

Activation of PPAR γ in bladder cancer via introduction of the long arm of human chromosome 9

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Abstract. Bladder cancer is divided into two molecular subtypes, luminal and basal, which form papillary and nodular tumors, respectively, and are identifiable by gene expression profiling. Although loss of heterozygosity (LOH) of the long arm of human chromosome 9 (9q) has been observed in the early development of both types of bladder cancer, the functional significance of LOH remains to be clarified. The present study introduced human chromosome 9q into basal bladder cancer cell line, SCaBER, using microcell-mediated chromosome transfer to investigate the effect of LOH of 9q on molecular bladder cancer subtypes. These cells demonstrated decreased proliferation and migration capacity compared with parental and control cells. Conversely, transfer of human chromosome 4 did not change the cell phenotype. Expression level of peroxisome proliferator-activated receptor (PPAR) γ , a marker of luminal type, increased 3.0-4.4 fold in SCaBER cells altered with 9q compared with parental SCaBER cells. Furthermore, the expression levels of tumor suppressor PTEN, which regulates PPAR γ , also increased in 9q-altered cells. These results suggested that human chromosome 9q may carry regulatory genes for PPAR γ that are involved in the progression of neoplastic transformation of bladder cancer.

Introduction

Bladder cancer (BCa) is one of the most prevalent types of malignant tumor of the urinary system (1). BCas are classified as superficial papillary or non-papillary carcinoma according to their constitution (2). Papillary carcinoma is usually non-invasive but may develop into non-papillary invasive carcinoma with a high histological grade, with repeated recurrence after treatment. Conversely, non-papillary invasive carcinoma usually has a high histological grade and poor clinical course. Various chromosomal aberrations are involved in the development and progression of these types of cancer. Generally, there are two types of chromosomal aberration: Primary, which is associated with tumor oncogenesis, and secondary, which is associated with tumor progression (3). Using comparative genomic hybridization and loss of heterozygosity (LOH) analysis, aberrations in urothelial carcinoma have been observed on human chromosomes 1p, 9 and 11p, which contain numerous onco- and tumor suppressor genes, such as runt-related transcription factor 3, cyclin-dependent kinase inhibitor 2A and cyclin D1 (4). Advanced BCa is accompanied by aberrations in human chromosomes 2q, 5q, and 8p (5-7). Furthermore, insights in the molecular pathology of BCa suggest two pathways for the development of BCa: Luminal and basal subtype (8). The q arm of chromosome 9 is deleted in both molecular subtypes, suggesting that it may be a primary event in the pathogenesis of BCa (9,10). Inactivating mutations of tuberous sclerosis 1, which is a key tumor suppressor gene on 9q, is found in 11-16% of BCa cases, regardless of stage (11,12). Mutations in Notch homolog 1, located on 9q, have also been identified in 18% of BCa (13). The aberration rates of these tumor suppressor genes are inconsistent with the degree of LOH in BCa, suggesting the presence of novel tumor suppressor genes that contribute to cancer development.

To understand the functional significance of the LOH at the 9q region in BCa development, human chromosome 9q was transferred to a BCa cell line using microcell-mediated

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chromosome transfer (MMCT) and examined for effects on phenotype.

Materials and methods

Cell lines and culture. SCaBER and RT4 cells were purchased from the American Type Culture Collection. T24 and 5637 cells were purchased from the RIKEN Cell Bank. Cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva), 100 U/ml penicillin and 100 μ g/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation). Mouse A9 cells containing human chromosome 9q or 4, respectively, tagged with neomycin resistance gene (neo) were maintained in DMEM supplemented with 10% FBS and 800 μ g/ml G418 antibiotic (Calbiochem; Merck KGaA) and used as chromosome donors. All cell lines were maintained at 37°C in a humidified incubator with 5% CO₂.

MMCT. Chromosome transfer via chromosome engineering was performed as previously described (14). A9(neo9q) or A9(neo4) cells were treated with 0.05 μ g/ml colcemid at 37°C for 48 h to induce formation of micronuclei, which were then purified by cytochalasin B (10 μ g/ml) digestion and centrifugation at 11,900 \times g for 60 min at 34°C. The isolated microcells were then resuspended in serum-free DMEM and filtered sequentially through 8, 5 and 3 μ m polycarbonate filters (Whatman plc; Cytiva). The purified microcells were collected by centrifugation at 400 \times g for 15 min at room temperature (RT) and resuspended in serum-free DMEM containing 50 μ g/ml phytohemagglutinin-P (FUJIFILM Wako Pure Chemical Corporation). The microcells were attached to the cell monolayer at 37°C for 15 min, fused with recipient cells in 47% polyethylene glycol solution for 1 min at RT, followed by washing with serum-free DMEM. Cells then maintained in non-selective medium (DMEM) for 24 h at 37°C, trypsinized and divided into six 100 mm dishes containing selection medium (containing 800 μ g/ml G418).

Acquisition of microcellular hybrid clones. The day after microcell fusion, culture medium was changed to selection medium (containing 800 μ g/ml G418). One week after fusion, no viable cells were observed. Two weeks after fusion, rapidly proliferating clones were isolated and maintained by serial passaging, as previously described (14).

Genomic PCR analysis. The presence of the 9p24.2-9q34.3 region on human chromosome 9q contained in A9(neo9) was verified by PCR using 21 specific sequence-tagged site (STS) markers (D9S54, 9p24.2; D9S268, 9p23; D9S285, 9p22.3; D9S165, 9p21.1; D9S200, 9p13.1; SHGC-103793, 9p12; UT801, 9p11.2; SHGC-141463, 9q12; SHGC-146514, 9q13; D9S15, 9q21.12; D9S1122, 9q21.2; D9S153, 9q21.31; D9S777, 9q22.1; D9S318, 9q22.2; D9S287, 9q22.32; D9S277, 9q31.1; D9S177, 9q33.1; D9S290, 9q34.11; D9S66, 9q34.2; and D9S158, 9q34.3). Primer sequences were obtained from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>). PCR was performed with 35 cycles of 30 sec at 94°C, 30 sec at 58-62°C and 30 sec at 72°C.

