

Original Research Article

Role of HEF1 Gene Expression in Prognosis of Urinary Bladder Transitional Cell Carcinoma

Athraa Falah Hasan^{1*} Alaa Salah Jumaah²

¹ College of Medicine, University of Babylon, Hilla, IRAQ

² College of Medicine, University of Kufa, , Kufa, Najaf, IRAQ

*E-mail: athraaashimerry@yahoo.com

Accepted 27 March, 2016

Abstract

Urinary bladder carcinoma is a common malignant tumor of the urogenital system worldwide. In 2015 ; it is the fourth most common cancer in men in the United State, while in Iraq it is one of the ten most common cancers in 2011. Human enhancer of filamentation 1 (HEF1) is a multidomain scaffolding protein of the Cas family; it is also an integral player in normal and pathological cell biology. The HEF1 protein has been implicated in the regulation of cell polarity, adhesion, motility, and invasion in multiple cell types. The main objective of the current study is to analyze HEF1 gene expression levels in urothelial carcinoma specimens and to study the impacts of HEF1 gene as genetic factors that contribute to development and prognosis of bladder cancer. Sixty samples of malignant bladder tumors as well as 60 samples of non-tumorous bladder tissues were investigated. Ages of patients were (62.95±12.839 s.d.) year. Total mRNA was extracted from FFPE blocks by using a specific kit. HEF1 gene expressions were estimated by using real-time PCR. Results were normalized to GAPDA gene as housekeeping gene. The gene expression data were analyzed in relevance to the patient's information obtained. Several statistical analyses were applied to analyze the data and found that the expression folds of HEF1 gene were found to be 11.219 folds in malignant bladder tumors in relation to non-tumorous bladder tissue. HEF1 genes were observed to be expressed excessively in high grade and advanced stage tumors which indicate that HEF1 gene may represent a novel bladder tumor marker with prognostic significance that could be introduced in plans of bladder cancer management.

Key words: urothelial carcinoma, HEF1 gene, Real time PCR

الخلاصة

يعتبر سرطان المثانة من اشهر أنواع الأورام السرطانية التي تصيب الجهاز البولي. ويعتبر السرطان الرابع الاكثر شهرة بين الرجال في الولايات المتحدة في 2015. وفي العراق فانه يعتبر واحد من أهم عشرة امراض سرطانية في 2011. وان بروتين التسقيل المتعدد المجالات HEF1 من عائلة CAS يلعب دورا تكامليا في علم الخلية الحيوي الطبيعي والمرضي. تهدف الدراسة الحالية الى تحليل التعبير الجيني لل HEF1 في انسجة سرطان المثانة لمعرفة تأثير هذه الطفرات على نشوء السرطان وتطوره لذلك تم دراسة 60 نسيج يعود لمرضى سرطان المثانة فضلا عن 60 نموذج لأنسجة المثانة الغير مصابة بالسرطان . كانت اعمار المرضى مساو الى 62.95 ± 12.839 s.d. سنة. تم استخلاص ال RNA من الأنسجة المدروسة ثم تحليلها بواسطة تفاعل البلمرة المتسلسل الكمي وتمت معايرة النتائج بالنسبة للتعبير الجيني لل HEF1 نسبة الى جين GAPDA بوصفه جين سيطرة داخلي. تم تحليل التعبير الجيني نسبة الى المعلومات المختلفة للمرض بواسطة عدة تحاليل احصائية. دلت

النتائج على زيادة تعبير جين HEF1 في الأنسجة الورمية بمقدار 11.219 مرة وكان التعبير الاكثر وضوحا هو في الاورام العالية التمايز والمتقدمة في مرحلة المرض. لذلك يمكن اعتبار جين ال HEF1 بوصفها كدالة ورمية سرطانية مثالية للاستدلال على تقدم المرض ويمكن اعتماده في خطط معالجة هذا الداء.

