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Molecular evaluation of cytokeratin 20 mRNA expression of transitional cell carcinoma cases

Tranzisyonel hücre karsinomlu olgularda sitokeratin 20 mRNA ekspresyonunun değerlendirilmesi

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Summary

Aim: Cytokeratins are multigenic proteins which consist of twenty polypeptides and are formed by intermediate filament of epithelial cells. The quantitatively stated difference in the level of Cytokeratin 20 mRNA expression is considerable in early detection of bladder carcinoma. This study aimed to evaluate the value of Cytokeratin 20 mRNA expression in tumor prognosis and recurrence.

Material and Methods: Thirty-four cases [Ta(2),T1(19),T2(11),T3(1),T4(1)] diagnosed as transitional cell carcinoma and 10 healthy volunteers as control group were recruited. The quantification of Cytokeratin 20 mRNA expression was performed by Reverse Transcriptase PCR.

Results: The average relative ratio of Cytokeratin 20 mRNA expression was 11.91±50.01 in 34 cases with transitional cell carcinoma and 0.07±0.12 in control cases. The highest expression of Cytokeratin 20 mRNA was found in T1/G2 as 25.62. No significant correlation was found between the Cytokeratin 20 mRNA expression and tumor grading. The results were determined according to the cut-off, specificity and sensitivity were 69.56% and 67.65%, respectively with positive, negative and total diagnostic values as; 85.19%,59.26% and 78.00%. The area under the Receiver Operating Characteristic curve was 0.917 ($p<0.0001$). The cut-off value of Cytokeratin 20 mRNA expression that had the sensitivity (82.4%) and the specificity (81.2%) was determined as 0.1224.

Conclusion: We suggest that the combination of increased Cytokeratin 20 mRNA expression and pathological tumor classification is an indicator of more aggressive tumor behavior and the cases should be monitored more closely. The evaluation of Cytokeratin 20 mRNA expression in urine samples will be quite useful in the diagnosis of increased Cytokeratin 20 mRNA expression in the cases with papilloma or tumor.

Key Words: Cytokeratin 20, Bladder Cancer, mRNA expression, Real Time online RT-PCR, Transitional Cell Carcinoma.

Özet

Amaç: *Sitokeratinler 20 polipeptitten meydana gelen ve epitel hücrelerinin intermediyal filamentlerinden şekil alan multigenik proteinlerdir. Sitokeratin 20 mRNA ekspresyon seviyesinin mesane kanserinin erken tanısında kantitatif farkı çok önemlidir.Bu çalışmada tümör prognozu ve rekürrensinde Sitokeratin 20 mRNA ekspresyonunun güvenirliliğinin araştırılması amaçlanmıştır.*

Yöntem ve Gereç: *Transizyonel hücre karsinomu tanılı 34 olgu [Ta(2),T1(19),T2(11),T3(1),T4(1)] ve kontrol olarak 10 sağlıklı gönüllü çalışmaya alınmıştır.Sitokeratin 20 mRNA ekspresyonunun kantifikasyonu Revers Transkriptaz PCR ile gerçekleştirilmiştir.*

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Bulgular: *Sitokeratin 20 mRNA ekspresyonunun ortalama rölatif oranı transizyonel hücre karsinomu tanılı 34 olguda 11.91±50.01 ve kontrol grubunda 0.07±0.12 olarak bulundu. En yüksek Sitokeratin 20 mRNA ekspresyon değeri 25.62 olarak T1/G2 grubunda görüldü.Sitokeratin 20 mRNA ekspresyonu ve tümör derecesi arasında anlamlı bir korelasyon bulunmadı.Pozitif ve negatif olgu sonuçları cut-off,spesifite ve sensitivite, metodun pozitif,negatif ve total tanı değerleri sırası ile %69.56, %67.65, %85.19, %59.26, %78.00 olarak hesaplandı. ROC (Receiver Operating Characteristic) eğrisi altındaki alan 0.917 (p<0, 0001) olarak bulundu.Sensitivitesi %82,4 ve spesifitesi %81,2 olan Sitokeratin 20 mRNA ekspresyonunun cut-off değeri 0,1224 olarak hesaplandı.*

Sonuç: *Artmış Sitokeratin 20 mRNA ekspresyonu ile birlikte tümörün patolojik sınıfı agresif tümör davranışının bir indikatörü olarak gözlenmiş ve bu hastaların daha yakından izlenmesi gerekliliğini ortaya koymuştur.İdrar örneklerindeki Sitokeratin 20 mRNA ekspresyonunun değerlendirilmesinin, papilloma veya tümörlü hastaların tanısında yardımcı olabileceğini düşünmekteyiz.*

