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# Methylation analysis of histone 4-related gene *HIST1H4F* and its effect on gene expression in bladder cancer

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ABSTRACT

Recently, aberrant DNA methylation of the *HIST1H4F* gene (encodes Histone 4 protein) has been shown in many types of cancer, which may serve as a promising biomarker for early cancer diagnosis. However, the correlation between DNA methylation of the *HIST1H4F* gene and its role in gene expression is unclear in bladder cancer. Therefore, the first objective of this study is to explore the DNA methylation pattern of the *HIST1H4F* gene and then further elucidate its effects on *HIST1H4F* mRNA expression in bladder cancer. To this end, the methylation pattern of the *HIST1H4F* gene was analyzed by pyrosequencing and the effects of the methylation profiles of this gene on *HIST1H4F* mRNA expression in bladder cancer were examined by qRT-PCR. Sequencing analysis revealed significantly higher methylation frequencies of the *HIST1H4F* gene in bladder tumor samples compared to normal samples ($p < 0{,}0001$). However, when we evaluated the correlations between hypermethylation of *HIST1H4F* and the clinicopathological parameters (tumor stage, tumor grade, lymph node metastasis, muscle-invasion), no significant difference was found between the groups ($p > 0.05$). In addition, we examined the role of hypermethylation of the *HIST1H4F* gene on *HIST1H4F* mRNA expression. We found that hypermethylation of *HIST1H4F* in the exon have no effect *HIST1H4F* mRNA expression in bladder cancer ($p > 0.05$). We also confirmed our finding in cultured T24 cell line which *HIST1H4F* gene is hypermethylated. Our results suggest that hypermethylation of the *HIST1H4F* seems to be a promising early diagnostic biomarker in bladder cancer patients. However, further studies are needed to determine the role of *HIST1H4F* hypermethylation in tumorigenesis.

## 1. Introduction

Bladder cancer is the most common type of urothelial cancer in men after prostate cancer. The survival rate of bladder cancer is low despite advanced surgery and chemotherapy (Harb-De et al., 2015). Therefore, the identification of effective biomarkers is essential not only for the diagnosis but also for the therapy of bladder cancer and healthcare (Chehab et al., 2015). Epigenetic mechanisms take part in an important role in the pathogenesis of bladder cancer (Coban and Varol, 2019). DNA methylation and histone modifications are the best-known epigenetic mechanisms affecting chromatin structure. DNA methylation plays an essential role and is intertwined with histone modifications in the regulating gene expression to maintain epigenetic memory. Aberrant DNA methylations and histone modifications are one of the cancer hallmarks (Llinas-Arias and Esteller, 2017; Coban and Varol, 2019).

Histone proteins are essential in formation chromatin architecture, packaging of genetic information, and play a vital role in the regulating DNA-dependent processes, such as transcription, repair, replication, and recombination (Buschbeck and Hake, 2017; Singh et al., 2018; Amatori et al., 2021). The accuracy of histone gene regulation is critical for chromatin integrity, genome replication, and stability (Ghule et al., 2014). The most conserved histone H4 protein is one of the histone

*Abbreviations:* Chr, Chromosome; HIST1H4F, Histone cluster 1 H4, family member F; H4, Histone-4; H4K20, Methylation of histone 4 Lysine 20; ROC, Receiver-operator characteristic curve; AUC, The area under the curve; TCGA, The Cancer Genome Atlas.

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octamer core proteins and is expressed in a replication-dependent. Replication-dependent histone genes are encoded by a family of genes, non-polyadenylated histone mRNA, and do not contain introns (van der Meijden et al., 1998; Singh et al., 2018). Most histone H4 genes are located on histone cluster 1 (Chr6p21), but also on histone cluster 2 (Chr1q21) and histone cluster 4 (Chr12p12) (Dong et al., 2019; Amatori et al., 2021).

Histone H4 is encoded by multiple genes (15 genes) at multiple loci that are coordinately regulated. However, each of these genes encodes the same protein (van Wijnen et al., 1992; Marzluff et al., 2002; Holmes et al., 2005). One of these genes is *HIST1H4F* gene (histone cluster 1 H4, family member F) and is located on chr6p21. Recent studies have shown that aberrant hypermethylation of the *HIST1H4F* is common in many type of cancers (Dong et al., 2019; Kitchen et al., 2016; Sandoval et al., 2013; Li (a) et al., 2021), therefore, it suggested that this methylation of this gene may serve as a promising biomarker for early diagnosis of cancer and others diseases (Asadikalameh et al, 2022; Zhang et al., 2022). For example, *HIST1H4F* gene is even recommended as a universal-cancer-only methylation marker for lung cancer (Dong et al., 2019).

