its very short time expression in tumor cells (24). In accordance with these observations, TWIST expression was common in tumor stroma, mesenchymal cells, young fibroblasts, and vascular endothelial cells in our study. The restricted stromal expression of TWIST1 was also explained in colorectal cancers (25).

TWIST1 which is extremely expressed in various tumor groups was also found to be correlated with cell proliferation, invasion, tumor grade, and the presence of metastasis in bladder cancer (26). Since TWIST1 is a direct positive or negative transcriptional regulator for many target genes, TWIST1 and the related molecular mechanisms in cancer researches must be investigated in detail.

In tissue samples from patients with pancreatic ductal adenocarcinoma; the immunohistochemical expressions of Snail, Slug, and Twist were reported as 78%, 50% and no or weak expression, respectively. In contrast to the studies showing overexpression in a variety of different tumors, this is an interesting fact since Twist showed no expression in 97% of human pancreatic cancer tissues and in all five human pancreatic cancer cell lines. Independently from hypermethylation, TWIST1 mRNA level was measurable in pancreatic cancer cell lines after 48 h of hypoxia (27). Our results share similarity with this data since TWIST1 expression at protein level was also relatively low (14.3%).

One important issue related with Twist1 expression

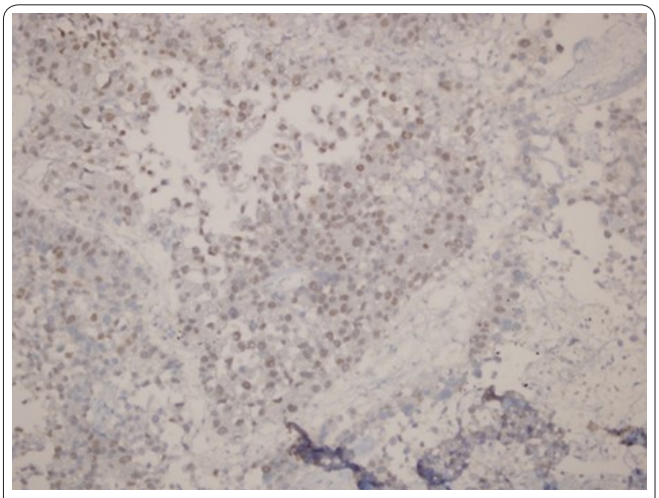

Figure 3. Positive staining of LASS2 in high grade invasive urothelial carcinoma, Score 6 (immunoperoxidase x400).