Anahtar Kelimeler: *Sitokeratin 20, Mesane kanseri, mRNA ekspresyonu, Real Time Online RT-PCR, Tranzisyonel Hücre Karsinomu.*

Introduction

Bladder cancer comprises 3% of cancers among women and 7% among men and is the second most common malignancy of the genitourinary system and the fourth most common cause of death from cancer in men, the eighth in women. Annually, about 66.000 (1) and 54.500 (2) new cases are diagnosed as bladder cancer in European countries in the United States respectively and most of them are diagnosed while the disease is superficial and have an excellent survival rate (3).

Bladder malignancies can be treated using different approaches such as transurethral resection for superficial tumors, intravesical chemotherapy and radical cystectomy for non-metastasized tumors, or systemic chemotherapy for locally advanced or metastasized tumors (4). Cystoscopy is the gold standard for diagnosis and routine follow up of the recurrence of bladder cancer. Since this method is considered to be invasive, investigators are still in search of unexpensive, non-invasive and reliable tests. Those tests are desired to provide rapid results as well as have high sensitivity and specificity.

Cytokeratins are formed by the intermediate filament of epithelial cells. They are multigenic proteins which consist of twenty different polypeptides. Cytokeratin 20 (CK 20) is a member of the intermediate filament protein family involved in cell structure and differentiation. The quantitative difference of Cytokeratin 20 mRNA (CK 20 mRNA) expression has been reported to be an important biological marker for the early detection of bladder carcinoma (4).

In this study, we aimed to evaluate the reliability of CK 20 mRNA expression in the development and recurrence of bladder tumors and to investigate the difference in the expression between the tumorous and normal urothelial cells. Highly sensitive real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was applied using the “LightCycler” to investigate CK 20 mRNA expression in voided urine from cancer patients. Exact quantification results of the mRNA expression in urine were then compared with the CK 20 mRNA expression pattern of urine from healthy volunteers.

Material and Method

Thirty-four bladder transitional cell carcinoma patients (2 Female and 32 Male) and 10 healthy volunteers (4 Female and 6 Male) were included in the study. Histopathological classification of tumor stage and grades of the cases were classified according to the TNM classification of the International Union against Cancer (5).

Fresh voided morning urine samples (10-50 ml) were collected from the patients and control group and 50 µl total RNA was extracted from urine samples using the RNA extraction kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions.

Twenty microliters (µl) complementary DNA (cDNA) was synthesized from 10 µl total RNA (1-5 µg final concentration for each case) with a reverse transcription kit (Reverse Transcriptase Master Mix, Roche Applied Science, Mannheim, Germany) in a total volume of 10 µl master mix.

The quantification of CK 20 mRNA expression was performed by Reverse Transcriptase PCR (RT-PCR) with LightCycler CK 20 mRNA Quantification Kit (CK20 Detection Mix, Roche Applied Science, Mannheim, Germany) in a total volume of 18 µl master mix with the addition of 2 µl cDNA. During PCR, a 124-bp fragment of CK20 encoding mRNA was amplified from the cDNA.

Relative CK 20 mRNA expression was evaluated by using real-time online LightCycler Quantitation Software (LCQuant, Roche Applied Science, Mannheim, Germany). The CK 20 mRNA copy number was calculated based on the crossing point (the point at

which PCR amplification begins its exponential phase) and it was divided to the housekeeping gene porphobilinogen deaminase (PBGD) mRNA copy number. The Relative ratio was adjusted according to the ratio of CK 20 mRNA by dividing PBGD in calibrator mRNA obtained from CK20 positive HT-29 colorectal cancer cell line.

Statistical comparisons between means were evaluated by one way analysis of variance (ANOVA) and the Receiver operating characteristic (ROC) curve analysis was defined to determine optimal cut-off values for CK 20 mRNA expression. All statistical analysis was performed with SPSS v15.0 (SPSS Inc., Chicago, IL, USA). A $p<0.05$ was assumed statistically significant.

Results

The mean age of the 34 cases diagnosed with transitional cell carcinoma (TCC) in the Urology Department of Ege University Medical Faculty was 67.59±10.44 years, while it was 52.9±7.69 years in the control group.