RNA isolation and reverse transcription-quantitative PCR. Total RNA was extracted using RNeasy Mini kit (Qiagen GmbH) from each cell line and treated with DNase I (FUJIFILM Wako Pure Chemical Corporation). First-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with random primers (Invitrogen; Thermo Fisher Scientific, Inc.), 5x First Strand Buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and dNTPs (Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol was 23°C for 12 min for primer annealing, followed by 42°C for 50 min for reverse transcription and then 95°C for 5 min for enzyme inactivation.

The mRNA expression of PPAR γ (encoding PPAR γ), forkhead box A1 (FOXA1) and GATA3 was analyzed using specific primers as follows: PPAR γ forward, 5'-GACAGGAAAGACAACAGACAAATC-3' and reverse, 5'-GGGGTGATGTGTTTGAACCTTG-3'; FOXA1 forward, 5'-AGGGCTGGATGGTTGTATTG-3' and reverse, 5'-ACCGGGACGAGGAGTAG-3' and GATA3 forward, 5'-GCTTCGGATGCAAGTCCA-3' and reverse, 5'-GCCCCACAGTTCACACACT-3'. cDNA was amplified using an Applied Biosystems StepOne thermal cycler system and SYBR Green PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). mRNA levels were normalized against GAPDH mRNA (PCR primers: Forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAAATCC-3') using the 2^{- $\Delta\Delta$ C_q} method (15). Thermocycling conditions were 10 min at 95°C for denaturation, followed by 15 sec at 95°C and 60 sec at 60°C for denaturation and annealing/extension for 40 cycles. The experiments were performed in triplicate.

Heatmap of gene expression. The expression levels of PPAR γ , FOXA1, and GATA3 of RT4, T24, SCaBER, SCaBER#4 and SCaBER9q were visualized using a freely available web server, Heatmapper (<http://www.heatmapper.ca>).

Fluorescence in situ hybridization (FISH). To identify successful transference of chromosome 9q in metaphase spreads, chromosomal FISH was performed using plasmid pSV2neo as a probe. The transferred chromosome 4 was identified using RP11-84C13 BAC DNA as a probe.

The preparation of chromosome slides, probe labeling, hybridization, washing and detection of signals were performed as previously described (16).

Cell proliferation assay. Cell proliferation assay was performed to evaluate the proliferation capability of cells. SCaBER, SCaBER#4, and SCaBER#9q cells were seeded at 1.0 \times 10⁵ cells in a 6 cm dish. All cells were cultured in DMEM supplemented with 10% FBS. Measurements were made using a hemocytometer on days 1, 2, 3, 4, and 5 after seeding. Cell counting was performed three times.

Migration assay. Wound healing assay was performed to evaluate the migration capability of cells. SCaBER, SCaBER#4 and SCaBER#9q cells were grown to 100% confluence in a 6-cm dish and a scratch was made with 200- μ l pipette tips. The cells were washed with PBS and placed in serum-free medium (DMEM). The wound width at 0 and 24 h was measured and evaluated using a digital

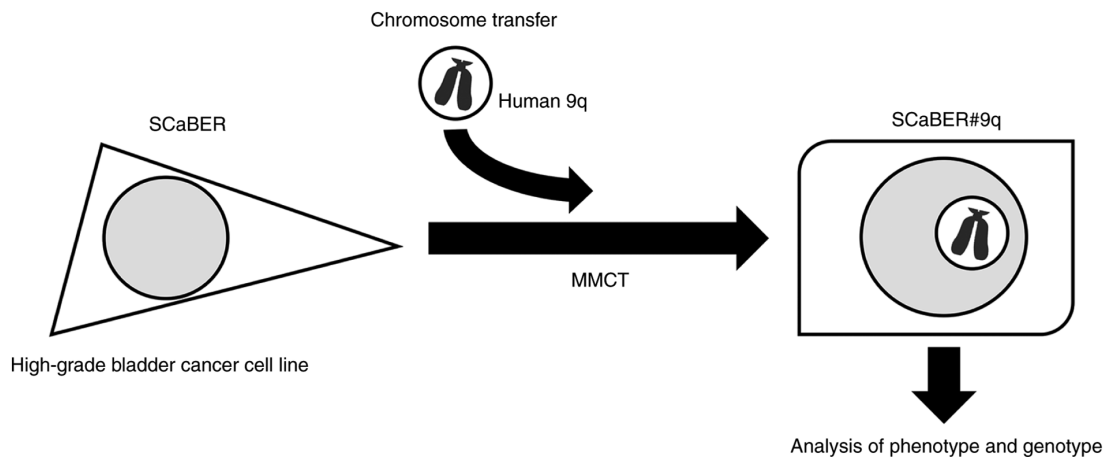


Figure 1. Long arm of human chromosome 9 was introduced into the bladder cancer cell line SCaBER via MMCT. MMCT, microcell-mediated chromosome transfer.

camera system (NIS-Elements Documentation, Ver.5.30; Nikon Corporation).

Western blotting. Western blotting was performed as previously described (17). Membranes were blotted with rabbit polyclonal antibodies against human PPAR γ (cat. no. #2430; 1:1,000; Cell Signaling Technology, Inc.), PTEN (cat. no. #9188; 1:1,000; Cell Signaling Technology, Inc.) or α -tubulin (cat. no. PM054-7; 1:5,000; Medical and Biological Laboratories Co., Ltd.) and anti-rabbit IgG horseradish peroxidase-linked antibody (cat. no. #7074; 1:2,000; Cell Signaling Technology, Inc.), according to the manufacturer's instructions. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (cat. no. 32106; PierceTM ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.). Protein bands on western blot films were quantified using ImageJ (ver.1.8.0; National Institutes of Health).

