Original Article

# The study of miRNA-200c expression and epithelial-to-mesenchymal transition-related transcription factors in the primary bladder urothelial carcinoma

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## Abstract

**Background:** Epithelial-mesenchymal transition (EMT) plays an important role in bladder carcinoma (BC) invasiveness and metastasis. Studies have shown that muscle-invasive BC (MIBC) and non-MIBC (NMIBC) are different at the molecular level owing to different EMT-related programming. Recent studies suggest that dysregulation of specific miRNAs is linked to EMT in BC. With this background, we aimed to study the immunoexpression of EMT-markers and its correlation with miRNA-200c expression in a series of MIBCs and NMIBCs.

**Materials and Methods:** Quantitative real-time-polymerase chain reaction for the quantification of miR-200c expression was performed on 50 cases of urinary BC obtained from transurethral resection of bladder tumor (TURBT), cystectomy specimens, and ten peritumoral bladder tissue. Immunohistochemistry for ZEB1, ZEB2, TWIST, E-cadherin, and β-catenin was performed on tumor and peritumoral bladder tissue.

**Results:** Thirty-five TURBT and 15 cystectomy specimens were assessed. Among MIBC, loss of expression of E-cadherin (72.3%), β-catenin (66.7%), and ZEB1, ZEB2, and TWIST2 immunoreactivity was noted in 53.3%, 86.7%, and 73.3% of cases, respectively. Among NMIBC, loss of expression of E-cadherin (22.5%), β-catenin (17.1%) and ZEB1, ZEB2, and TWIST immunoreactivity was noted in 11.5%, 51.4%, and 91.4% of cases, respectively. Upregulation of miRNA-200c was noted in cases with retained E-cadherin and negative TWIST expression. Downregulation of miRNA-200c expression was noted in all the cases showing loss of E-cadherin, β-catenin, and in cases immunoreactive for ZEB1, ZEB2, and TWIST in MIBC. Downregulation of miRNA-200c expression was also noted in cases of MIBC with retained β-catenin and those immunonegative for ZEB1 and ZEB2. A similar trend was noted in NMIBC. Median miRNA-200c expression was low in both high-grade and low-grade NMIBC compared to peritumoral bladder tissue and was not statistically significant.

**Conclusion:** This study for the first time explores the relation of miR200C with E-cadherin, b-catenin, and its direct transcriptional regulators, namely Zeb1, Zeb2, and Twist in the same cohort of BC. We observed that miRNA-200c is downregulated in both MIBC and NMIBC. We identified novel expression of TWIST in cases of BC showing downregulation of miR200Cs suggesting that it is one of the protein targets of altered

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miRNA-200c expression contributing to EMT and can serve as a promising diagnostic marker and therapeutic target. Loss of E-cadherin and ZEB1 immunoexpression in high-grade NMIBC suggests an aggressive clinical behavior. However, ZEB2 heterogeneous expression in BC limits its diagnostic and prognostic utility.

**Keywords:** Bladder carcinoma, miRNA-200c, TWIST, ZEB1, ZEB2

## INTRODUCTION

Epithelial-to-mesenchymal transition (EMT) is a highly dynamic process which not only operates in normal physiological processes but plays an important role in tumor invasiveness and distant metastases.[1] The phenomenon of switch in gene expression from epithelial to mesenchymal and mesenchymal to epithelial phenotype cumulates in series of events leading to the activation of transcriptional repressors viz. ZEB1, ZEB2, and TWIST, chromatin remodeling and epigenetic modification rendering stemness to the tumor cells.[1,2] Muscle-invasive bladder carcinoma (MIBC) and non-MIBC (NMIBC) are distinct at the molecular level,[3] have different prognosis, therapeutic management, and clinical outcome.[4] Approximately 45% of stage T1 NMIBC progresses to MIBC,[5] and currently, there are no biomarkers available which can distinguish high risk from low-risk NMIBC. MicroRNAs are a class of noncoding RNA molecules which negatively modulate protein and gene expression.[6] Studies have shown that miRNAs can act as novel therapeutic, diagnostic, and prognostic markers in BC.[7-10] However, data available regarding aberrant miRNA-200c expression in BC is conflicting.[7-10] Some authors have reported miRNA-200c to be upregulated[8,9] while others found it to be downregulated compared to the normal urothelium[10,11] in BC. Overexpression of miRNA-200c represses ZEB1, ZEB2, TWIST, etc., and retains E-cadherin expression preventing BC invasiveness.[12-15] Expression profile of EMT markers and miRNAs status is one of the major determinants deciding tumor invasiveness, recurrence, resistance to radiation, and chemotherapeutic agents postsurgery which directly impacts patients' selection for therapy and overall survival outcome.[16-18] With this background, we aimed to study the immunoexpression of EMT-markers and its correlation with microRNA-200c expression in a series of MIBC and NMIBC cases to evaluate their role as potential complementary biomarkers in the grading of BC.