The stages and grades of the 34 cases with TCC in our study group were as follows; Ta/G1 (1 case), Ta/G2 (1 case), T1/G2 (16 cases), T2/G2 (3 case), T1/G3/ (3 cases), T2/G3 (8 cases), T3/G3 (1 case) and T4/G3 (1 case).

The adjusted average relative ratio of CK 20 mRNA expression in 34 TCC series was 11.91±50.01. The median value was as 0.50 (range 0.005-305.41). The average relative ratio of CK 20 mRNA expression in the control group was 0.07±0.12. The median value was 0 (range 0-0.35). The relative ratio (RR), stage and grade of cases with TCC were given in (Table-1).

The average of relative ratio (0.07±0.12) plus two-fold standard deviations (0.31) of the control group was considered as the cut-off value. Twenty two cases were found within the upper borderline limits and evaluated as positive for CK20 mRNA expression.

The highest expression of CK20 mRNA was found in T1/G2 group as 25.62 (Table-2).

No significant correlation was found between CK20 mRNA expression and tumor grading ($p>0.05$). However, low CK20 mRNA expression was observed in high grade and high stage cases.

The positive and negative results were determined according to the cut-off, and the specificity and sensitivity of the method were 69.56 % and 67.65 %, respectively, with positive, negative and total diagnostic values of 85.19%, 59.26%, 78.00%.

The Receiver Operating Characteristic (ROC) curve was constructed from the sensitivity and specificity values. The area under the ROC curve (AUC) was 0.917 ($p<0.0001$). By using ROC analysis; the cut-off value of CK20 expression that had the highest sensitivity (%82.4) and the specificity value (%81.2) was determined as 0.1224 (Figure-1).

Table 1. Clinical, gene expression and demographic features of the cases.

Case	Sex	Age	Stage	Grade	CK20 RR
1	M	55	T1	G2	0.00574
2	M	44	T3	G3	0.00598
3	M	61	T2	G3	0.01889
4	M	76	T2	G3	0.01970
5	M	75	T1	G2	0.08106
6	F	57	T1	G2	0.11860
7	M	73	T4	G3	0.12315
8	M	84	T1	G3	0.19283
9	M	65	T2	G3	0.23754
10	M	61	T2	G3	0.24061
11	M	82	T2	G3	0.29664
12+	M	75	Ta	G2	0.32110
13+	M	75	T2	G2	0.32369
14+	M	78	T2	G2	0.48891
15+	M	64	T2	G2	0.50611
16+	M	67	T1	G2	0.52294
17+	M	80	T1	G3	1.37873
18+	M	59	T1	G2	1.63773
19+	M	46	T1	G2	2.49738
20+	M	61	T2	G3	2.83191
21+	M	53	T1	G2	2.95051
22+	M	83	T1	G2	3.32635
23+	M	52	T1	G3	3.90000
24+	M	59	Ta	G1	4.23480
25+	M	71	T1	G2	4.30375
26+	M	79	T1	G2	5.01571
27+	F	74	T1	G2	6.02583
28+	M	69	T2	G3	9.49015
29+	M	65	T2	G3	10.06826
30+	M	74	T1	G2	13.18848
31+	M	72	T1	G2	13.22526
32+	M	75	T1	G2	24.06143
33+	M	67	T1	G2	27.52294
34+	M	67	T1	G2	305.41010
	Mean	**67,59**			**10.25887**
	SD	**10,44**			**46.48719**

Table 2. CK20 mRNA expression by grade and stage.

		CK20 Relative Ratio	
Stage / Grade	**Case**	**Mean**	**Standard Deviation**
Ta	2	2.28	2.77
G1	1	4.23	
G2	1	0.32	
T1	19	21.86	69.12
G2	16	25.62	75.10
G3	3	1.82	1.89
T2	11	2.23	3.82
G2	3	0.44	0.10
G3	8	2.90	4.35
T3 /G3	1	0.05	
T4 / G3	1	0.123	

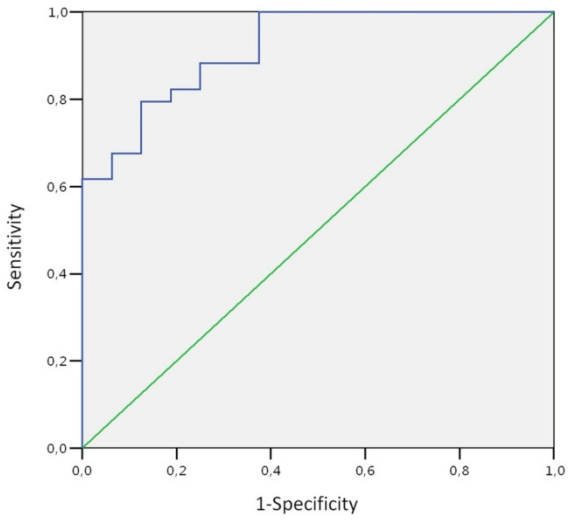

Figure 1. The relative ratio of CK 20 mRNA expressions of the cases according to stages.

