ORIGINAL ARTICLE

Correlation of REG1A, Claudin 7 and Ki67 expressions with tumor recurrence and prognostic factors in superficial urothelial urinary bladder carcinomas

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ABSTRACT

Aım: Superficial urothelial urinary bladder tumors are neoplasms that frequently recur and have a potential for invasion and metastasis. REG gene family is composed of various acute phase reactants, lectins, antiapoptotic factors, and growth factors that are effective on pancreatic island cells, neural cells, and epithelial cells. REG1A and REG1B are two forms of the human REG1 gene. It is reported that they are expressed in several cancers and are correlated with the prognosis of the patient. Claudins are integral transmembrane proteins that interconnect cells. However, their role in human tumorigenesis is extremely controversial. The aim of this study is to evaluate the relationship of REG1A, claudin 7 protein expressions, and Ki67 proliferation index in superficial urothelial urinary bladder tumors with well-known parameters of prognosis and tumor recurrence, and also to clarify whether these parameters are independent prognostic factors or not. **Materials and Methods:** A hundred and eleven patients diagnosed with superficial urothelial carcinoma between 2011 and 2016 years in our hospital and followed up in our urology clinic were included in this study. The slides prepared from paraffin blocks were immunohistochemically stained with REG1A, claudin 7, and Ki67 antibodies. **Results:** REG1A showed positive staining in 37 (33%) and negative staining in 74 (67%) of urothelial tumors. Claudin 7 was positive in 24 (22%) and negative in 87 (78%) cases. REG1A expression showed a positive correlation with tumor stage and tumor recurrence; a high Ki67 proliferation index was positively correlated with tumor stage and grade. The loss of claudin 7 expression was related to tumor recurrence. Besides, tumors with REG1A expression and claudin 7 loss had a shorter survival independent of recurrence. **Conclusıon:** In urothelial tumors, REG1A expression and loss of claudin 7 might be significant markers of prognosis that predict tumor recurrence.

KEY WORDS: Claudin 7, prognosis, REG1A, urothelial tumors

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INTRODUCTION

Urinary bladder cancer is the second most common malignancy of urogenital system,[1] and it covers 90%–95% of primary urothelial tumors.[2-4] Tumor grade and stage are considered to predict prognosis and decide treatment in urothelial tumors. Fifty percent of the initially noninvasive tumors show recurrence, and 5%–10% progress to invasive carcinoma.[1,5] Superficial urothelial tumors are neoplasms that frequently recur and that have an invasion and metastasis potential. Therefore, factors that influence recurrence and progression are thoroughly investigated, and various results have been reported in the literature. Despite a consensus on the significance of certain factors, there is an ongoing requirement for novel parameters.

Ki67 proliferation index is an independent disease-free survival marker

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in urothelial carcinoma of the urinary bladder.[6] Also, in advanced metastatic urothelial cancers, it has a significant correlation with tumor metastasis, invasion, and recurrence.[7] Regeneration gene (REG) was initially described during the reproduction of complementary DNA (cDNA) archive obtained from pancreatic islands of a 90% depancreatized mouse.[8] REG gene family is composed of various acute phase reactants, lectins, antiapoptotic factors, and growth factors that affect pancreatic island cells, neural cells, and epithelial cells of the gastrointestinal system.[9,10] Till now, 17 REG family genes have been identified including REG1A and REG1B, which are the two forms of the human REG1 gene.[11] REG1 mRNA was first discovered in the pancreas and in less amount, in gastric mucosa and kidneys.[12] Relation between REG1A expression and prognosis has been investigated in stomach, colon, gall bladder, and esophagus cancers; however, its prognostic effect is under discussion. REG1A expression in urothelial cancers is occasionally studied.[13-18] Geng *et al*.[19] found that a diminished REG1α expression lessened tumor growth, migration, invasion, and angiogenesis. In patients with high levels of REG1α, earlier recurrence and inferior survival were observed. Claudins are transmembrane proteins that interconnect cells, of which, 24 members have been identified in human tissues and expression profiles of various claudin types are tissue- and organ-specific. Claudins' role in human tumorigenesis is extremely questionable. Especially in pathological conditions such as tumorigenesis, there is evidence that tight junctions alter the molecular constitution.[20-23] Data on claudin expression profile in various premalignant and malignant alterations suggest that claudins might not only be diagnostic but also prognostic markers.[24] However, data on claudin expression in urothelial carcinomas are limited.[19,25,26]