## MATERIALS AND METHODS

A total of 50 cases of primary bladder urothelial carcinoma (UC) which included muscle-invasive ($n$ = 15) and nonmuscle-invasive ($n$ = 35) bladder carcinoma (BC) were retrieved from Department of Pathology between August 2016 and April 2017. Transuretheral resection of bladder tumor (TURBT) and cystectomy specimens with UC from patients were included admitted under the Department of urology with no prior history of local or systemic therapy were included. Peritumoral bladder tissues from cystectomy specimens were included as control. For both malignant and normal bladder tissue, 2–5 mg of fresh tissue was collected in a tube containing RNA and stored at −20°C. The rest of the tissue was formalin-fixed and paraffin-embedded (FFPE) for preparation of hematoxylin- and eosin-stained sections and immunohistochemistry. The study was in compliance with ethical standards.

### RNA extraction and reverse transcription and quantitative real-time polymerase chain reaction

RNA isolation was done using TRIzol Reagent (Thermofisher scientific Pvt Ltd). The integrity of extracted RNA pool was checked on 1 × MOPS-formaldehyde agarose gel. The intact form of the total RNA pool was confirmed on 1 × MOPS-formaldehyde agarose gel. Further, low-molecular-weight RNAs were eluted using AmbionmirVana miRNA Isolation Kit as per manufacturer instructions and checked on 8 mM Urea denaturing PAGE. The RNA concentration and purity were determined spectrophotometrically by measuring A260/A280 ratio using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Expression levels of the miRNA-200c were determined by quantitative real-time polymerase chain reaction (PCR) (Biorad C96f Real-Time PCR machine) using Taqman microRNA reverse transcription kit and Syber Green qPCR Master Mixes (Biorad) with selected microRNA primer assay miR-200c (Sigma). The small nuclear RNA U6 was used as an endogenous control (RNU6B). The reactions were performed in a 96-well optical plate in two cyclic programs at 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s. All reactions were run in independent duplicates. Normalized expression ($2^{-\Delta\Delta Ct}$) was calculated by normalizing the threshold cycle (Ct) value for each sample by using the comparative Ct method where the amount of targeted miRNA was normalized to the endogenous control sample. The Ct was defined as the fractional cycle number at which the fluorescence passed a fixed threshold.

### Immunohistochemistry

Five-microns-thick sections cut from FFPE tissue blocks were immunolabeled with the following antibodies using standard protocol: ZEB1 (Polyclonal [HPA027524]; dil 1:1000; Sigma Life Sciences), ZEB2 (Polyclonal [SAB4503710]; dil 1:400; Sigma Aldrich), TWIST (Monoclonal [Clone 10E4E6]; dil 1:200; LifeSpanBioScience) E-cadherin (Monoclonal [SP64]; dil 1:400; Spring, βcatenin (Monoclonal [EP35]; dil 1:100 Bio SB).Peritumoral bladder tissue was used as control.

Tissue sections were scored semiquantitatively using the immunoreactive score (IRS) scale. Percentage of positive cells; 0: No positive cells; 1: <10% of positive cells; 2: 10%–50% of the positive cells; 3: 51%–80% of the positive cells; 4: >80% of the positive cells and staining intensity score of 0–3 was applied; 0: No staining; 1+: Mild reaction; 2+: Moderate reaction; 3+: Strong reaction. The final IRS score was calculated as percentage of positive cells multiplied staining intensity. IRS score ranged from 0 to 12 (0–1 = negative; 2–3 = mild; 4–8 = moderate; 9–12 = strongly positive).[19] Score ranging from 4 to 12 was considered positive. Loss or retained membranous staining pattern for E-cadherin and β-catenin was noted.