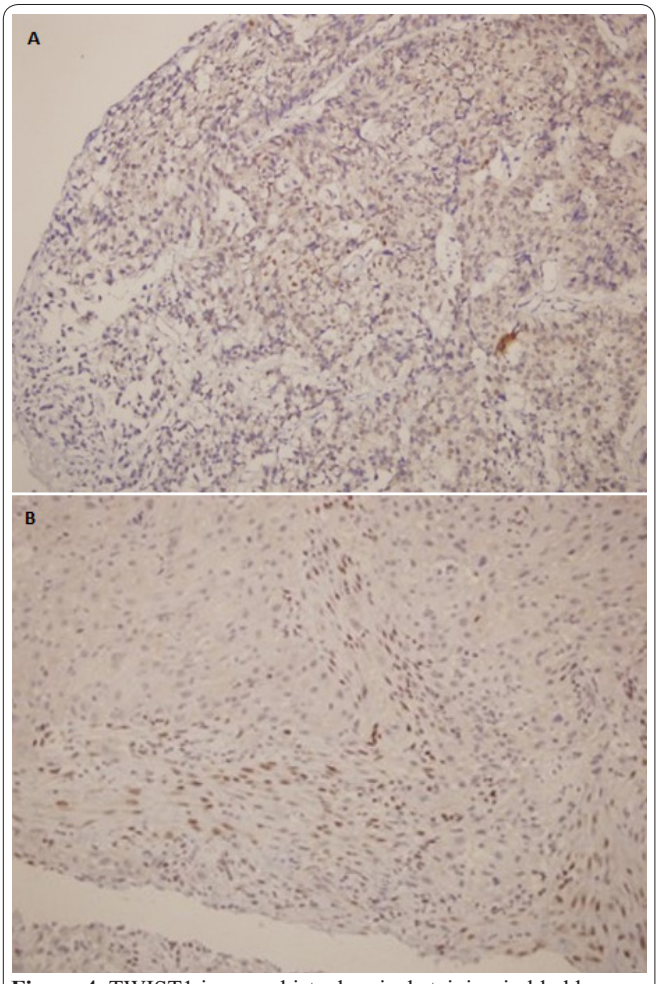

Figure 4. TWIST1 immunohistochemical staining in bladder cancer tissues. (**A**) Positive staining of TWIST1 in high grade invasive urothelial carcinoma, Score 3 (immunoperoxidase x200), (**B**) Negative immunostaining for TWIST1 in high grade invasive urothelial carcinoma (Diffuse and strong TWIST1 staining in stromal cells (immunoperoxidase x200).

can be the evaluation of the efficiency of the antibody used since the cellular location of Twist1 protein expression was reported in different studies; cytoplasmic in some studies, and nuclear in the others. Sung et al. investigated Twist1 expression patterns in gastric stromal fibroblasts and cancer cells using a monoclonal Twist1 antibody after validating the effectiveness of four commercial Twist1-specific antibodies (H81: Rabbit polyclonal, Santa Cruz Biotechnology; Ab50887: Mouse monoclonal, Abcam; Ab50581: Rabbit polyclonal, Abcam; 4119S: Rabbit polyclonal, Cell Signaling Technology). Of the four Twist1 antibodies used for immunostaining in FFPE tissues, only the ab50887 antibody resulted with the selective nuclear staining in SNU484 gastric cancer cell line. The other antibodies (ab50581, 4119S, H81) stained not only the cell block section from SNU484 but also sections from other cell lines with mainly cytoplasmic staining, with or without additional nuclear staining. Moreover, Western blot analysis revealed that all four antibodies recognized Twist1, but nonspecific bands appeared when ab50581 and H81 antibodies were used (28). In a more recent study in gastric cancer, Twist and matrix metalloproteinase-9 (MMP-9) expressions were evaluated with immunohistochemistry and the researchers reported that Twist expression was significantly increased in gastric cancer cases with lymph node metastasis. Differently from Sung et al., the researchers used ab50581 antibody and reported the expression mainly in the cell cytoplasm as a diffuse staining (29). Thus, in the light of the literature data, the differences reported in Twist expression in carcinoma cases may also be somewhat related with the different antibodies used and depending upon different antibody selections, data with high percentages that do not reflect selective nuclear staining may affect the expression standardization. As reported by Sung et al., the fact of intensive Twist expression in stromal cells rather than the gastric cancer cells and that Twist1 is a molecular marker of altered fibroblasts in gastric cancer (28) merits further investigations since stromal staining was also shown in our urothelial carcinoma study.

Immunohistochemical co-expression of TWIST1 and ZEB2 in oral squamous cell carcinoma was reported to be significantly associated with poorer Overall survival, particularly in patients without detectable lymph node metastasis (30). Claudins are essential tight junction proteins forming the backbone of the blood-brain-barrier and the expressions of claudins have been shown to be down- or up-regulated in various human tumors. The immunohistochemical expressions of claudins 2-5, 7, and 10 were investigated in ependymomas and a relationship was found only between TWIST and CLDN3; grade II ependymomas negative for TWIST were more often negative for CLDN3. Moreover, a strong nuclear staining was detected for TWIST in the majority of tumor cells (31). At the immunohistochemical level, the overexpressions of Slug and Twist with down-regulation of E-cadherin were reported as characteristic findings in hemangiopericytomas and solitary fibrous tumors (32). High nuclear expression of Twist1 was reported to be an independent poor prognostic factor in multiple myeloma patients (33). Wushou et al. performed a systematic meta-analysis to determine the role of Twist-1 in the prognosis of carcinoma patients and this analysis included 17 studies: four studies evaluated lung cancer, three evaluated head and neck cancer, two evaluated breast cancer, two evaluated esophageal cancer, two evaluated liver cancer and one each evaluated osteosarcoma, bladder, cervical and ovarian cancer. Expression of Twist-1 was associated with worse 3-year survival in carcinoma (34). As in other studies, the limitation of this study is the exclusion of the measures of TWIST1 mRNA levels by focusing on immunohistochemical level.