الكلمات المفتاحية: سرطان المثانة الانتقالي, جين ال HEF1 , تفاعل البلمرة المتسلسل الكمي.

Introduction

Bladder cancer is one of the most common cancers of urogenital system. Transitional cell carcinoma (Urothelial carcinoma) represents approximately 90% of all primary neoplasms of bladder [1]. Transitional cell carcinoma has become a common disease worldwide, for example, in the United States there is approximately; 74,000 new cases (56,320 in men and 17,680 in women) and 16,000 deaths (11,510 in men and 4,490 in women) and it is number 4th among most common cancer in men, after prostate, lung, and colorectal cancer and number 11th in women in American cancer society 2015 [2]. While in Iraq, urothelial carcinoma represents the fifth most common cancer after carcinoma of breast, lung, leukemia and brain; and it is the second most frequent cancer in males and the tenth one in females according to Iraqi cancer registry [3].

Bladder cancer has several known risk factors, age is the major risk factor. The median ages of males and females diagnosed with BC are 72 and 74 years, respectively [4]. Cigarette smoking is another most important risk factor for urothelial carcinoma, accounting for approximately 50% of cases [5]. Other risk factors include many chemicals (aniline dyes and aromatic amines) [6], urinary tract infection, chronic irritation [7], and exposure to pelvic radiation [8].

At presentation, approximately 70% of urothelial carcinoma are non-muscle-invasive (stage Tis, Ta, T1) and 30% muscle-invasive (stage T2, T3, T4). 50-70% of the non-muscle-invasive neoplasms will recur despite transurethral resection and intravesical immunotherapy or chemotherapy [9]. One-third of recurrent cancers may demonstrate progression of tumor into a higher grade and/or high stage of disease. Fifty percent of muscle-invasive tumors of those treated locally for invasive tumors will relapse with metastatic disease within 2 years of treatment. These data reflect the malignant

potential and heterogeneous nature of urothelial carcinoma of the bladder [10]. Bladder carcinoma requires lifelong surveillance, once diagnosed because of this natural history of recurrence and progression. Really, this management contributes to bladder carcinoma carrying the highest cost from diagnosis to death of all cases, ranging from \$96,000 to \$187,000 (2001 values) in the United States [9]. Consequently, there is a need for an accurate marker of disease in order to decrease the cost associated with surveillance. An accurate marker would also have the added benefit of improving quality of life by potentially minimizing the number of invasive endoscopic evaluations [9].

Human enhancer of filamentation 1 (HEF1), is a multidomain scaffolding protein of the Cas family [11,12]. The HEF1 protein was implicated in the regulation of cell process including adhesion, polarity, motility, and invasion in several cell types [13]. It acts as a scaffold protein and belongs to a family of Crk-associated substrates that regulates protein complexes controlling differentiation and invasion of cancer [14].

Materials and Methods

Sixty cases of paraffin embedded tissue (14 females and 46 males) with the urothelial carcinoma were included in this retrospective study, their ages were ranging from 23 to 90 years. Sixty specimens of non-tumorous bladder lesions were considered as control group. Histologically, the tumors were classified, based on a consensus reached at a conference by International Society of Urological Pathology (ISUP) in 1998 and adopted by the WHO in 2004 [15], into 25 high-grade and 35 low-grade papillary urothelial carcinoma. Tumors were staged according to the American Joint Committee of 2002 TNM classification was updated in 2009(7th version) [15], into 7 cases stage Ta, 24 cases stage T1 and 29 cases stage T2.