Discussion

Cytokeratins are divided into two groups: Type I cytokeratins, between CK9 and CK23 and Type II cytokeratins, between CK1 and CK8 (6) and they are considered as important identifiers of the differentiation in the epithelium. In the cases with nacre diseases or epithelial tumor hyper-proliferation, CK6 and CK16 are expressed. CK 20 is expressed in gastric, intestinal epithelium, urothelium, epithelium and merkel cells, and can be detected in colon, gastric, pancreatic adenocarcinomas and transitional cell carcinomas (7).

Expression of CK 20 was determined by immunohistochemical or RT-PCR studies in the exfoliated fresh voided morning urine cells by different groups (8, 9, 10, 11).

Dysregulation of CK20 expression is established as an early event in urothelial tumor development in especially high grade dysplasia (12).

By using the RT-PCR technique, Buchumensky et al. reported that TCC cells can be fixed by the CK 20 mRNA method even if at doses as low as 1/1.000.000. They supported the CK 20 mRNA test as advantageous in the determination of low grade cancers (13).

In their study, Rotem et al determined CK20 expression in 86.7% of TCC cases and just 3.3% of healthy volunteers (14).

In a study by Eissa et al. with the voided urine samples, they found the positivity rates for CK 20 mRNA of the control, benign and malignant groups as 0 (0%), 1/34 (2.9%) and 51/63 (82.3%), respectively (chi-square = 98.83, P = 0.000) (15).

CK 20 mRNA expression was detected in all TCC cases (100%) and 4 of our control group (40%). Determination of expression can be actualized in normal cells as in the study of Southgate et al (16). However, because the quantitative expression is compared with the CK 20 mRNA expression in HT29 colorectal cell line (calibrator RNA), the existence of the expression is not expounded as positive but the cases above the cut-off value are accepted as positive. In this study, the cut-off value is 0.31 and the expression is three times less than calibrator RNA. The cases above this value are evaluated as positive.

Harnden et al found that, if CK 20 expression was initially negative, no tumor recurrence was developed in a 5-year retrospective follow-up series of superficial tumors. They hypothesized that tumors occur or recur once CK 20 expression is deregulated or increased (12).

In tumor tissue and urine of TCC patients, CK 20 mRNA expression was expressed in higher levels than normal urine samples or in normal urothelial tissue. Christoph et al reported that when compared with specimens from normal tissue or urine, CK 20 expression was about 8 times higher in tumor tissue and 13 times higher in exfoliated cells than urine of tumor patients. When normal and tumor patients' urines are compared, a statistically significant difference between CK 20 mRNA expressions was determined and they proposed that this difference could help to detect bladder cancer at an early stage or earlier during follow-up (4).

In this study, CK 20 mRNA expression was found to be increased 33 times when compared to normal urine samples in the cases with TCC. In 47% of our cases included in tumor group (T1), an invasion is observed to the sub-epithelium connective tissue and CK 20 mRNA expression in this group was found to be increased 73 times compared to the control group and 22 times for the calibrator RNA group.

In a study by Christoph et al, it is shown that CK 20 mRNA expression is significantly lower in Ta than T1 tumors. In some Ta tumors, CK 20 mRNA expression could be determined in spite of being low. In addition, they reported that CK 20 mRNA expression is lower in high stage tumors such as T2 or higher stages tumors (4).

Analogously to the article, in our study the highest expression was observed in the T1 group. In light of our data, we are of the opinion that high CK 20 mRNA expressions are especially descriptive in the T1 tumor phase. As was seen in the study by Christoph et al, in our study, while CK 20 mRNA expression was determined in T2 and higher grade tumors, their

expression levels were in lower levels to the T1. As Harnden et al (12) proposed, in high grade tumors dysregulation of CK 20 mRNA expression was not observed and this data correlated to the study by Christoph et al. (4)

Although there was no correlation between tumor grade and CK 20 mRNA expression in our study, Rotem et al, determined a strong correlation between CK 20 mRNA expression and tumor grades. They found grade 3 and 4 tumors positive (100%) in terms of CK 20 mRNA expression (14).