Survival analysis. The Cancer Genome Atlas (TCGA) (18) and National Cancer Institute Genomic Data Commons (19) public cancer genome bladder urothelial carcinoma (BLCA) dataset (accessed 20 June, 2021) was visualized and analyzed using UCSC Xena (xena.ucsc.edu). Overall survival curve was obtained using the Kaplan-Meier method with the median expression of each gene as the cutoff (PPARG: <19.31 vs. \geq 19.31; FOXA1: <18.80 vs. \geq 18.80; GATA3: <20.52 vs. \geq 20.52), and differences in survival were evaluated with the log-rank test.

Statistical analysis. Data from triplicate experiments are presented as the mean \pm standard error of the mean. Data were analyzed using one-way ANOVA with post-hoc Tukey's honestly significant difference test. All statistical analysis was performed using SPSS Statistics software (version 24.0; IBM Corp.) $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of mouse A9/human mono-chromosomal hybrids. The strategy for investigation of tumor suppressor effect of

human chromosome 9 in BCa is shown in Fig. 1. We previously generated a library of A9 hybrid cells, each containing one of the human chromosomes (except Y) (20). To confirm the status of human chromosome 9 in A9(neo9) cells, PCR analysis using 21 STS markers located on human chromosome 9 was performed (Fig. 2). PCR analysis showed deletion of the short arm loci of human chromosome 9 (#9delp12-pter) in A9(neo9) cells (Fig. 2). FISH analysis was performed in A9(neo9) cells using neo-plasmid probe. The pSV2neo probe for #9delp12-pter was randomly integrated into two regions, #9p12 and #9q34.3 (Fig. 3A). Additionally, the long arm of human chromosome 9, was independently retained in mouse A9 cells.

Introduction of human chromosome 9q into SCaBER cells. Human chromosome 9q was transferred into SCaBER cells using MMCT. Microcell hybrid cells were isolated via three successive chromosome transfer experiments and analyzed to confirm the presence of transferred #9delp12-pter tagged with pSV2neo by FISH. Transferred #9delp12-pter was stably retained in the microcell hybrid clone (SCaBER#9q; Fig. 3B). Microcell hybrids with introduced chromosome 4 (SCaBER#4) were used as a control. The presence of transferred chromosome 4 in SCaBER#4 was confirmed by FISH analysis using a PR11-q4C13 BAC probe containing the 4q22.1 genomic DNA region. The parental SCaBER cells had two copies of chromosome 4, whereas SCaBER#4 microcell hybrid clones had three copies of chromosome 4; this confirmed the presence of the transferred chromosome (Fig. 3C and D).

The morphological features of microcell hybrid clones generated via introduction of a human chromosome were microscopically examined. SCaBER#9q cells were flatter and larger than parental SCaBER and SCaBER#4 cells (Fig. 3E). Thus, human chromosome 9q may have carried genes that regulated this transformed phenotype in SCaBER cells.

Effect of chromosome 9q introduction on cell proliferation and migration ability. The proliferation rate of microcell hybrid clones was examined to determine the effects of chromosome transfer on cell proliferation. SCaBER#9q cells exhibited significantly decreased proliferation compared with control SCaBER#4 and parental cells at days 3-5 (Fig. 4A).