A total of 50 cases of UC including 15 cystectomies and 35 TURBTs were identified. Among these, 30% ($n$ = 15) were MIBC and 70% ($n$ = 35) of cases were NMIBC. Among MIBC, 80% of cases were cystectomies and 20% were TURBT specimens, while 91% were TURBT and 9% were cystectomies among NMIBC. NMIBC were stage Ta (60%; $n$ = 21) and T1 (40%; $n$ = 14) while among MIBC, 53.3% T2 ($n$ = 8), 26.6% T3 ($n$ = 4) and 20% ($n$ = 3) stage T4. Male preponderance was noted in both groups with a male: female ratio of 14:1 and 6:1 in MIBC and NMIBC, respectively ($P$ = 0.306). The mean age was 57.4 years and 55.5 years for MIBC and NMIBC, respectively ($P$ = 0.404).

### Statistical analysis

The immunohistochemistry and miRNA expression along with histopathological reports were entered in Microsoft Excel spreadsheet. Categorical variables were compared using the Pearson Chi-square test. Statistical significance was taken as $P < 0.05$. Data were analyzed using IBM SPSS Statistics Software (version 20.0 Chicago, IL, USA).

## RESULTS

### Histopathology

All 15 cases (100%) of MIBC showed histomorphological features of high grade (HG) UC, while 57.3% of NMIBC were low grade (LG) and 42.8% showed HG morphology. LG NMIBC were stage Ta, while HG NMIBC were predominantly stage T1 [Table 1]. Papillary architecture was the dominant histological pattern in majority (88%; $n$ = 38) while 12% ($n$ = 6) were other histological variants. Variant included were small-cell carcinoma (16.6%; $n$ = 1), plasmacytoid (16.6%; $n$ = 1), sarcomatoid (16.6%; $n$ = 1), and HG papillary UC with squamous differentiation (33.3%; $n$ = 2), of which all were MIBC except for small-cell variant.

### Immunohistochemistry

#### *Expression of E-cadherin in muscle invasive bladder carcinoma and nonmuscle invasive bladder carcinoma*

Loss of membranous expression of E-cadherin was noted in 73.3% ($n$ = 11/15) of MIBC. Among NMIBCs, loss of E-cadherin expression was noted in 22.9% ($n$ = 8/35) of cases ($P$ = 0.001). E-cadherin expression was retained in the peritumoral bladder tissue [Figure 1].

#### *Expression of β-catenin in muscle-invasive bladder carcinoma and nonmuscle invasive bladder carcinoma*

Loss of membranous expression for β-catenin was noted in 66.7% (10/15) of MIBC, while only 17.1% ($n$ = 6/35) of NMIBC showed loss of β-catenin immunoexpression ($P$ = 0.002). β-catenin immunoexpression was retained in peritumoral bladder tissue [Figure 1].

#### *Expression of ZEB1 in the muscle-invasive bladder carcinoma and nonmuscle-invasive bladder carcinoma*

Among MIBC, 13.3% ($n$ = 2/15) showed nuclear immunoreactivity for ZEB1 while among NMIBC, 8.57% ($n$ = 3/35) of cases were positive ($P$ = 0.629) [Figure 2].

**Table 1: Clinical, immunohistochemical and miRNA-200c expression profile of high grade and low grade nonmuscle invasive bladder carcinoma**

| | HG NMIBC ($n$=15), $n$ (%) | LG NMIBC ($n$=20), $n$ (%) | $P$ (HG vs. LG NMIBC) |
|---|---|---|---|
| Stage Ta | 2 (13.4) | 19 (95) | 0.001 |
| Stage T1 | 13 (86.6) | 1 (5) | |
| Median miRNA-200c | 0.097 | 0.11 | 0.546 |
| Immunohistochemistry | | | |
| E-cadherin | | | |
| Loss | 8 (53.3) | 0 | 0.001 |
| Retained | 7 (46.6) | 20 (100) | |
| β-catenin | | | |
| Loss | 5 (33.3) | 1 (5) | 0.064 |
| Retained | 10 (66.7) | 19 (95) | |
| ZEB1 | | | |
| Positive | 3 (20) | 0 | 0.07 |
| Negative | 12 (80) | 20 (100) | |
| ZEB2 | | | |
| Positive | 5 (33.3) | 2 (10) | 0.112 |
| Negative | 10 (66.6) | 18 (90) | |
| TWIST | | | |
| Positive | 14 (93.3) | 10 (50) | 0.009 |
| Negative | 1 (6.7) | 10 (50) | |