Total RNA (total ribonucleic acid) was extracted from tissues using (TRIzol® reagent kit. Bioneer. Korea) according to the manufacturer's instructions, and the concentration was determined by 260/280 nm absorbance using a Nanodrop spectrophotometer (Thermo Scientific, U.K.). For removing the trace amounts of contaminating genomic DNA, the extracted total RNA were treated with DNase I enzyme by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions. Then we used the M-MLV Reverse Transcriptase kit (Bioneer. Korea) containing random hexamer primer to synthesize cDNA from total RNA. Using mRNA molecule as a template, reverse transcriptase synthesizes a single-stranded DNA in the presence of random hexamer primer (annealing to total RNA). The first new strand DNA molecule can then be used as a template for double-stranded DNA synthesis. The cDNA (complementary deoxyribonucleic acid) was then subjected to real-time polymerase chain reaction with specific primers for HEF1. A 5 µL cDNA, 2.5 µmol/L forward primer and 2.5 µmol/L reverse primer 2.5 Taq Man probe 2.5 DEPC water were added to Accu Power® Plus Dual Star™ qPCR Pre Mix (Bioneer. Korea) in a total volume of 50 µL. RT reaction was done as follows: denatured at

95°C for 5 min, followed by 95°C for 20 min and 60°C for 30 seconds. The sequence of the forward primer for HEF1 was 5'- ATCTTGGCCATCAACAAGCC -3', and that of the reverse primer was 5'- TGCGTTGGTGTGATGGTTG -3'. The sequence of the primers used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was 5'- ACGACCACTTTGTCAAGCTC -3' (forward) and 5'- TTCCTCTTGTGCTCTTGCTG-3' (reverse); (Bioneer company, Korea). The relative amount of FOXM1 messenger RNA (mRNA) to GAPDH was calculated as the average $2^{-\Delta Ct}$ where ΔCt (cycle threshold) = $Ct - Ct_{GAPDH}$

Statistical Analysis

Data were summarized, presented and analyzed using two software programs. These were Microsoft Office Excel 2007 and the statistical packages for social sciences (SPSS 18) using T.test, one way ANOVA test, Chi square test at the level of significant $\alpha \leq 0.05$.

Results

1 :Clinico-pathological analysis:

Grading of the presented malignant cases revealing that low grade was reported in 35 (58.33%) cases while those of high grade were 25 (41.67%) cases (Figure 1, Figure 2).

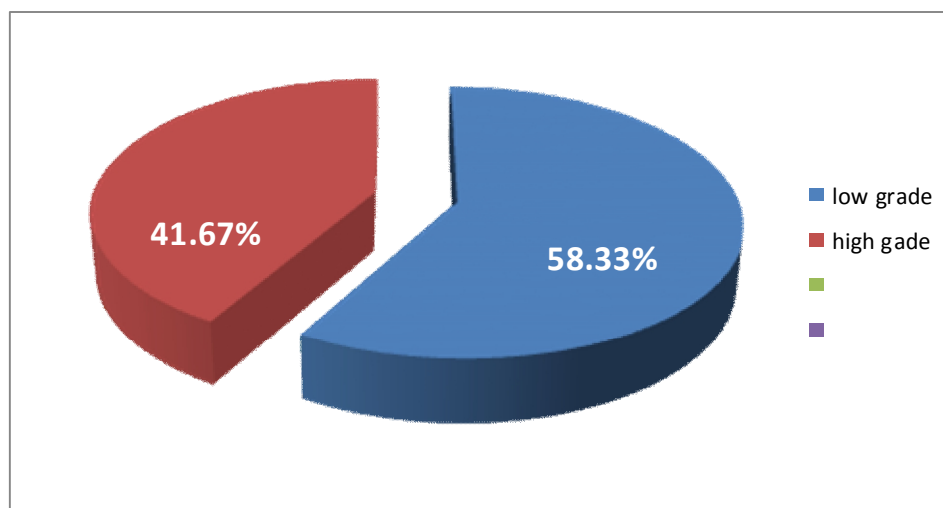


Figure 1: A pie chart showing the percentage of grade of urothelial carcinoma.