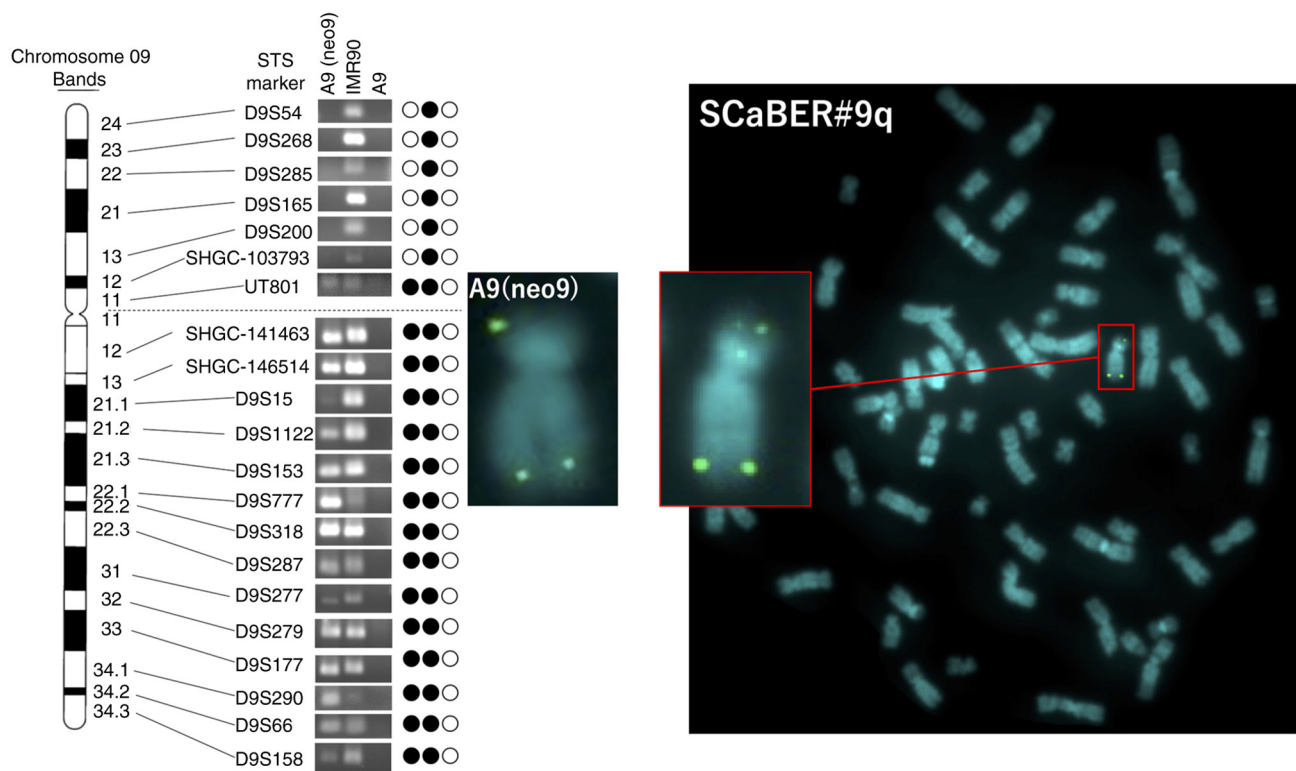


Figure 2. Presence or absence of the region 9p24.2-9q34.3 on human chromosome 9q contained in mouse A9(neo9) was verified by PCR. PCR analysis showing deletion of short arm loci of human chromosome 9 (#9del p12-pter) in mouse A9(neo9) cells. IMR90, a normal human fibroblast cell line, was used as a positive control. STS, sequence-tagged site.

To evaluate the role of chromosome 9q in regulation of malignant potential of basal BCa, wound healing assay was performed using SCaBER microcell hybrid clones. Compared with parental SCaBER and SCaBER#4 cells, SCaBER#9q cells showed decreased mobility (Fig. 4B and C). This indicated that tumor suppressor genes involved in cell proliferation and migration in SCaBER cells were present on 9q.

Expression profile analysis of luminal markers. The luminal subtype of BCa has a lower proliferative and migratory potential than the basal subtype and is less malignant (8). GATA3, FOXA1, and PPAR γ are specifically upregulated in the luminal subtype (as previously determined by RNA sequencing analysis) (21). Furthermore, overexpression of GATA3 and FOXA1, which are tumor suppressive, is accompanied by activation of PPAR γ in transformation of basal into luminal BCa cells (22-26). Additionally, LOH on the long arm of chromosome 9 is commonly observed in premalignant lesions of bladder carcinogenesis, suggesting that chromosome 9q encodes tumor suppressor genes that serve a key role in development and progression of BCa. The expression levels of luminal markers (FOXA1, GATA3 and PPARG) was examined; compared with SCaBER cells, SCaBER#9q cells exhibited a 2.4-, 1.9- and 5.2-fold increase in the expression of the aforementioned luminal markers (Fig. 5A-C).

A heatmap of differential molecular subtypes was used to evaluate the relative expression of luminal markers (Fig. 5D). The relative expression level of luminal markers was high in RT4 cells (typical luminal subtype), whereas SCaBER (basal) and T24 cells (non-type) exhibited low expression levels (27).

Only expression of PPARG was higher in SCaBER#9q cells than in parental, SCaBER#4 and RT4 cells (Fig. 5D). The expression profiles of three luminal marker genes in BCa was further investigated using BLCA dataset. Higher expression of all luminal marker genes was associated with improved survival in patients with BCa (Fig. 6). These results suggested that tumor suppressor genes on 9q may serve an important role in determining the molecular subtype of BCa, which is associated with development of malignancy.

Analysis of protein expression of luminal markers. Western blotting was performed to confirm that expression of luminal markers (GATA3, FOXA1, and PPARG) was also increased at the protein level. Only PPAR γ was significantly increased in SCaBER#9q cells (3.0-4.4-fold) compared with parental cells (Figs. 7A and B and S1). PPAR γ inhibits proliferation, metastasis and invasion of cancer by induction of PTEN expression (28). The expression levels of PTEN were also increased in SCaBER#9q cells (Fig. 7A and B). This suggested that chromosome 9q carried genes that regulate luminal marker of BCa.

Discussion

BCa drug treatment has been based on cisplatin-based chemotherapy for 30 years but the development of immunotherapy (pembrolizumab) using immune checkpoint inhibitors has presented a change for BCa treatment. However, pembrolizumab only has a 21.1% response and 7.0% complete response rate (29). Therefore, there is a need for precision medicine,