MIBC: Muscle invasive bladder carcinoma, NMIBC: Non-MIBC, HG: High grade, LG: Low grade

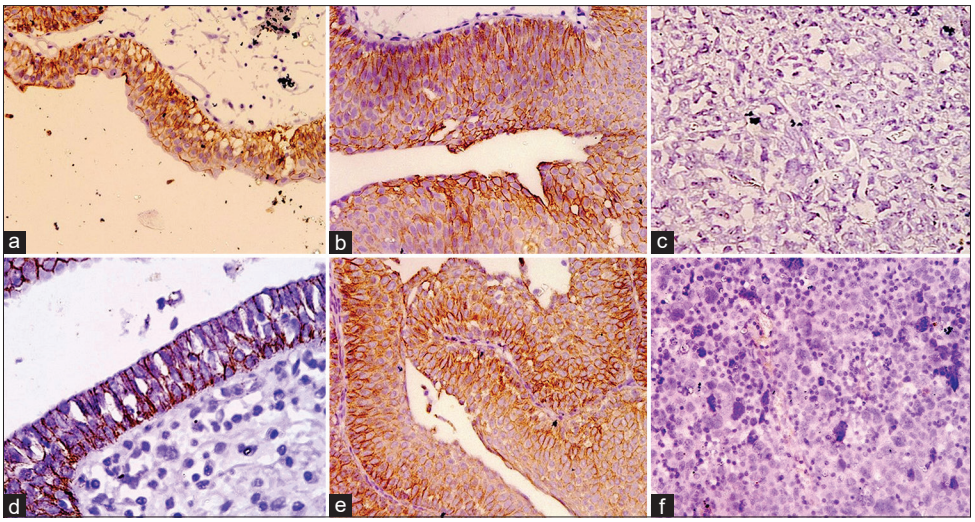



**Figure 1:** Peritumoral bladder urothelium displaying membranous staining pattern of E-cadherin (a: H and E, ×200). Retained E-cadherin immunoexpression in NMIBC (b: H and E, ×200). Loss of E-cadherin in MIBC (c: H and E, ×200). Peritumoral bladder urothelium displaying membranous staining pattern of β-catenin (d: H and E, ×200). Retained β-catenin immunoexpression in NMIBC (e: H and E, ×200). Loss of β-catenin immunoexpression in MIBC (f: H and E, ×200). MIBC: Muscle invasive bladder carcinoma, NMIBC: Non-MIBC

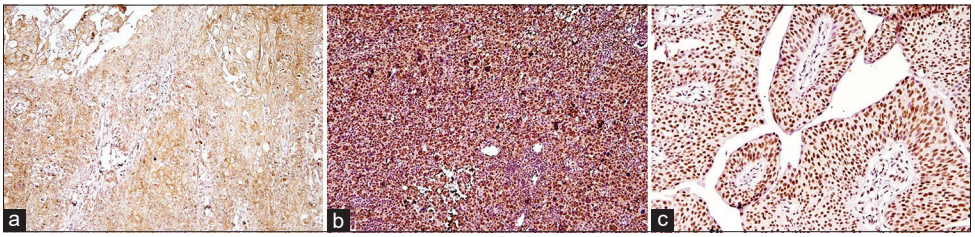



**Figure 2:** Photomicrograph showing cytoplasmic immunopositivity for ZEB 2 in MIBC (a:x200). Nuclear ZEB1 immunopositivity in MIBC (b: x200). Nuclear TWIST positivity in NMIBC (c: ×200). MIBC: Muscle invasive bladder carcinoma, NMIBC: Non-MIBC

### *Expression of ZEB2 in the muscle-invasive bladder carcinoma and nonmuscle invasive bladder carcinoma*

Among MIBC, 60% ($n = 9/15$) showed cytoplasmic ZEB2 immunoreactivity. Among NMIBC, 20% ($n = 7/35$) showed cytoplasmic ZEB2 immunoreactivity ($P = 0.009$) [Figure 2].

### *Expression of TWIST in muscle-invasive bladder carcinoma and nonmuscle invasive bladder carcinoma*

Among MIBC cases, nuclear TWIST immunopositivity was noted in 80% ($n = 12/15$) of cases. Among NMIBC, immunopositivity was noted in 69% ($n = 24/35$) of cases ($P = 0.507$). None of the control peritumoral bladder tissue sections ($n = 10$) showed immunopositivity forZEB1, ZEB2, and TWIST [Figure 2].