The correlation between the methylation profiles of *HIST1H4F* and gene expression at transcriptional level has not been investigated in bladder cancer. In the present study, we first determined the DNA methylation profile of the *HIST1H4F* gene in bladder cancer tissues. Secondly, we aimed to quantify and characterize the *HIST1H4F* methylation pattern in bladder cancers considering tumor grade, tumor stage, lymph node metastasis, and muscle invasion. Finally, the effect of hypermethylation of the *HIST1H4F* gene on *HIST1H4F* mRNA expression in in bladder cancer tissues and T24 cells was investigated in a time-dependent manner.

## 2. Materials and Methods

### 2.1. Tissue specimens

This single-center study was performed on primer tumor specimens from 38 bladder cancer patients and 9 healthy controls admitted to the Department of Urology, Afyonkarahisar Health Science University (Afyonkarahisar, Turkey). All specimens were histologically classified as normal bladder urothelium and primer bladder cancer tissues. The clinicopathologic data of the 47 cases are shown in Table 1. The study was approved by the Kocatepe University Ethics Committee (approval number 2017/1–13) and conducted accordance with the Declaration of Helsinki.

**Table 1**
Clinical characteristics of patients and controls.

| | Patients n = 38 (%) | Controls n = 9 |
|---|---|---|
| Sex | | |
| Male | 33 | 9 |
| Female | 5 | 0 |
| Age, year | | |
| Median | 68,7 | 65,5 |
| Range | 41–88 | 36–82 |
| Tumor stage, n(%) | | |
| pTa | 11 (28,9%) | – |
| pT1 | 12 (31,6%) | – |
| pT2 | 13 (34,2%) | – |
| pT3 | 2 (5,3%) | |
| Lymph node | | |
| Negative | 10 (66,7%) | |
| Positive | 5 (33,3%) | |
| Tumor grade n(%) | | |
| Low | 14 (36,8%) | – |
| High | 24 (63,2) | – |
| Muscle invasive | | |
| + | 15 | |
| – | 23 | |

### 2.2. Cell culture

The human bladder cancer cell line T24 was obtained from American Type Culture Collection (ATCC). T24 cells were cultured in McCoy's 5A supplemented with 10 % FBS and 1 % antibiotics in 5 % $CO_2$ at 37 °C and tested negative for *Mycoplasma* by qPCR. To determine time-dependent *HIST1H4F* gene expression, cells were first deprived of serum for 72 h to induce quiescence and then re-stimulated with serum to enter the cell cycle. Simultaneously, cells were harvested at 0, 4, 8, 12, 16 and 24 h after stimulation (Holmes et al., 2005).

### 2.3. DNA Extraction, bisulfite Modification, and methylation status

Genomic DNA from tissues and cell cultures were extracted using QIAamp DNA Mini Kit (Qiagen, Germany). 500 ng of DNA was modified by bisulfite conversion by using the EZ DNA Methylation Gold (Zymo Research, USA). The bisulfite-treated DNA served as the PCR template. PCR was performed under standard conditions with biotinylated primers, and PCR products were subjected at agarose gel electrophoresis before sequencing. The methylation profile of fifteen CpG loci in *HIST1H4F* gene (chr6:26,240,743–26,240,793) was determined by pyrosequencing (Fig. 1). Primers in coding regions were designed according to the regions presented in other studies (Sandoval et al., 2013) using PyroMark Assay Design Software 2.0.01.15 (Qiagen, Germany). The sequencing primer sequences of *HISFT1H4F* were TTGGTAGAGGTAAAGGTGGTAAA and biotin-AACAACATCCATTACAATAACTATCTTAC and GTAAAGGTT-TAGGAAAGGGAG for the sequencing primer. Pyrosequencing reaction and quantification of methylation at each CpG loci were performed using a Pyromark Q24 System version 2.0.6 (Qiagen, Germany).