The aim of this study is, in superficial urothelial tumors, to evaluate the relationship of REG1A and claudin 7 protein expression as well as Ki67 proliferation index with well-known prognostic parameters and tumor recurrence, and also, to assess their roles as independent prognostic factors.

MATERIALS AND METHODS

Selection of patient group and methods

A hundred and eleven patients who were diagnosed and followed up by the Urology Clinic of our hospital and whose pathological archival data were available were included in this study (Ethics Committe Approval Number 1, Date: 24/04/2012). The patients were classified into two groups as "without recurrence, Group 1" (patients who had no lesion in at least 24 months after curative treatment, $n = 38$) and "with recurrence, Group 2" (patients with any lesion in the urinary bladder histopathologically approved as cancer, detected after ≥3 months from curative treatment, $n = 74$). "Survival independent of recurrence" was time from treatment initiation to detection of the first recurrence in the urinary bladder in "with recurrence" group and to last clinical evaluation in "without recurrence" group. Initial resection materials of all patients in the study group that were diagnosed as urothelial urinary bladder tumors were histopathologically re-examined. During the examination, paraffin blocks with sufficient tumor density were chosen for immunohistochemical (IHC) analysis. Cases with muscle invasion in recurrences, cases not followed-up, and cases without sufficient tissue for IHC analysis were excluded.

Immunohistochemical evaluation

A total of 3-micron slides were prepared, deparaffinized in 60°C incubator, and relevant steps were performed with the tissue microarray (TMA) technique (Leica Bond Max, Germany). To block endogen peroxide, the slides were placed into 0.5% hydrogen peroxide for 15 min. Then, the slides were incubated with rabbit polyclonal anti-REG1 alpha antibody (Abcam, Code ab47099, 1/400 dilution, UK); rabbit claudin 7 antibody (Spring, Code E10591, ready to use, CA); and liquid mouse monoclonal antibody Ki67 antigen (Leica, Code NCL-L-Ki67-MM1, 1/100 dilution, UK) for 30 min. Respectively, post-primary antibody, polymer antibody, and 3,3'-diaminobenzidine (DAB) mixtures (Leica; LOT 11776) were applied for 10 min; the slides were contrast-stained with Mayer hematoxylin and properly covered. As positive tissue control, urothelial carcinoma with positive staining for REG1A, colon adenocarcinoma for claudin 7, and tonsilla tissue for Ki67 were used.

For REG1A protein, strong cytoplasmic staining of >10% of tumor cells was considered positive (18). Claudin 7 positivity was evaluated with an immunohistochemical score (immunohistochemical score = percentage of claudin 7 positive tumor area × staining severity of tumor cells). Percentage of tumor positive area was scored between 0 and 100 (0 = no staining, 1 = 1%–25% staining, 2 = 26%–50% staining, 3 = ≥51% staining), and staining severity was scored between 0 and 3 (No staining = 0, mild staining = 1, moderate staining = 2, strong staining = 3). The results were 0 = negative, 1–2 = mild, 3–6 = moderate, and 7–9 = strong staining (23). Then, mild and moderate staining was considered negative and strong staining considered positive staining. Only, membranous staining was taken into account. For Ki67, <15% nuclear staining of tumor cells was considered negative and ≥15% positive.[27]

Statistical analysis

Prism 5.0 (Graphpad Software Inc.) program was used for statistical analysis of the data. To assess the variables, the unpaired *t*-test, Chi-square test, and McNamer test; to assess survival independent of recurrence, the Mantel–Cox test was used. $P < 0.05$ was considered statistically significant.