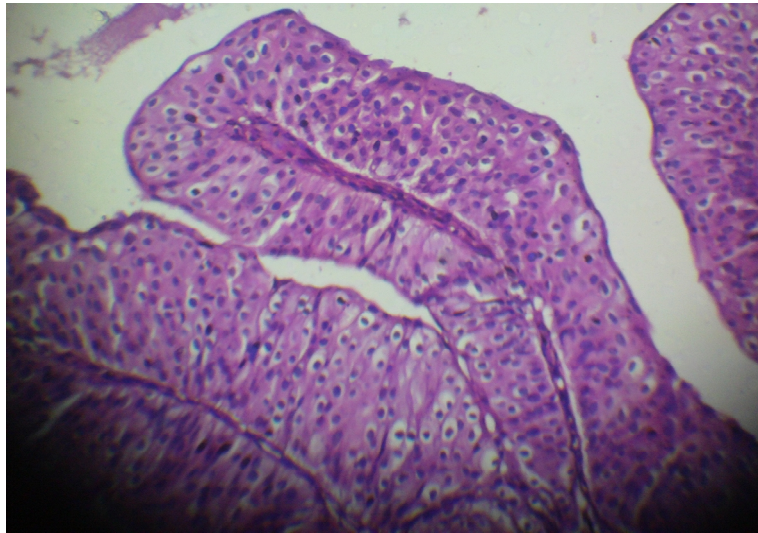


Figure 2: Low grade urothelial carcinoma

Assessment of the stage (T) of the 60 cases of transitional cell carcinoma revealed that ; 7 (11.67%) cases were of

Ta, 24 (40%) of T1, and 29 (48.33%) of T2 (figure 3, figure 4) .

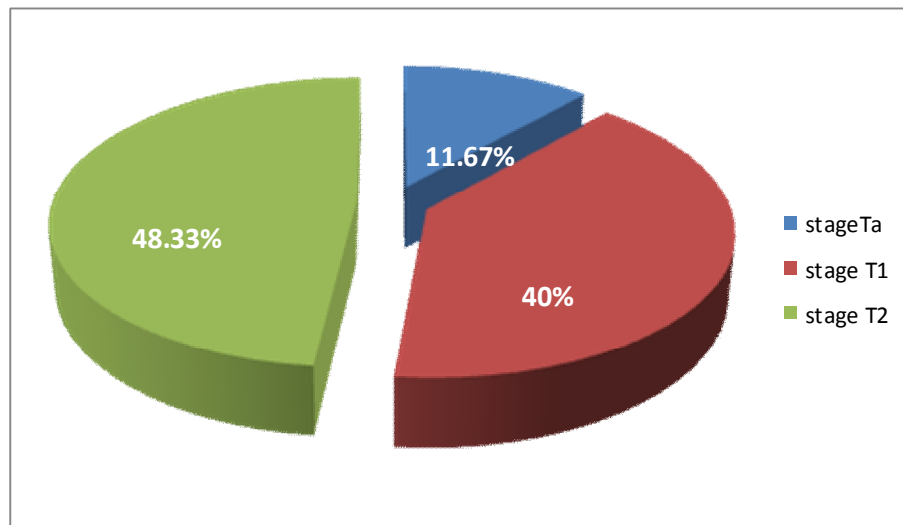


Figure 3: A pie chart showing the percentage of stages of urothelial carcinoma.