### 2.4. RNA extraction and Quantitative real-time PCR

Total RNA from tissue and cell culture were extracted using QIAzol (Qiagen, Germany) and reverse transcribed by $RT^2$ HT First Strand kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed on RotorGene Q using qPCR SYBR Green Master Mix (Qiagen, Germany). qPCR primers are GGAAAGG-GAGGCGCCAAG and GCCCGAAATGCGTTTCACG for *HIST1H4F,* AAGGT GAAGGTCGGAGTCAA and GGAAGATGGTGATGGGATTT for *GAPDH* (Dong et al., 2019).

### 2.5. Statistical analysis

GraphPad Prism 9 was used to analyze methylation status in tumor and normal tissues using Fisher's exact test (frequency of methylation) and Student's t-tests (mean level of methylation and expression). In addition, receiver operating characteristic (ROC) was applied to the methylation percentage to determine the sensitivity and specificity of *HIST1H4F* methylation with the optimal cut-off value for discriminating between bladder cancer patients and controls. Correlations between DNA methylation and gene expression was tested using Spearman's rank correlation coefficient. A *p-value* of $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Methylation pattern of HIST1H4F in bladder cancer

A locus containing fifteen CpG dinucleotides at the exon region was selected for DNA methylation analysis of *HIST1H4F* gene (Fig. 1). Methylation of *HIST1H4F* gene in bladder cancer and healthy tissues showed a characteristic receiver-operator characteristic curve (ROC), distinguishing bladder cancer from healthy control ($p < 0.001$). The optimal cut-off value of the methylation pattern of *HIST1H4F* was calculated using the analysis ROC to maximize sensitivity and specificity as a biomarker and was set at 11,95 %. The area under the curve (AUC)

H4 SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG
H4F SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG

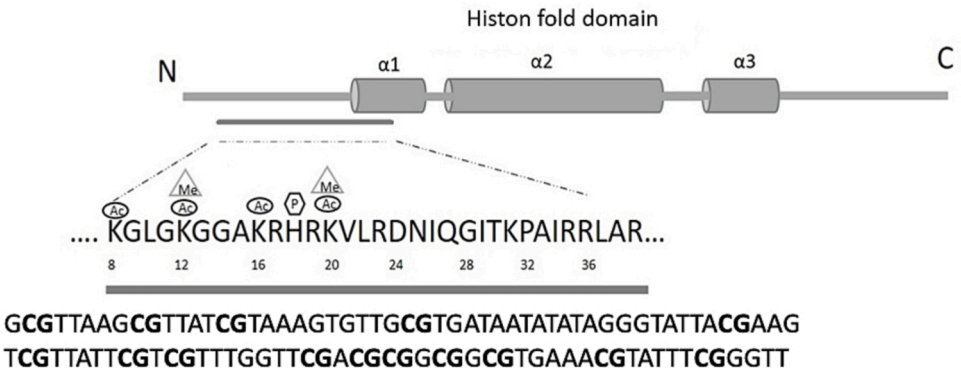


**Fig. 1.** Schematic diagram showing the location of the HIST1H4F region analyzed.

was 0.899 for *HIST1H4F*. Specificity and sensitivity were found as 88,9% and 88,64 %, respectively (Fig. 2A).

*HIST1H4F* hypermethylation in the exon region was observed in 33 (86,8%) of 33 bladder cancer patients, whereas any of the control samples (0/9) not detected hypermethylation in the *HIST1H4F* gene. Our results showed that the *HIST1H4F* gene was significantly hypermethylated in bladder cancer tissues (Fig. 2 and Fig. 3A, $p < 0.0001$). The clinical characteristics and DNA methylation profiles of fifteen CpG dinucleotides are shown in Table 2. There was no significant difference between the methylation percentages of fifteen CpG dinucleotides, almost all CpG dinucleotides were methylated in hypermethylated samples considering the cut-off value. Clinicopathological characteristics of the tumor samples were summarized in Table 2. There was no significant correlation between mean methylation level of *HIST1H4F* and tumor grade ($p > 0.05$), tumor stage ($p > 0.05$), muscle-invasion (+/-) ($p > 0.05$), lymph node metastasis (+/-) ($p > 0.05$)(Fig. 4, $p > 0.05$). Our results indicate that the *HIST1H4F* gene is frequently methylated in bladder cancer tissues and that hypermethylation of *HIST1H4F* can be used as a potential early diagnostic biomarker for bladder cancer, as this gene exhibits hypermethylation at all stages of bladder cancer.