## MiRNA-200c expression in muscle invasive bladder carcinoma and nonmuscle invasive bladder carcinoma

Median value of expression of miR-200c was 0.04 (range: 0.003–0.73) and 0.38 (range: 0.0055–0.900) in MIBC and NMIBC, respectively, while it was 0.3011 for peritumoral bladder tissue. Within the MIBC group, median values for miRNA-200c were 0.26 and 0.25 respectively for MIBC (Stage Ta and T1) and MIBC (Stage T3 and T4).

### *Correlation of miRNA-200c expression in muscle-invasive bladder carcinoma with E-cadherin, β-catenin, ZEB1, ZEB2, and TWIST expression*

The median value of miRNA expression was 0.06 and 0.33 in cases showing loss and retained E-cadherin expression, respectively ($P = 0.076$). The median value of miRNA was 0.06 and 0.073 in cases showing loss and retained β-catenin expression ($P = 0.877$). The median value of miRNA was 0.02 and 0.095 in cases immunoreactive and immunonegative for ZEB1 ($P = 0.076$). The median value of miRNA was 0.076 and 0.006 in cases immunoreactive and immunonegative for ZEB2 ($P = 0.242$). The median value of miRNA was 0.06 and 0.22 in cases immunoreactive and immunonegative for TWIST ($P = 0.554$). None were statistically significant [Table 2 and Graph 1].

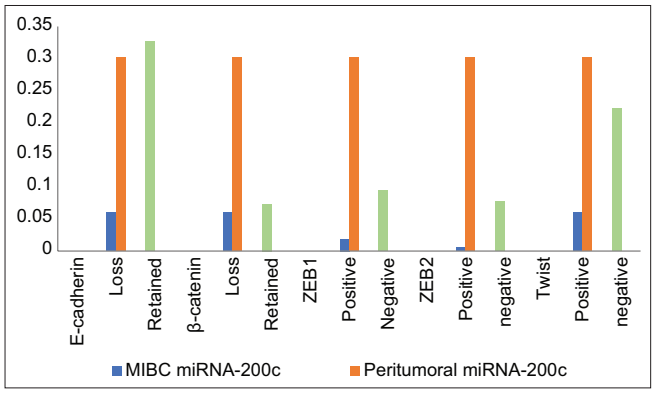
### *Correlation of miRNA-200c expression in nonmuscle invasive bladder carcinoma with E-cadherin, β-catenin, ZEB1, ZEB2, and TWIST expression*

The median value of miRNA expression was 0.42 and 0.09 in cases showing loss and retained E-cadherin expression, respectively ($P = 0.151$). The median value of miRNA was 0.27 and 0.103 in cases showing loss and retained

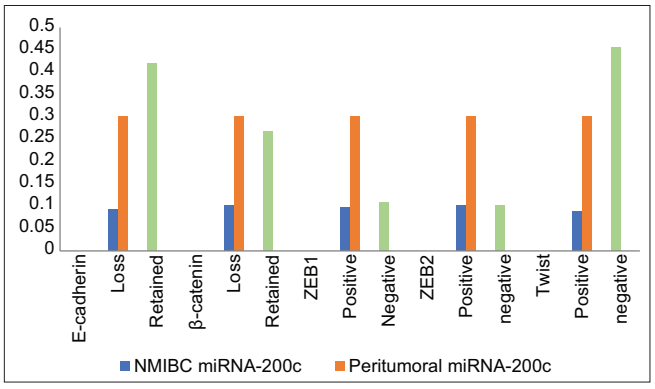
**Table 2: Immunohistochemical and miRNA-200c expression profile of muscle invasive bladder carcinoma and nonmuscle invasive bladder carcinoma**