RESULTS

A hundred eleven patients (93 male (84%), 18 female (16%), and female/male ratio; 1/5.1) with primary urothelial urinary bladder carcinomas were included in the study. The mean follow-up of the patients' was 28.84 ± 1.05 (24–48) months in Group 1 and 19.25 ± 14.51 (4–56) months in Group 2. The mean age of patients was 62.18 ± 1.18 (31–94) years. Fifty two patients (47%)

were stage pTa, 59 were (53%) stage pT1. In 47 patients (42%), high-grade tumor and in 64 patients (58%), low-grade tumor were found [Table 1].

Correlation between REG1A, Claudin 7, Ki67 expressions, and tumor recurrence

The correlation of REG1A, claudin 7, and Ki67 expressions with tumor recurrence is investigated and summarized in Table 2. REG1A expression and claudin 7 loss were significantly correlated with tumor recurrence (P = 0.001, P = <0.0001), whereas Ki67 and tumor recurrence was not significantly related (P = 0.841).

Table 1: Clinicopathological characteristics of the groups

	Group 1 (n=38)	*Group 2 (n=73)*
Age (mean±sd)	57.58±2.27	64.58±1.28
Gender		
Male	27 (71%)	66 (90%)
Female	11 (29%)	7 (10%)
Stage		
pTa	23 (61%)	36 (49%)
pT1	15 (39%)	37 (51%)
Grade		
Low	23 (61%)	41 (56%)
High	15 (39%)	32 (44%)

sd: Standard deviation

Table 2: REG1A, claudin 7, and Ki67 expressions and tumor recurrence

	Group 1 (n=38)	*Group 2 (n=73)*	*P*
REG1A			
+	5 (13%)	32 (44%)	*P*=0.001
-	33 (87%)	41 (56%)	
Claudin 7			
+	19 (50%)	5 (7%)	*P*=<0.0001
-	19 (50%)	68 (93%)	
Ki67			
+	22 (58%)	40 (55%)	*P*=0.841
-	16 (42%)	33 (45%)	

REG1A expression

REG1A was positive in 37 (33%) and negative in 74 (67%) of urothelial tumors [Figure 1a]. The relation between REG1A expression and clinicopathological parameters is summarized in Table 3. There was a statistically significant relation of REG1A expression with pathological stage and gender (respectively, P = 0.044, P = 0.031) whereas not with histological grade and age (P = 0.840, P = 0.128).

Claudin 7 expression

Claudin 7 was positive in 24 (22%) and negative in 87 (78%) of urothelial tumors [Figure 1b]. The relation between claudin 7 expression and histopathological parameters is investigated and summarized in Table 3. There is a statistically significant relation between claudin 7 expression and gender (P = 0.003), whereas not with pathological stage, histological grade, and age (respectively, P = 1, P = 1, P = 0.823).

Relationship between immunohistochemical markers

There was a statistically significant relation between REG1A and Ki67 expressions (P = 0.001) [Table 4], and between claudin 7 and Ki67 expressions (P = 0.0001) [Table 4]. However, no statistically significant relationship was observed between REG1A and claudin 7 expressions (P = 0.0801) [Table 5].

Relation of survival independent of recurrence with REG1A, claudin 7, and Ki67 expressions

There was a significant difference between REG1A positive and negative groups [Figure 2a] and claudin 7 positive and negative groups [Figure 2b] regarding survival independent of recurrence (respectively, P = 0.0002 and P = <0.0001). However, no significant difference between Ki67 expression (+) and (-) groups was observed on that parameter (P = 0.8763) [Figure 2c].