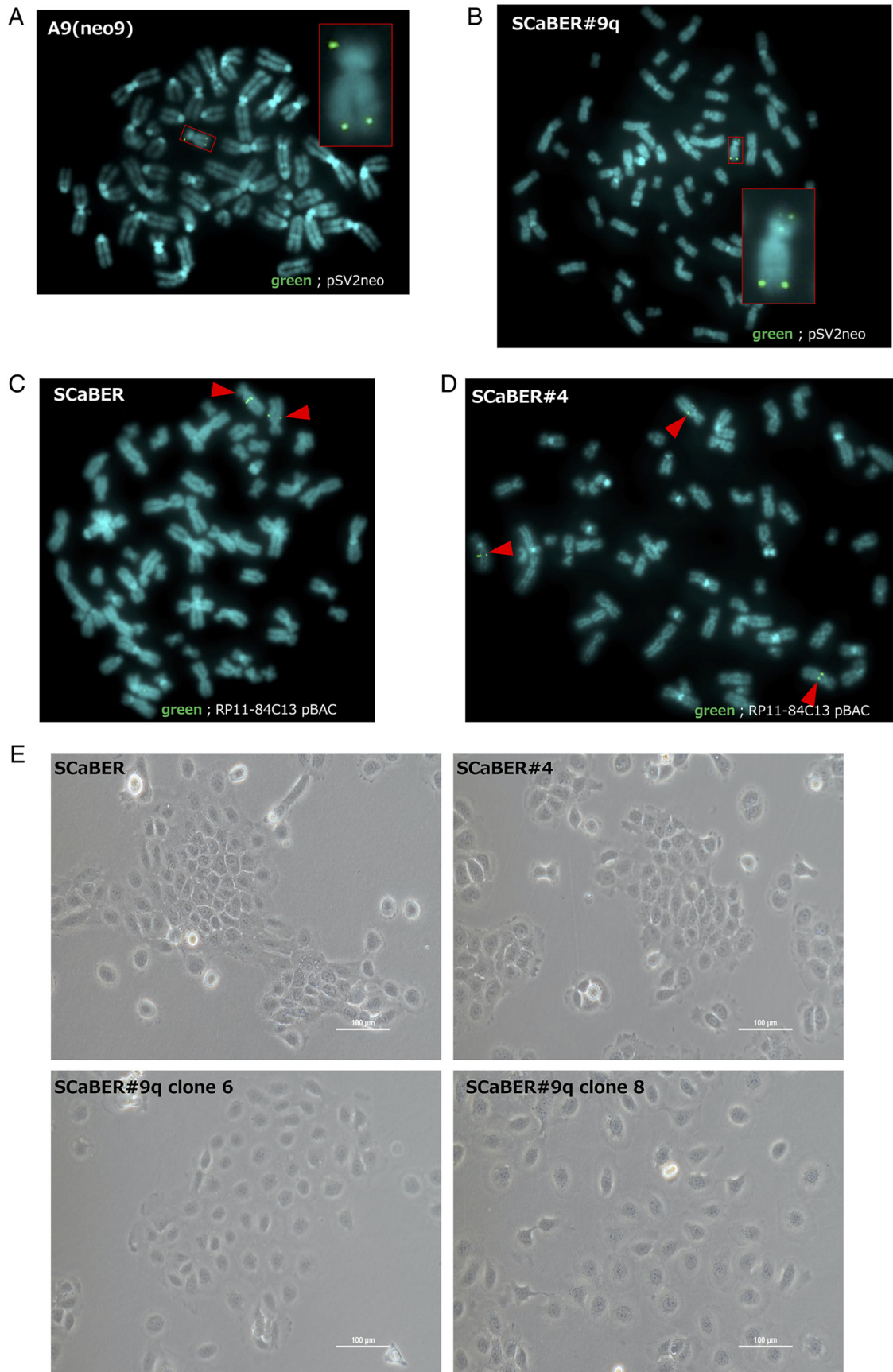


Figure 3. Chromosomal FISH identified successful transference of chromosome 9q in metaphase spreads and morphological change. (A) Human chromosome 9 in A9(neo9) cells and (B) transfer of 9q to SCaBER cells by MMCT from mouse A9(neo9) cells were confirmed by FISH (probeDNA: pSV2neo). (C) Three copies of chromosome 4 in SCaBER cells and (D) transfer of human chromosome 4 (Chr.4) to SCaBER by MMCT from mouse A9(neo4) cells were confirmed by FISH (probeDNA: RP11-84C13 pBAC) and arrowheads indicate chromosome 4. (E) Compared with SCaBER (parental strain), SCaBER#9q showed a decrease in nuclear-cytoplasmic ratio and altered cell morphology. Magnification, x200. FISH, fluorescent *in situ* hybridization; MMCT, microcell-mediated chromosome transfer.