| Immunohistochemistry | MIBC (*n*=15), *n* (%) | NMIBC (*n*=35), *n* (%) | *P* | Median smiRNA-200c MIBC | *P* | Median miRNA-200c NMIBC | *P* |
|---|---|---|---|---|---|---|---|
| E-cadherin | | | | | | | |
| Loss | 11 (73.3) | 8 (22.8) | 0.001 | 0.06 | 0.4298 | 0.092 | 0.476 |
| Retained | 4 (26.6) | 27 (77.2) | | 0.33 | | 0.421 | |
| β-catenin | | | | | | | |
| Loss | 10 (66.6) | 6 (17.2) | 0.002 | 0.06 | 0.8774 | 0.103 | 0.3981 |
| Retained | 5 (33.3) | 29 (82.8) | | 0.073 | | 0.268 | |
| ZEB1 | | | | | | | |
| Strongly positive | 2 (13.3) | 1 (2.8) | 0.629 | 0.02 | 0.0757 | 0.097 | 0.4571 |
| Moderate | 0 | 2 (5.7) | | | | | |
| Mild | 6 (40) | 0 | | 0.095 | | 0.108 | |
| Negative | 7 (46.6) | 32 (91.4) | | | | | |
| ZEB2 | | | | | | | |
| Strongly positive | 2 (13.3) | 1 (3) | 0.009 | 0.006 | 0.2416 | 0.103 | 0.8658 |
| Moderate | 7 (46.6) | 6 (17) | | | | | |
| Mild | 4 (26.6) | 11 (31.4) | | 0.076 | | 0.102 | |
| Negative | 2 (13.3) | 17 (48.6) | | | | | |
| TWIST | | | | | | | |
| Strongly positive | 5 (33.3) | 13 (37) | 0.507 | 0.06 | 0.5537 | 0.090 | 0.117 |
| Moderate | 7 (46.6) | 11 (31.4) | | | | | |
| Mild | 1 (6.6) | 8 (23) | | 0.22 | | 0.466 | |
| Negative | 2 (13.3) | 3 (8.6) | | | | | |

MIBC: Muscle invasive bladder carcinoma, NMIBC: Non-MIBC




**Graph 1:** Comparing median miRNA-200c expression in MIBC in relation to peritumoral bladder tissue and immunohistochemical correlation. MIBC: Muscle invasive bladder carcinoma




**Graph 2:** Comparing median miRNA-200c expression in NMIBC in relation to peritumoral bladder tissue and immunohistochemical correlation. NMIBC: Nonmuscle invasive bladder carcinoma

β-catenin expression ($P$ = 0.398). The median value of miRNA was 0.097 and 0.108 in cases immunoreactive and immunonegative for ZEB1 ($P$ = 0.457). Median value of miRNA was 0.103 and 0.102 in cases immunoreactive and immunonegative for ZEB2 ($P$ = 0.866). Median value of miRNA was 0.09 and 0.47 in cases immunoreactive and immunonegative for TWIST ($P$ = 0.117). None were statistically significant [Table 2 and Graph 2].

### *Correlation of miRNA-200c expression in recurrent bladder carcinoma with E-cadherin, β-catenin, ZEB1, ZEB2, and TWIST expression*

None of the cases which recurred showed loss of E-cadherin or β-catenin expression and neither ZEB1 nor ZEB2 immunoreactivity. TWIST immunoexpression was noted in 55.5% of cases ($n$ = 5/9). Median miRNA-200c expression was 0.007 and 0.435 for TWIST-positive and negative cases, respectively, not statistically significant ($P$ = 0.472).

### *Correlation of miRNA-200c expression in high grade nonmuscle invasive bladder carcinoma and low grade nonmuscle invasive bladder carcinoma with E-cadherin, β-catenin, ZEB1, ZEB2 and TWIST expression*

TWIST immunoreactivity was present in 93.3% of HG NMIBC while only in 50% of LG NMIBC which was statistically significant ($P$ = 0.009). E-cadherin loss was noted in 53.3% of HG cases while none of the LG showed loss and was statistically significant ($P$ = 0.001). Immunoreactivity of ZEB1, ZEB2, and β-catenin was not statistically significant among the two groups. Median miRNA-200c was equal among both group and lower than peritumoral bladder tissue [Table 1 and Graph 3].