Table 3: REG1A and claudin 7 expression and relation with clinicopathological parameters in urothelial carcinomas

	REG1A			*Claudin 7*		
	REG1A (+) (n=37)	*REG1A (-) (n=74)*	*P*	*Claudin 7 (+) (n=24)*	*Claudin 7 (-) (n=87)*	*P*
Age (mean±sd)	64.73±1.89	60.9±1.49	*P*=0.128	61.58±2.27	62.23±1.38	*P*=0.823
Gender						
Male	35 (95%)	58 (78%)	*P*=0.031	15 (63%)	78 (90%)	*P*=0.003
Female	2 (5%)	16 (22%)		9 (37%)	9 (10%)	
Stage						
pTa	14 (38%)	44 (59%)	*P*=0.044	13 (54%)	46 (53%)	*P*=1
pT1	23 (62%)	30 (41%)		11 (46%)	41 (47%)	
Grade						
Low	22 (59%)	42 (57%)	*P*=0.840	14 (58%)	50 (57%)	*P*=1
High	15 (41%)	32 (43%)		10 (42%)	37 (43%)	

sd: Standard deviation

Table 4: Relation of REG1A and claudin 7 expression with Ki67 expression in urothelial carcinoma

	REG1A (+) (n=37)	*REG1A (-) (n=74)*	*P*	*Claudin 7 (+) (n=24)*	*Claudin 7 (-) (n=87)*	*P*
Ki67 (+) (*n*=62)	23 (62%)	39 (53%)	*P*=0.001	14 (58%)	48 (55%)	*P*=0.0001
Ki67 (-) (*n*=49)	14 (38%)	35 (47%)		10 (42%)	39 (45%)	

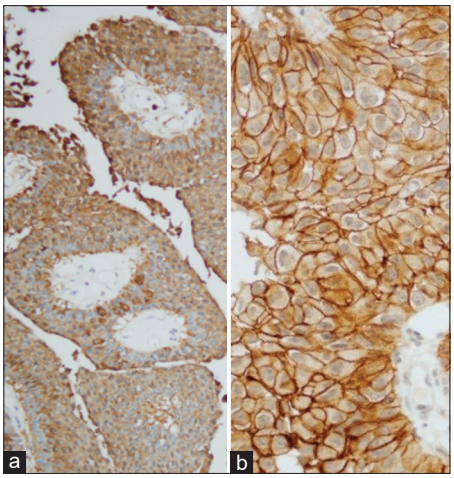

Figure 1: In urothelial carcinomas: (a) Positive cytoplasmic staining with REG1A (×200) (b) Strong membranous staining with claudin 7 (×400)

Table 5: Relation between REG1A and claudin 7 expression in urothelial carcinomas

	REG1A (+) (n=37)	*REG1A (-) (n=74)*	*P*
Claudin 7 (+) (*n*=24)	7 (19%)	17 (23%)	*P*=0.0801
Claudin 7 (-) (*n*=87)	30 (81%)	57 (77%)	

DISCUSSION

It is shown that REG1 has an important role in the regeneration of several tissues such as the stomach, pancreas, and kidney. In previous studies, REG1A expression was observed in various human cancer types such as gastric cancer, colorectal cancer, pancreas cancer, cholangiocarcinoma, and hepatocellular cancer.[28] In these studies, REG1A expression was positively correlated with a decrease in cancer differentiation. Kimura *et al*.[29] reported that in pulmonary adenocarcinoma and squamous cell carcinoma, REG1A expression was a poor prognostic factor. In a study of Yamagishi *et al*. including patients with gastric carcinoma, there was significant relation of REG1A expression with tumor stage and venous invasion, and they reported that REG1A was effective on tumor progression because of trophic and/or anti-apoptotic effect on gastric cancer cells.[30] As staging is an important factor in urothelial carcinomas for treatment and prognosis,[3,31] Geng *et al*.[18] investigated REG1A expression in urothelial tumors, but found no relation between REG1A expression and stage in 110 patients with superficial urothelial cancers. However, in our study, in superficial urothelial tumors, REG1A expression was higher in stage pT1 tumors than stage pTa tumors. This result, similar to the study by Yamagishi *et al*. supports the trophic and antiapoptotic effect of REG1A on tumor cells.