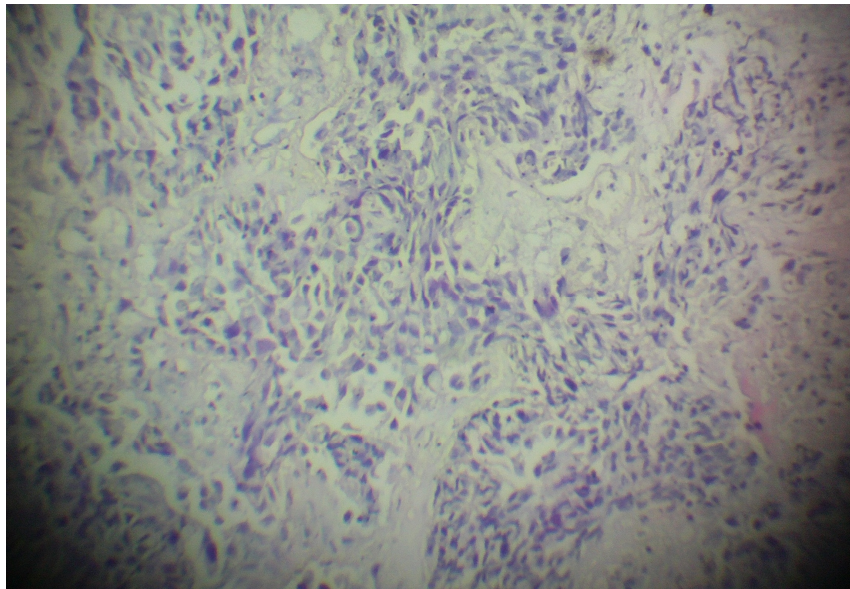


Figure 4: Stage T2 urothelial carcinoma

2- Results of HEF1 gene expression

2.1. HEF1 genes expression in urothelial carcinoma and non-tumorous bladder tissues

HEF1 gene expressions(fold change) were found to be 3.072 ± 0.868 s.d. in non-

tumorous bladder tissue and 11.219 ± 9.48 s.d. in malignant tissue, so HEF1 gene expression significantly ($p < 0.0001$) raised in urothelial carcinoma in relative to non-tumorous bladder tissues (table.1),(Fig 5).

Table 1: Relative expression results of FOXM1 and HEF1 gene in both non-tumorous and malignant bladder tissues

Gene	Fold change in non-tumorous bladder tissue	Fold change in urothelial carcinoma	P value
HEF1	3.072 ± 0.868	11.219 ± 9.48	< 0.0001

$P < 0.05$ Highly significant difference

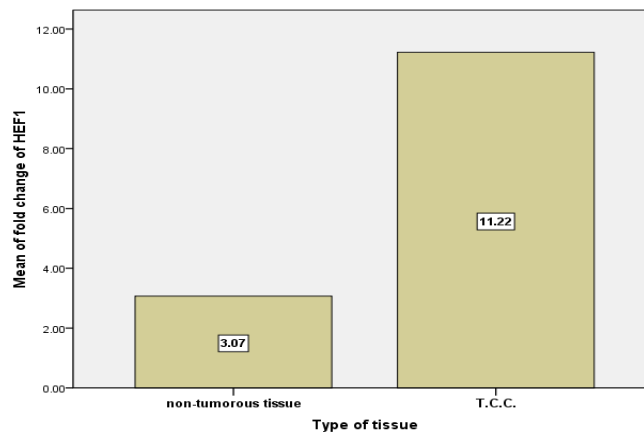


Figure 5: HEF1 expression gene level (mean fold change) in non-tumorous tissue and T.C.C.

2.2. Relevance of age of patients with HEF1 expression

The mean fold change of the up-regulated cases was 11.388 ± 4.859 s.d. for the age group ≤ 50 years old, and was $13.121 \pm$

10.543 s.d. for the age group > 50 years old (fig 6), there was no significant difference between these fold change ($p=0.456$) (Table 2).

Table 2: HEF1 gene expression level (mean of fold change) in relation to age group

(I) sage2	(J) sage2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
≤ 50	> 50	-1.73178-8.31812*	2.31613 2.23760	.456 .000	-6.3232-3.8823	2.8597 12.7539
> 50	≤ 50	1.73178 10.04989*	2.31613 1.33722	.456 .000	-2.8597-7.3990	6.3232 12.7008
non-tumorous tissue	≤ 50	-8.31812-*	2.23760	.000	-12.7539-	-3.8823-
	> 50	-10.04989-*	1.33722	.000	-12.7008-	-7.3990-

*. The mean difference is significant at the 0.05 level.