Inoue et al determined a correlation between tumor grade (G1/G2 versus G3) and CK 20 mRNA expression in tissue samples and declared that poorly differentiated tumors are more aggressive and have greater recurrence rates than differentiated tumors (9).

While a correlation was observed between CK 20 mRNA expression in G1 and G2 cases, CK 20 mRNA expressions in G3 cases were low despite the increase of the grade. Since follow-up cases are a great part of G3 cases, we consider that CK 20 mRNA expression of this group was found to be low as a consequence of the therapy that the patient had. According to the threshold value that we determined, CK 20 mRNA expression with mean ± 2 standard deviation in the control group, specificity of our study was detected as 69,56%, the sensitivity was detected as 67,65% and total diagnostic value was found to be 78,00%.

In a group of 62 cases, Eissa et al found the overall sensitivity and specificity in benign and malign groups to be 82.3% and 98.8% respectively for CK 20 mRNA (15).

Buchumensky et al found an overall sensitivity of CK 20 expression by using RT-PCR versus regular cytology in malignant urothelial epithelium 91% and 56% respectively (13). Southgate and Harnden reported that CK 20 is expressed in vivo in superficial normal urothelium and proposed that tumor occurrence or recurrence cannot be concluded from just the detection of CK 20 in the urine (16).

Along with having partially lower sensitivity and specificity values according to the values in the work by Eissa, (17), these values were detected as statistically significant in ROC analysis ($p<0,005$). According to the ROC analysis, as we accept the cut-off value of relative ratio of CK 20 mRNA expression as 0.1224, the sensitivity and specificity of our method were determined to be 82.4% and 81.2%, respectively. Additionally, urinary CD44 was investigated as a second marker, the positivity rate of CD44 was found significantly associated with urine cytology. They suggested that urinary CD44 and CK20mRNA had higher sensitivities compared to voided urine cytology (17). Similar results were found in Guo's studies (18). New urine markers survivin and mucin combined with CK20. Pu et al found that the sensitivity and specificity were 90.4 and 94.7% for survivin, 82.6 and 97.4% for CK-20, 62.6 and 94.7% for mucin and 46.0 and 100% for voided urine cytology. The combined sensitivity of voided urine cytology with the three biomarkers together was higher than either the combined sensitivity of voided urine cytology with one of the biomarkers than that of the biomarker alone (19). It can be concluded that these sensitivity and specificity values are in an acceptable range for a diagnostic test. However, because of the insufficient number of cases, the sensitivity and specificity values of grades could not be calculated since they do not reflect the actual levels.

In addition, there are several studies regarding to the application of our method as a diagnostic test in other cancer types with high CK 20 mRNA expressions.

In a study by Chausovsky, expression of CK 20 mRNA was determined in the blood cells and 14 of the 22 cases with metastatic colon carcinoma found to be CK 20 positive (sensitivity of 63.6% and specificity of 92.3%), in a group of 28 cases with pancreatic carcinoma, 22 showed positive CK 20 expression, and 12 of 18 patients with gastric carcinoma showed positive CK 20 expression. Both 12 of 13 patients with metastatic lung carcinoma and 12 of 13 patients with colonic carcinoma with no known metastases were negative and in all 22 patients with no known malignant diseases, CK 20 results were found negative (20). CK 20 mRNA expressions were not evaluated in blood samples of our cases.

As the study of Chausovsky (20) pointed out, we think that CK 20 mRNA can be a potential tumor marker in colon, gastric and pancreas cancers. We think that it might contribute to the analysis of CK 20 mRNA expression in peripheral blood of the cases with bladder cancer.

In conclusion, a low or high level of CK 20 mRNA expression may be a potential marker for the diagnosis and follow-up of bladder cancer cases. In addition, this method may extend the range for the cystoscopy follow-up period, which is still being used as a standard reference for monitoring bladder cancer.

We suggest that the combination of increased CK 20 mRNA expression and pathological tumor classification is an indicator of more aggressive tumor behavior and the cases should be monitored more closely. The evaluation of CK 20 mRNA expression in urine samples will be quite useful in the diagnosis of increased CK 20 mRNA expression in the cases with papilloma or tumor.

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