Dhar *et al*. evaluated REG1A expression in a study including 68 gastric adenocarcinomas, and found that REG1A expression was higher in poorly differentiated adenocarcinomas, and REG expression was negatively correlated with cellular differentiation level.[13] On the contrary, Geng *et al*.[18] did not find a relation between REG1A expression and histological grade in superficial urothelial tumors. Similarly, in our study, we did not observe any significant relation between REG1A expression and histological grade.

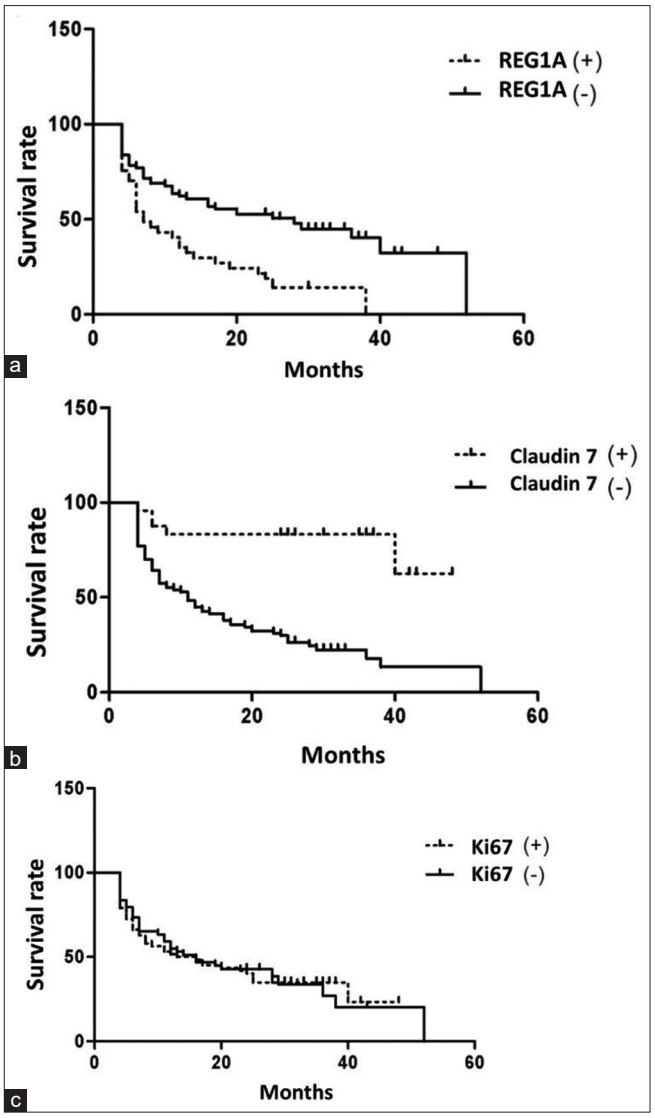

Figure 2: Relation between survival independent of recurrence and (a) REG1A expression, (b) claudin 7 expression, and (c) Ki67 expression

It was shown that REG1A expression might be a poor prognostic marker for surgically treated colon, pancreas, and stomach cancers.[13-16,28,32,33] Similar to gastrointestinal tumors (like stomach, colon, biliary tractus cancers), REG1A expression was related to poor prognosis in a study including 150 breast cancers. Besides, REG1A-positive patients were more likely to be HER2 positive than REG1A negative ones, and REG1A-negative group had better disease-dependent survival after surgical intervention than the positive group.[34] In a study on urinary bladder cancers, patients with REG1A expression had more tumor recurrence than patients without REG1A expression.[18] Similarly, in our study, REG1A expression was significantly higher in the group with recurrence than without recurrence. Therefore, REG1A might be a factor that predicts tumor progression. In our study, the

relationship between survival independent of recurrence and REG1A was also evaluated. The fact that REG1A positive patients have a shorter survival independent of recurrence supports its significance as a prognostic marker in urothelial urinary bladder tumors.