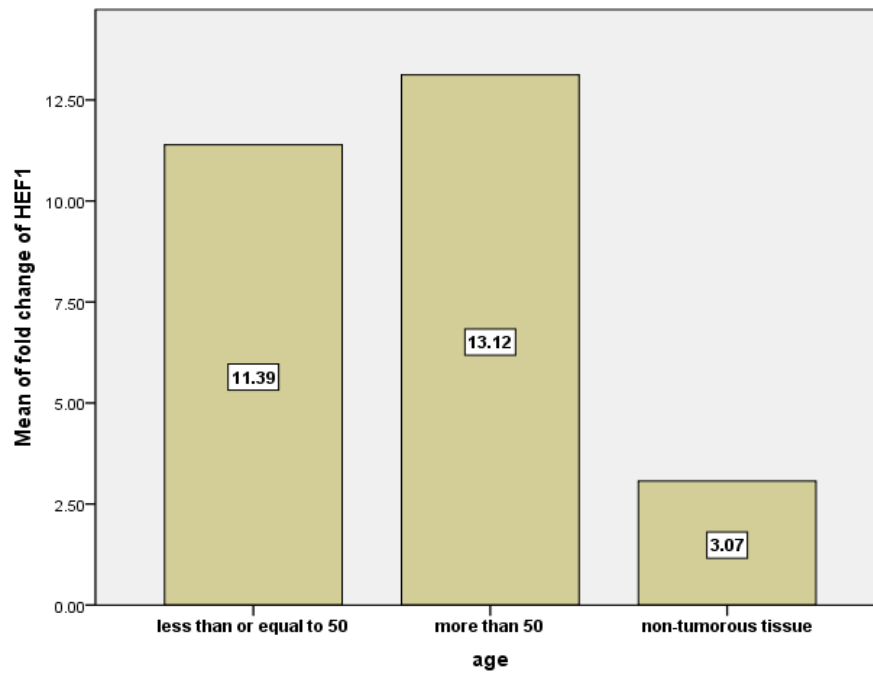


Figure 6: HEF1 gene expression level (mean of fold change) in relation to age group.

2.3. The relevance of bladder cancer grade with HEF1 gene expression

The mean fold change of the up-regulated cases was 10.443 ± 5.269 for low grade, and 15.739 ± 12.866 s.d. for high grade

(fig 7) . According to one-way ANOVA test there is significant difference between these groups P value=0.004 (Table 3).

Table 3: HEF1 gene expression level (mean of fold change) in relation to grades of bladder cancer

(I) grade	(J) grade	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
low grade	high grade	-5.29669*	1.79984	.004	-8.8647-	-1.7287-
	non-tumorous tissue	7.37300*	1.44586	.000	4.5067	10.2392
high grade	low grade	5.29669*	1.79984	.004	1.7287	8.8647
	non-tumorous tissue	12.66968*	1.57456	.000	9.5483	15.7911
non-tumorous tissue	low grade	-7.37300*	1.44586	.000	-10.2392-	-4.5067-
	high grade	-12.66968*	1.57456	.000	-15.7911-	-9.5483-

*. The mean difference is significant at the 0.05 level.