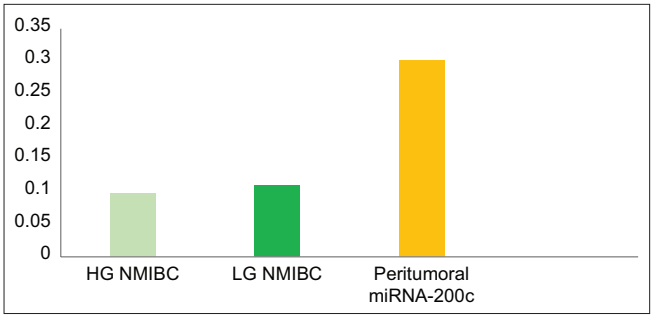
*Correlation of miRNA-200c expression in histological variants of urothelial carcinoma with E-cadherin, β-catenin, ZEB1, ZEB2, and TWIST expression*

E-cadherin loss was noted among all the variants (100%; $n = 6/6$), while the β-catenin loss was seen only in small cell and sarcomatoid variant of UC. Strong immunoreactivity for ZEB1 was noted among small cell and sarcomatoid variant of UC while other variants were immunonegative. ZEB2 expression was variable. TWIST immunoreactivity was noted in all the variants described. MiRNA-200C expression was downregulated among all except for small-cell variant which showed high-miRNA-200c levels compared to peritumoral bladder tissue [Graph 4].

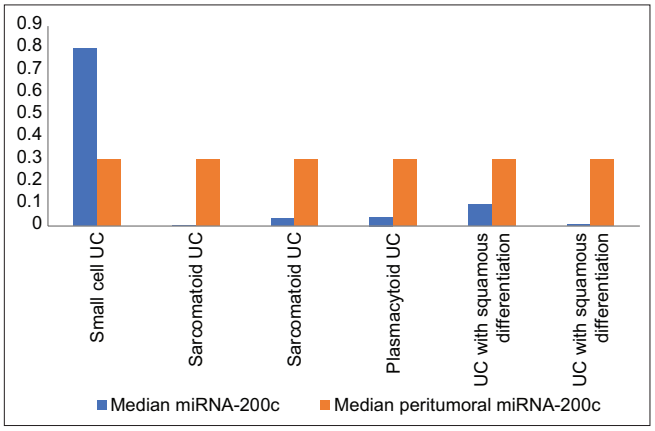
## DISCUSSION

Data regarding aberrant miRNA-200c expression in BC and its role in regulating EMT is conflicting. Some authors have reported miRNA-200c to be upregulated,[8,9] while others found it to be downregulated compared to the normal urothelium[10,11] in BC. Our study revealed downregulation of miRNA-200c expression in cases showing loss of E-cadherin, β-catenin and in cases immunoreactive for ZEB1, ZEB2, and TWIST in MIBC. Notably, ZEB1-positive cases showed markedly low median miRNA-200c expression. However, downregulation of miRNA-200c expression was also noted in cases of MIBC with retained β-catenin and those immunonegative for ZEB1 and ZEB2. Similar trend was noted in NMIBC. Interestingly, retained E-cadherin and negative TWIST were associated with higher miRNA-200c compared to peritumoral normal urothelium.

With growing knowledge regarding potential role of miRNAs in EMT in different tumors, studies done on select cancer cell lines viz. lung, breast etc., have demonstrated that upregulation of miRNA-200c favors epithelial phenotype while downregulation is associated with expression of markers of mesenchymal phenotype. Hurteau *et al*.,[12] using lung and breast cancer cell lines found that loss of E-cadherin is the initial event in process of EMT and overexpression of miRNA-200c in these cell lines leads to the reversal of mesenchymal phenotype. Burk *et al*.,[13] described that ZEB1 directly inhibits the transcription of miRNA-200c in pancreatic, colorectal, and breast cancer cells. Gregory *et al*.,[14] found that inhibition of miRNA-200c is sufficient to induce EMT in metaplastic breast cancer specimens lacking E-cadherin. Korpal *et al*.,[15] using *in vitro* model assay in murine mammary epithelial cell lines demonstrated loss of expression of miRNAs represses E-cadherin by initiating expression of ZEB1/ZEB2 during EMT contributing to migration and invasion in the tumor cells. The present study confined on human BC demonstrates a similar trend described in other cancer cell lines;[12-15] upregulation of miRNA-200c favors epithelial phenotype and downregulation is associated with the appearance of mesenchymal phenotype. Previously, Kenny *et al*.,[20] on MIBC and NMIBC demonstrated increased ZEB1 expression linked to altered miRNA-200c expression similar to our result. Our study describes the novel inverse correlation between miRNA-200c and TWIST expression in MIBC and HG NMIBC which has not been previously described. Regarding cadherin-catenin pathway markers; Erdemir *et al*.,[21] Khorrami *et al*.,[22] Breyer *et al*.,[23] demonstrated that loss of E-cadherin is associated with a higher rate of tumor progression and recurrences in NMIBC.Furthermore, Bilim *et al*.,[24] demonstrated that reduced membranous immunoreactivity of β-catenin in TCC was associated with advanced stage and tumor multifocality. However, recently Poletajew *et al*.,[19] and Zhao *et al*.[25] described that expression of E-cadherin and β-catenin cannot reliably predict prognosis and survival in a subset of high-risk NMIBC. We found loss of E-cadherin in MIBC and HG MIBC which correlated with advanced