Geng *et al*.[18] investigated the relation between REG1A expression and proliferative activity in urothelial tumors and found no significant relation. However, in gastric carcinomas, cases with REG1A expression had a higher proliferation index.[14,35,36] In our study, the relation between REG1A expression and Ki67 proliferative index was investigated in urothelial carcinomas, and similar to gastric carcinomas, tumors with REG1A expression were found to have a higher Ki67 proliferation index. Therefore, REG1A expression might be a poor prognostic marker in urothelial carcinoma.

Claudin and occludin are two transmembrane proteins that connect the membranes.[37,38] Although both are involved in tight junctions, claudins have a more active role and directly affect the selective permeability of epithelial cells.[39] It is not clear how higher claudin levels affect neoplastic progression. Presumably, aberrant expression or upregulation of claudins modify normal tight junctions and lead to neoplastic processes. *In vitro* study of Furuse *et al*.[40] showing that claudin 2 upregulation in Madin-Darby Canine Kidney (MDCK) cells led to the decrease in tight junction function, supports this theory. A study in gastric biopsies that investigate claudin 7 expression showed that claudin 7 expression was 30% in metaplasia, 80% in dysplasia, and 70% in adenocarcinoma, and reported that claudin 7 expression was effective on tumor progression and tumorigenesis.[41] There are several studies suggesting that claudin expression profiles in premalignant and malignant lesions might be used not only as diagnostic but also as prognostic markers.[24] Decreased claudin 7 expression in prostatic adenocarcinomas is found to be related with high tumor grade and decreased claudin 1 expression with high tumor grade and recurrence whereas claudin 3 expression is related with advanced tumor stage and recurrence, and claudin 4 expression with advanced tumor stage.[42] In an *in vitro* study by Hoevel *et al*.[43] Claudin 1 re-expression led to apoptosis in breast carcinoma which clarified another mechanism of claudin loss leading to tumor progression. It is well known that tight connections of epithelium are significant for urothelial integrity, and inhibition of urothelial carcinoma recurrence. Törzsök *et al*.[44] in a study including 86 urothelial carcinoma and nontumoral urothelium, showed less claudin 7 expression in high-grade tumors than low-grade tumors, however, did not investigate its relation with stage. In a study with 129 upper urinary system urothelial carcinoma, claudin 7 and occludin levels were not related with stage and histological grade.[26] We also did not find a correlation of claudin 7 expression with stage and histological grade in our study.

In a study evaluating claudin 1 expression in colonic cancers, low levels of claudin expression were related to high tumor grade, recurrence, and shorter survival, and authors reported that loss of claudin 1 expression led to disease progression and poor differentiation.[45] Melchers *et al*.[46] found that in oral squamous cell carcinoma, claudin 7 loss could be used to identify patients with increased regional recurrence risk. In our study, we evaluated the relation of claudin 7 expression with recurrence and recurrence-independent survival. In the group with recurrence, claudin 7 expression was lower, and the patients with a lower claudin 7 expression had a shorter recurrence-independent survival. So, claudin loss which is a result of loss of tight junctions between cells might be a poor prognostic marker. Any significant relation between claudin 7 and REG1A expression was not found.

Studies showed that Ki67 expression in urothelial carcinomas was related to tumor grade, muscle invasion, stage, and recurrence in urothelial carcinomas.[47] In our study, when we evaluated the relation between Ki67 and claudin 7, it was found that as the Ki67 proliferation index increased, claudin 7 expression decreased, and a negative correlation was observed.

CONCLUSION

Our results showed that REG1A expression, claudin 7 expression loss, and high Ki67 proliferative index might be poor prognostic markers in urothelial tumors. As recurrence-independent survival of REG1A positive, claudin 7 negative patients were shorter, REG1A and claudin 7 might be prognostic parameters in urothelial tumors of the urinary bladder.

As well, in the group with recurrence, REG1A expression and claudin 7 expression loss were significantly higher than the group without recurrence and therefore, REG1A and claudin 7 might be important factors that predict tumor progression.

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Conflicts of interest
There are no conflicts of interest.

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