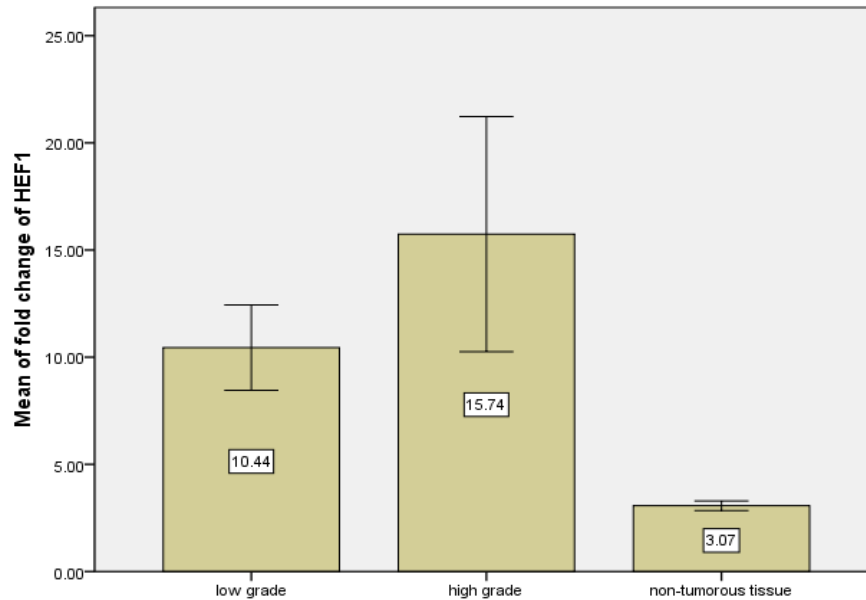


Figure 7: HEF1 gene expression level (mean of fold change) in relation to grades

2.4. The relevance of bladder cancer stage with HEF1 gene expression.

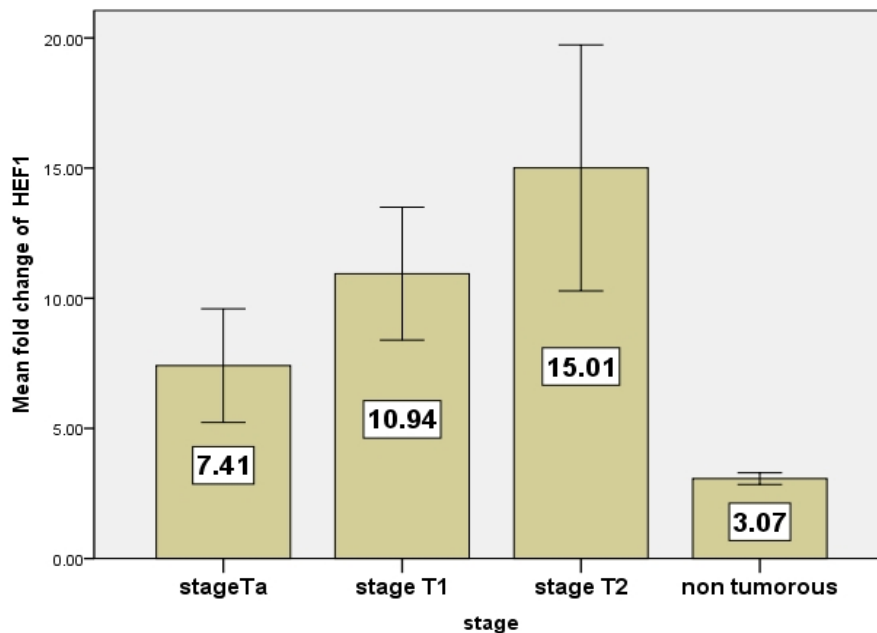
To clarify the relevance of urothelial carcinoma stage with HEF1 gene expression the one way ANOVA test was used to analyze the data, there is no significant difference between stage Ta

and stage T1 ($P=0.314$) and stage Ta and non-tumorous tissue ($p=0.19$), but there is significant difference between stage T1 and stage T2 ($P=0.034$) and between stage T2 and stage Ta ($p=0.029$) (table 4). The mean fold change of the up-regulated cases was 7.413 ± 2.180 for stage Ta, 10.943 ± 5.705 for stage T1 and 15.006 ± 12.045 s.d. for stage T2 (fig 8:).