In our study, TWIST1 expression was evaluated both at mRNA and immunohistochemical level and mRNA levels in bladder tumor tissues were significantly found lower than the adjacent normal cells. Moreover, the relationship between TWIST1 mRNA decrease and stage/grade in tumor cells may refer to the potential role of TWIST1 as an invasion marker and its potential use as a molecular marker in urothelial carcinoma. However, it may be much better if future studies aim to evaluate both the mRNA and methylation profiles of TWIST1 gene since the finding of TWIST1 downregulation offers an unexpected role figure for a proto-oncogene.

The studies evaluating TWIST1 expression in bladder cancer are quite few. Tang et al., investigated Twist1 expression both at mRNA and protein level in bladder urothelial carcinoma samples. The expression levels of TWIST1 mRNA levels in cancerous mucosas were significantly lower than the non-cancerous mucosas as in our study. Twist protein expression in cancerous tissues was significantly higher than the non-cancerous mucosas and mainly distrubuted in the nucleus and expressed in the mesenchymal cells of several specimens. Twist1 protein displayed a distinct expression tendency from mRNA and Twist1 protein was not associated with TNM stage and grade and both mRNA and protein expressions were not correlated with age, gender, smoking history (22). The lack of association of Twist1 protein with mRNA levels may be explained with post-transcriptional regulations. Indeed, our previous study in which we investigated TWIST methylation profiles in both the tumor tissues and urines of bladder cancer patients, focused on this debate and possibility of gene regulation at post-transcriptional level since the hypermethylated profile of TWIST1 was somehow conflictive for a gene that functions as an oncogene and thus is expected to be highly expressed (35). Verifying our hypothesis in our previous study, TWIST1 mRNA levels in tumor tissues was low constituting a contradiction for an oncogene role. Besides, TWIST1 expression was also low at immunohistochemical level.

In a recent bladder cancer study, Krüppel-like factor 4 (KLF4) expression was positively correlated with TWIST1 and vimentin, inversely correlated with E-cadherin expression and metastasis-free survival was poorest in KLF4/TWIST1 coexpression group. Thus, KLF4/TWIST1 coexpression can be offered as a potential biomarker to improve the risk stratification in urothelial carcinoma of bladder (36).

Though the studies evaluating LASS2 expression in cancer are much more limited when compared with TWIST, LASS2 has been reported to correlate with the degree of invasion and recurrence in carcinomas of prostate, liver, breast, cervix, ovarian, and pancreas (6). Fan et al., reported that LASS2 expression was signifi-

cantly lower in drug-resistant Michigan Cancer Foundation-7/adriamycin (MCF-7/ADR) human breast cancer cells than the drug-sensitive MCF-7 cells and low LASS2 expression was associated with poor prognosis in patients with breast cancer. The importance of this study is noteworthy in terms of the prediction of the chemoresistance and the involvement of low LASS2 expression in chemotherapeutic outcomes (37). Ke et al., reported the downregulation of LASS2 at both qPCR and immunohistochemical level in high-grade meningiomas in comparison to the grade I or normal brain and found associations between low LASS2 expression and clinico-pathological parameters such as tumor size, brain invasion and tumor recurrence, and thus indicated LASS2 as a new promising marker in meningiomas compared with routinely applied clinical markers (38). Xu et al., demonstrated LASS2 as a new tumor suppressor gene by reporting that LASS2 exhibited its effect via V-ATPase activity and silencing of LASS2 with small hairpin RNA (shRNA) in low metastatic human prostate carcinoma cell line PC-3M-2B4 enhanced cell proliferation, cell survival, and cell invasion via increasing V-ATPase activity, extracellular hydrogen ion concentration and in turn the activation of secreted MMP-2 and MMP-9 (10). Su et al., reported that LASS2 acts as a metastasis suppressor gene by inducing apoptosis via a caspase-dependent mitochondrial pathway and thus it can be a novel therapeutic target in anticancer gene therapy (39). The interaction of LASS2 with V-ATPase activity has also been shown in hepatocellular carcinoma. The combination of the asialoglycoprotein receptor (ASGR) which is mainly expressed in hepatocytes and LASS2 was emphasized as a prognostic predictor in hepatocellular carcinoma patients (40). In an other hepatocellular carcinoma study, the correlation between LASS2 and transforming growth factor-β1 (TGF-β1) at immunohistochemical level was analyzed and this two-gene combination was represented as a novel prognostic biomarker for hepatocellular carcinoma patients since the low expressions of LASS2 and TGF-β1 contributed to the aggressiveness and poor prognosis (41). As it is evident from the literature data, LASS2 is a quite promising gene in terms of its association with cancer. Thus, searching the molecular mechanisms in more detail via the investigation of LASS2 not alone but also with other related genes may allow the representation of more efficient molecular marker candidates in anticancer approaches.