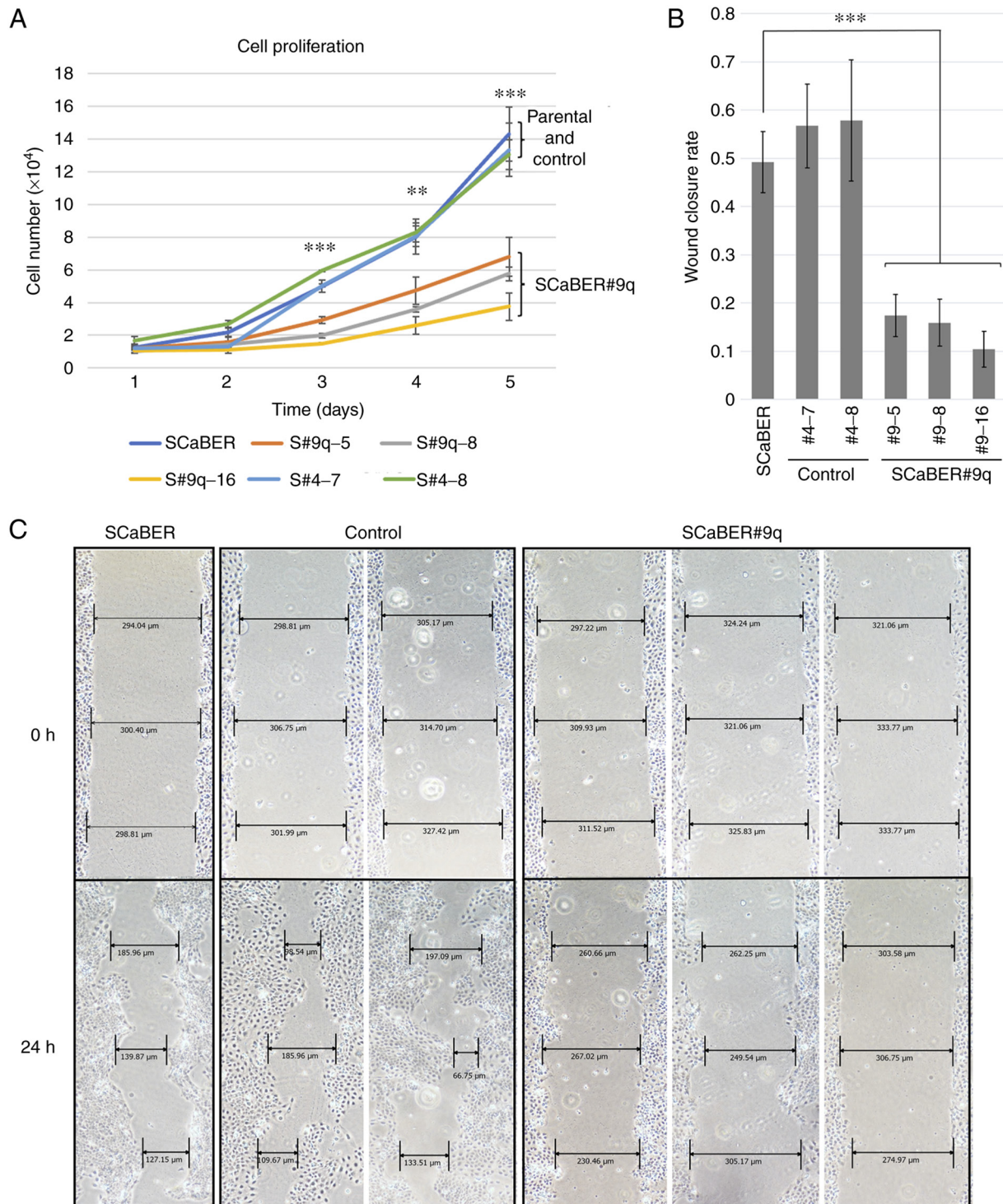


Figure 4. 9q-transfected clones showed decreased cell proliferation and migration ability. (A) Cell proliferation assay showed a significantly decreased proliferation rate in human bladder cancer cells transfected with the long arm of chromosome 9 (SCaBER#9q) compared with control SCaBER#4 and parental cells. ** $P < 0.01$, *** $P < 0.001$. (B) Wound closure rate showed a significant decrease in migration ability of SCaBER#9q. Data are presented as the mean \pm SEM of triplicate experiments. *** $P < 0.001$. (C) The cells scratched by 200- μ l pipette tips were placed in serum-free medium (DMEM) and measured and evaluated using a digital camera system (NIS-Elements Documentation, Ver.5.30; Nikon Corporation) at 0 and 24 h. Magnification, $\times 80$.

identification of biomarkers that can be used as predictors of therapeutic efficacy and development of novel therapies. Greater understanding of the molecular mechanisms underlying the development and progression of BCa is therefore key. Identification of novel tumor suppressor genes involved in progression of BCa may clarify the mechanism of its development and lead to identification of new therapeutic targets. The

present study showed that malignant phenotypes, such as cell proliferation and migration, are suppressed in the SCaBER human high-grade basal BCa cell line following introduction of the long arm of human chromosome 9, resulting in enhanced expression of the luminal marker PPAR γ . This suggested that chromosome 9q carried genes that directly or indirectly regulated the PPAR γ luminal gene in SCaBER cells.