Table 4: HEF1 gene expression level (mean of fold change) different stages of bladder cancer and in non-tumorous bladder tissue

(I) stage	(J) stage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
stage T a	stage T1	-3.53075-	3.49030	.314	-10.4506-	3.3891
	stage T2	-7.59408*	3.42252	.029	-14.3796-	-.8086-
	non-tumorous tissue	4.34232	3.29069	.190	-2.1818-	10.8664
Stage T 1	Stage T a	3.53075	3.49030	.314	-3.3891-	10.4506
	stage T2	-4.06333*	1.89531	.034	-7.8210-	-.3057-
	non-tumorous tissue	7.87307*	1.64534	.000	4.6110	11.1351
Stage T 2	stage Ta	7.59408*	3.42252	.029	.8086	14.3796
	Stage T 1	4.06333*	1.89531	.034	.3057	7.8210
	non-tumorous tissue	11.93639*	1.49620	.000	8.9700	14.9028
non-tumorous tissue	Stage T a	-4.34232-	3.29069	.190	-10.8664-	2.1818
	stage T1	-7.87307*	1.64534	.000	-11.1351-	-4.6110-
	Stage T 2	-11.93639*	1.49620	.000	-14.9028-	-8.9700-

*. The mean difference is significant at the 0.05 level.

**Figure 8:** Mean fold change of HEF1 gene expression level (mean of fold change) in relation to stages

Discussion

There is a progressive increase in the incidence and death rates from cancer over the world including urinary bladder cancer, in Iraq the incidence of B.C. increase from the fifth one in 2010 [16] to the fourth one in 2011 and regarded as one of the commonest ten cancers [3].

Human enhancer filament is an intermediate in very important signaling pathways in the cellular processes like proliferation, migration, survival and others [17-19]. Human enhancer filament is a multidomain scaffolding protein of the Cas family; it is also has an integral role in normal and pathological cell biology [20,21]. Elevated expression of HEF1 has been detected in a variety of tumor types, including melanoma, lung cancer, glioblastoma and breast cancer [26-29]. These data support the fact that HEF1 is a tumor-promoting factor, and elevated expression of HEF1 in tumors correlates with poor prognosis and treatment resistance [20,21].

In the current study, we observed up-regulation of HEF1 in urothelial carcinoma with 11.219 folds compared to the normal epithelial tissues (3.072 folds) (table 1) (fig 5), indicating that the expression level of HEF1 correlates with the tumorigenesis of urothelial carcinoma. Findings of the up-regulation of HEF1 expression in urothelial carcinoma patients are in concordance with the data of Qi Zhang et.al [30].

HEF1 expression has been found to be more frequently up-regulated with advancing age as in fig 6 but this was statistically not significant (table 2).

Our results were in concordance with the data of Qi Zhang et.al [30] which show no significant difference of HEF1 expression between two age groups.

In the current study and according to Analysis of variance (ANOVA) test, there is significant difference ($P=0.004$)(table.3) between low grade and high grade in which the expression level (fold change) was more expression in high grade (15.74) when compare to low grade (10.44) (fig

7), indicating that up-regulation of HEF1 has a role in bladder cancer tumorigenesis and prognosis. Our results were in concordance with the data of Qi Zhang et.al [30].

A significant ($P=0.029$) rise of HEF1 gene expression fold was observed in those of stage T2 when they were compared with those of stage Ta. Similarly HEF1 gene expression was evident to be increased significantly ($P= 0.034$) in tumor of stage T2 with respect to those of stage T1 (Table 4) (fig 8).

In the current study HEF1 expressions were found to elevate as the stages were advanced. These results are in consistence with previous reports. Qi Zhang et.al [30] have pointed out significant correlation of HEF1 expression with advancing of bladder cancer stages.

Conclusion

HEF1 gene up-regulation were detected significantly in transitional cell carcinoma. HEF1 expression status is closely correlated with important histopathologic characteristics (grades and stages) indicating that the tumor with overexpression of HEF1 gene are biologically aggressive and associated with poor prognosis and indicate the progression of cancer. These findings further support the role of HEF1 gene in the carcinogenesis of bladder cancer regarding evolution, behavior, and aggressiveness of transitional cell carcinoma and thus HEF1 gene could be considered as bad prognostic parameters in bladder cancer.

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