The first study investigated LASS2 expression at mRNA and protein level in bladder cancer tissues (6) was continued by the same research team in human bladder cancer cell lines BIU-87, T24, EJ ve EJ-M3. Though LASS1 and LASS3 mRNA expression levels were not correlated with clinical parameters, LASS2 mRNA expression level was significantly correlated with diverse proliferation, metastasis and invasion. Moreover, it was observed at the protein level that the more aggressive the cancer cell line, the lower the LASS2 protein expression level. This is the first study in bladder cancer supporting the role of LASS2 as a tumor suppressor gene and implying its use as a clinical prognostic marker (7). To the best of our knowledge, there is no other study since then evaluating LASS2 expression in bladder cancer and thus our study is important in terms of enriching the literature data. In recent bladder cancer studies, the effects of microRNAs in LASS2 regulation have also gained importance; miR-9 downregulated LASS2 expression by directly targeting its 3'-UTR (42), miR-93 played an important role in the chemo-sensitivity of bladder cancer cells and could be involved in regulating LASS2 (43). Considering the fact that approximately 30% of all genes are regulated by miRNAs, it may be a reasonable approach for future studies to evaluate LASS2 expression with the potential miRNAs that can regulate oncogenes or tumor suppressor genes. Suitable non-invasive sources for diagnostic purposes in bladder cancer have also focused on cell-secreted extracellular vesicles since they are a source of low invasive disease markers and can be found in many body fluids including urine. Using microarray technology, mRNAs associated with urinary vesicles for diagnosis of bladder cancer were assembled and the fact that one of the genes whose presence in urine differed between cancer and non cancer patients was LASS2 (44) is also important in terms of the future potential of our study.

The fact that LASS2 mRNA levels in tumor tissues show a significant decrease compared to the normal adjacent tissues in our study supports the role of LASS2 as a tumor suppressor gene in carcinoma. However, lack of association between LASS2 mRNA and protein levels and the investigated clinical parameters (stage, grade, recurrence, metastasis) reflects the need of future studies related with this gene in larger cohorts. In our study, TWIST1 mRNA levels showed a decrease contrasting with a proto-oncogene role and thus indicating the potential transcription regulation mechanisms. Since the downregulation of TWIST mRNA is also significantly associated with the stage and the grade, this may refer to the applicability of this gene as a potential molecular marker in bladder cancer. Immunohistochemical detection of TWIST reflects a low positivity mainly focusing on tumor stroma and thus TWIST might be beneficial as stromal marker. Nevertheless, we encourage more studies on the detection of TWIST1 immunohistochemical analysis in bladder tumors to enrich our knowledge in the light of the contradictory results in the literature. To the best of our knowledge, this is the first study evaluating the combined roles of TWIST1 and LASS2 both at mRNA and immunohistochemical level in bladder cancer. Future studies targeting some other genes that may interact with these two genes will be contributory in terms of extending the signalling pathways and developing combined molecular markers in bladder cancer. We also encourage future studies evaluating the expressions of these two genes with miRNAs and/or methylation profiles which have the potential of emphasizing transcriptional regulation mechanisms to have a deeper insight.

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Authors' contributions

ZY designed the project, performed RNA experiments, and was a major contributor in writing the manuscript. OA developed the protocols and performed the immunohistochemical analysis of the genes investigated. HK analyzed and interpreted data. RB collected the clinical samples and supervised all the operations. OA, HK, and RB reviewed the manuscript. All authors read and approved the final version of manuscript.

Ethics approval

Institutional review board of Ondokuz Mayis University Medical Center approved this study (Decision number: 2013/390).

Disclosure statement

The authors declare that they have no competing interests.

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