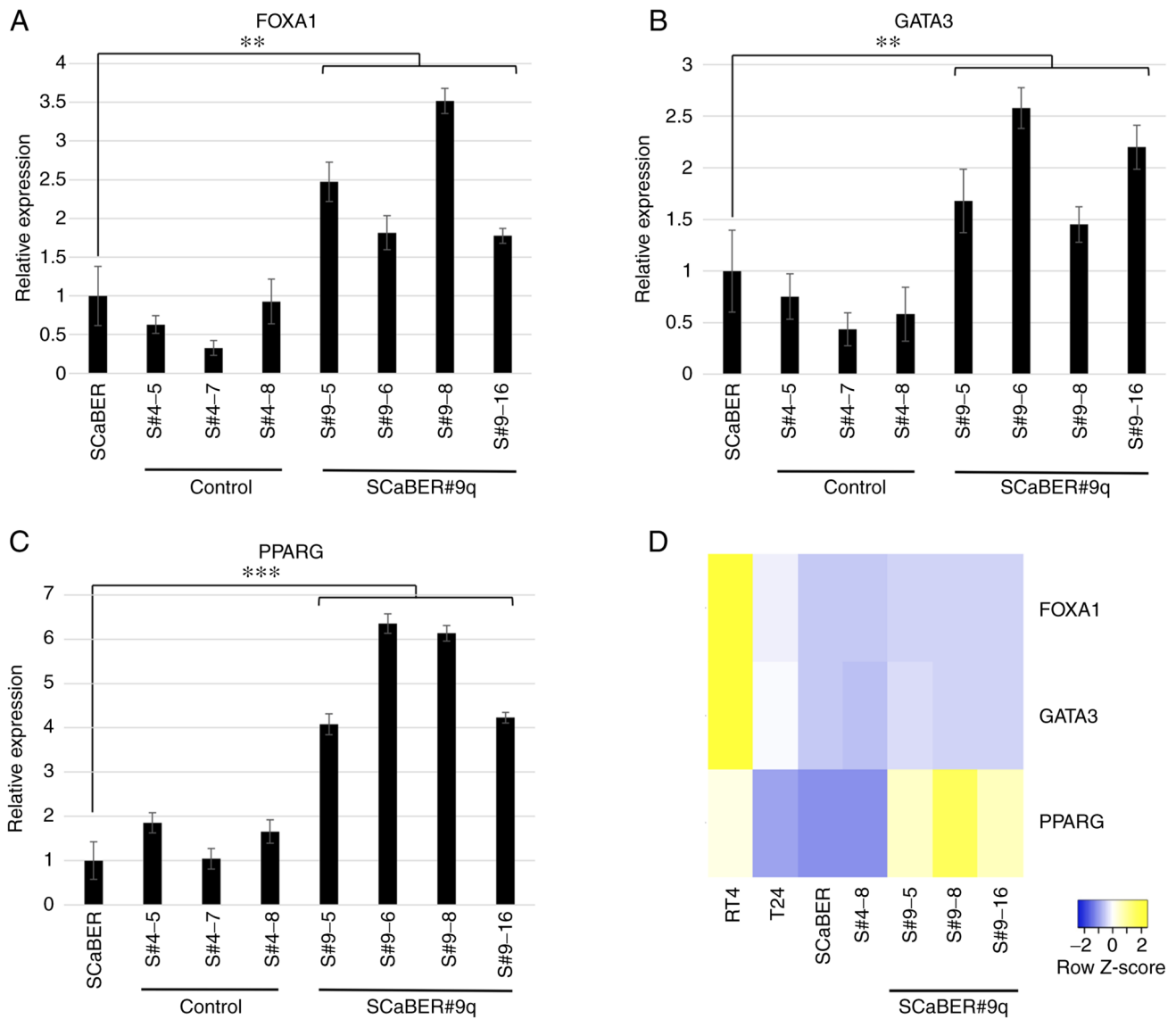


Figure 5. Reverse transcription-quantitative PCR of the luminal markers FOXA1, GATA3 and PPARG from cells transferred with 9q and control cells. Human bladder cancer cells transferred with long arm of chromosome 9 (ScaBER#9q) showed 2.4-, 1.9-, and 5.2-fold increase in (A) FOXA1, (B) GATA3 and (C) PPARG expression, respectively, compared with ScaBER and ScaBER#4 cells. Data are presented as the mean \pm SEM of triplicate experiments. ** $P < 0.01$, *** $P < 0.001$. (D) Heatmap of expression levels of FOXA1, GATA3 and PPARG compared with other cell lines (RT4, luminal; T24, non-type; 5637, basal). Gene expression levels were normalized against GAPDH mRNA using the $2^{-\Delta\Delta C_q}$ method. FOXA1, forkhead box A1.

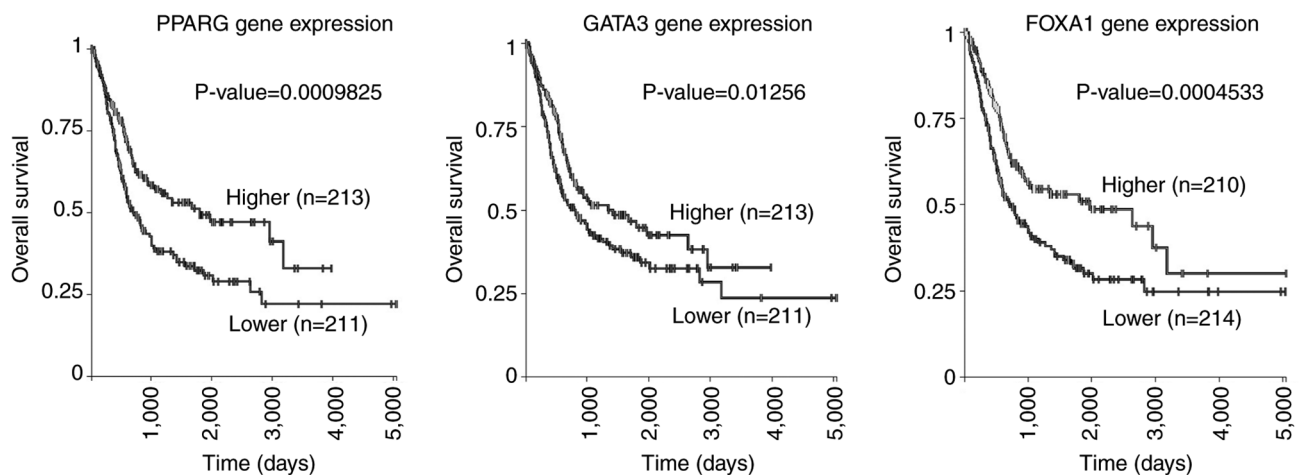


Figure 6. Expression profiles of three luminal marker genes in BCa was investigated using public datasets. Association between expression levels of PPARG, GATA3 and FOXA1 and overall survival from The Cancer Genome Atlas and the National Cancer Institute Genomic Data Commons database.

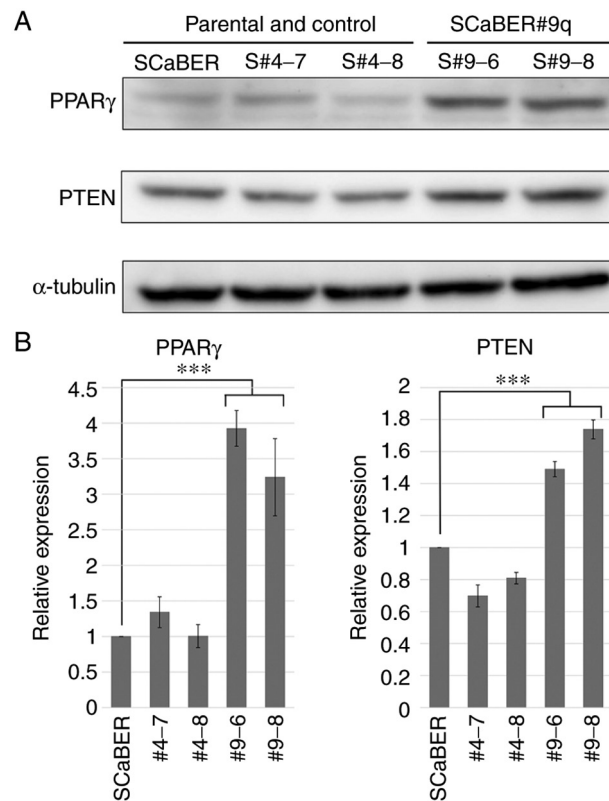


Figure 7. Western blotting of PPAR γ and PTEN from cells transfected with 9q and control cells. (A) Human bladder cancer cells transfected with long arm of chromosome 9 (SCaBER#9q) cells showed increased PPAR γ and PTEN expression compared with SCaBER and SCaBER#4 cells. (B) Relative expression was calculated by normalizing against α -tubulin. Bars correspond to the mean \pm SEM of triplicate experiments. ***P<0.001.