**Graph 3:** Comparing median miRNA-200c expression among HG and LG NMIBC in relation to peritumoral bladder tissue. NMIBC: Nonmuscle invasive bladder carcinoma, HG: High grade, LG: Low grade




**Graph 4:** Comparing median miRNA-200c expression among histological variants of UC in relation to peritumoral bladder tissue and immunohistochemical correlation. UC: Urothelial carcinoma

tumor grade and stage reflecting an altered cadherin-catenin pathway. Aggressive histological variants of BC similarly displayed loss of E-cadherin. However, cases in our cohort showing recurrences did not reveal loss of E-cadherin or β-catenin, and majority showed upregulated miRNA-200c levels. Hence, loss of E-cadherin expression though associated with higher grade and aggressive phenotype in BC is not a reliable marker to predict recurrence and other regulators in complex EMT pathway are likely to be operating in NMIBC cases showing recurrences.

Kenny *et al*.,[20] in a larger cohort described ZEB1 immunoexpression in cases of NMIBC and MIBC and further demonstrated that restoration of miRNA-200c levels is associated with reduced ZEB1 expression. Similar to Kenny *et al*., the present study found ZEB1 positive cases were associated with the downregulation of miRNA-200c expression in MIBC and HG NMIBC. A similar trend was noted in HG sarcomatoid and small-cell variants of UC. These findings support the hypothesis that dysregulated miRNA-200c targets ZEB1 and is involved in invasive tumor phenotype.[25] Lee *et al*.[26] in a comprehensive study included 120 different bladder tumors including papillary urothelial neoplasm of low malignant potential, LG, HG papillary UC and infiltrating carcinoma (IC). The study demonstrated different expression profiles in different groups. MiRNA-200cexpression was significantly lower in HG as compared to LG and significantly higher in IC than in HG. ZEB1 was more frequently expressed in HG as compared to LG and IC. ZEB2 immunoreactivity was more in IC than HG. Study also described higher miRNA-200c levels in ZEB1 negative than positive cases while no significant difference was noted among ZEB2 positive and negative tumors. In the present study, we found median miRNA-200c expression was lower in both ZEB1 and ZEB2 positive and negative cases. Notably, markedly lower miRNA-200c was noted in MIBC as compared to HG and LG NMIBC which points toward its role in tumor invasiveness.

Zhao *et al*.[25] described higher TWIST expression in HG BC as compared to LG BC. We found similar results with higher TWIST expression in HG MIBC and NMIBC as compared to LG NMIBC. Wishahi *et al*.,[17] in a comprehensive study found higher TWIST expression in HG T1 and Ta tumors compared to LG T1 and Ta. Similar to this study, we found a higher TWIST score in HG T1 NMIBC as compared LG Ta and T1 NMIBC but higher scores were also prevalent in lower grades of Ta and T1 NMIBC. Among cases showing recurrences, consistent immunoexpression of TWIST was noted, unlike E-cadherin and β-catenin. None of the studies have previously explored the correlation of miRNA-200c expression with TWIST immunoexpression. TWIST overexpression was seen in all the aggressive histological variants. Overall, we found a strong inverse correlation between miRNA-200c expression and TWIST in MIBC, HG NMIBC and aggressive histological variants of BC which suggests that TWIST is one of the protein targets of altered miRNA-200c expression contributing to EMT in BC.

## CONCLUSION

The present study explores the relation of miRNA-200c associated regulation of different proteins implicated in EMT pathways in bladder cancer. Novel expression of TWIST in cases showing downregulation of miRNA-200c suggests it is one of the protein targets of altered miRNA-200c expression contributing to EMT and can serve as a promising diagnostic biomarker and a potential therapeutic target. However, more studies in larger cohorts with survival data analysis are needed to validate the role of EMT related biomarkers as potential targets for possible therapeutic intervention.

### Compliance with ethical standards

Informed consent was obtained from the patient. No animals were involved in the study.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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