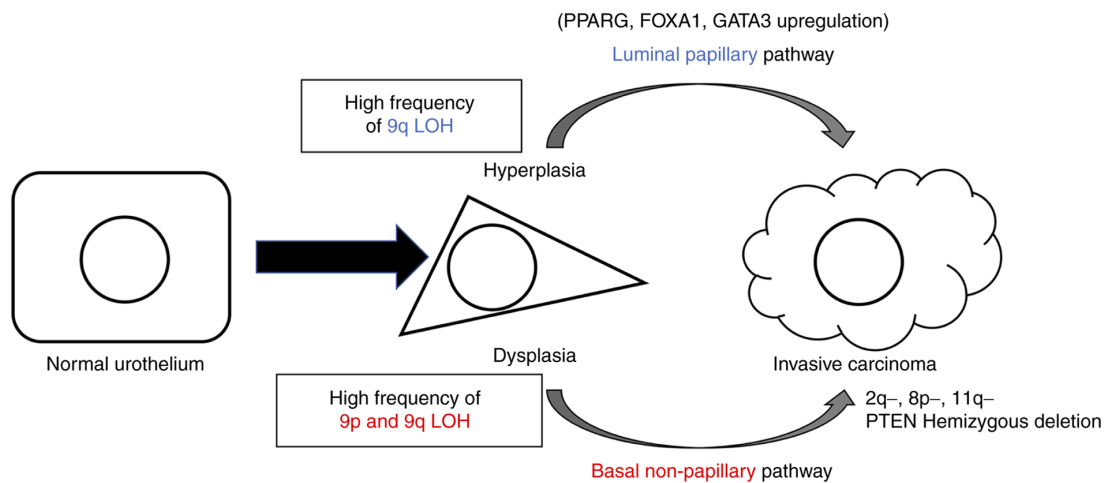


Figure 8. Schematic representation of carcinogenesis and progression of bladder cancer. High frequency of LOH in 9q is a common early event and deletion of 2q, 8p and 11q are late events in bladder cancer carcinogenesis. GATA3, FOXA1, and PPAR γ are upregulated in the luminal pathway of bladder cancer progression. LOH, loss of heterozygosity; FOXA1, forkhead box A1.

TCGA, MD Anderson Cancer Center and other research groups have reported that urothelial carcinoma can be classified into basal and luminal subtypes by gene expression profiling (30,31). Based on this classification and pathological characteristics, two pathways of carcinogenesis have been proposed: Papillary/luminal pathway, which leads to hyperplasia, papillary non-invasive and invasive carcinoma, and the non-papillary/basal pathway, which leads to flat dysplasia, carcinoma *in situ* and non-papillary invasive carcinoma

(Fig. 8) (8). GATA3, FOXA1, and PPAR γ are upregulated in the luminal pathway and overexpression of GATA3 and FOXA1 and PPAR γ activation drive transdifferentiation from the basal to luminal phenotype (23). In the present study, transcription levels of PPAR γ , FOXA1 and GATA3 were upregulated in SCaBER#9q cells compared with parental and SCaBER#4 cells. However at the protein level, only PPAR γ was upregulated in SCaBER#9q cells. Translation is affected by various regulatory factors, such as the cap-binding protein

eIF4E and microRNAs (32,33). Therefore, the discrepancy between mRNA and protein expression of luminal genes in SCaBER#9q cells may be because of specific regulatory factors of translation.

PPARs, which are members of the nuclear receptor superfamily, can be divided into three subtypes: PPAR α , β and γ (34). Previous studies have shown that PPAR γ serves a key role in occurrence and progression of BCa via regulation of proliferation, apoptosis, metastasis, reactive oxygen species and lipid metabolism (26,35-38). High expression of PPAR γ indicates better prognosis for patients with more differentiated, non-invasive tumors with low proliferative potential (39).

Aberration of chromosome 9, including deletions and LOH, is frequently observed in both non-muscle and muscle invasive BCa (>50%). In particular, p16, a tumor suppressor gene located on chromosome 9p, plays a key role in the progression of non-muscle invasive BCa (40-44) but the functional role of novel suppressor genes on 9q in BCa remains unclear. In the present study, expression levels of PPAR γ and PTEN were significantly increased by introduction of 9q to basal BCa cells (SCaBER), suggesting a transformation from poorly differentiated to more highly differentiated, low-grade BCa. The proliferative and migratory ability of SCaBER#9q cells also decreased. These results support a previous study showing that PPAR γ inhibits proliferation, metastasis and invasion of cancer by inducing PTEN expression (28). However, there are conflicting reports on the association between PPAR γ and PTEN in BCa (17,45). The present findings may provide insight into PTEN regulatory pathways.

In conclusion, the present study provided evidence that the long arm of human chromosome 9 contained genes that regulate PPAR γ . We previously identified paired-like homeodomain 1 as a novel tumor suppressor gene on human chromosome 5 that regulates telomerase activity chromosome transfer and gene expression profiling analysis (46). Future identification and characterization of putative PPAR γ regulatory genes on 9q should facilitate understanding of the molecular mechanisms involved in the development of BCa.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

TYa, TO and HK designed the experiments and analyzed the data. TYa, TO and RS performed experiments. TO and HK wrote the manuscript. TYu, NY, HI, SM, KH and MH analyzed data. TO and HK confirm the authenticity of all the raw data.

HK and AT conceived and supervised the project. All authors revised and edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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