### 3.2. Effect of hypermethylation of HIST1H4F on mRNA expression

It is well known that DNA methylation is one of control mechanisms for the gene expression, and DNA hypermethylation usually has a negative effect on gene expression. It is unclear how *HIST1H4F* methylation affects gene expression. Therefore, we next set out to elucidate the effects of hypermethylation of *HIST1H4F* on gene expression at the transcriptional level in bladder cancer. When we analyzed the expression of *HIST1H4F* in bladder tissue, the results showed that *HIST1H4F* was expressed in both normal and tumor tissues, but there were no significant differences between normal and tumor tissues (Fig. 3B, $p > 0.05$). Consequently, we did not detect any correlation between methylation and mRNA expression for the *HIST1H4F* gene in bladder cancer tissues (Spearman's rank correlation coefficient [ρ] = 0.4938, $p > 0.05$). In addition, we checked whether there was a correlation between hypermethylation of *HIST1H4F* and gene expression in the bladder cancer cell line T24. First, our results showed that similar to tumor samples *HIST1H4F* is hypermethylated in T24 cells, and the gene is expression at the mRNA level. Since it has been shown that the Histone gene transcription is increased 2- to 5-fold and peaks within 1–3 h when DNA synthesis is initiated (Ewing et al., 1994) and regulated in a replication-dependent manner (Amatori et al., 2021). We examined

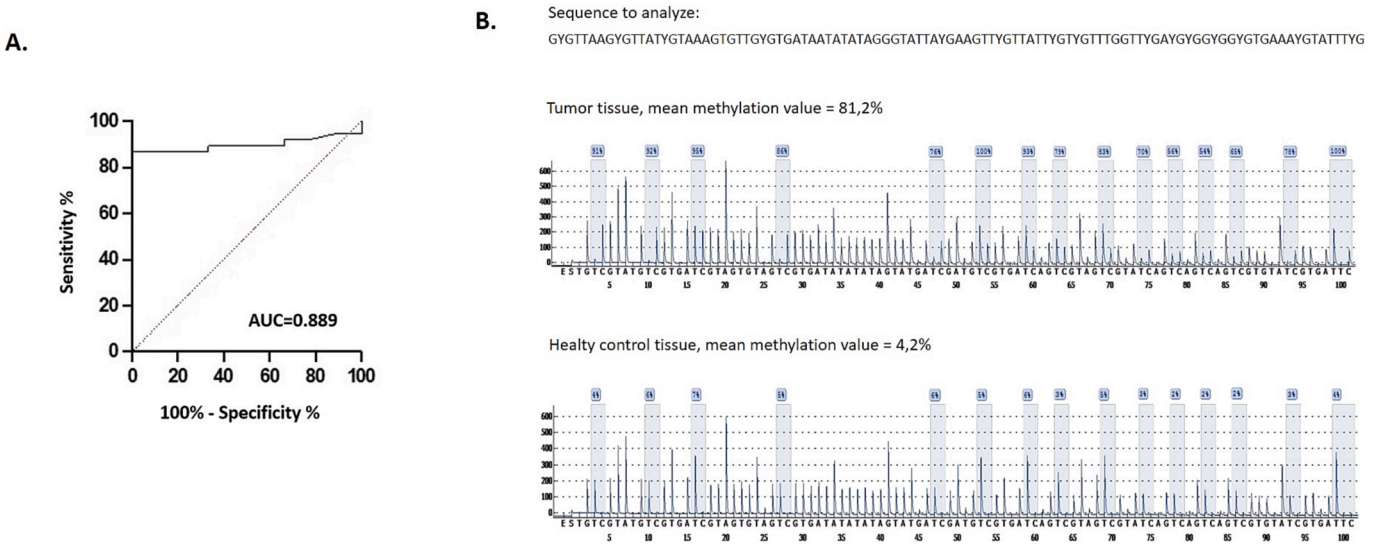


**Fig. 2. A.** ROC curve of *HIST1H4F* methylation in bladder cancer patients. Mean *HIST1H4F* methylation levels are able to predict the risk of bladder cancer. **B.** DNA methylation analysis by pyrosequencing. A pyrogram of the exon region of the *HIST1H4F* gene in bladder cancer and healthy tissues.

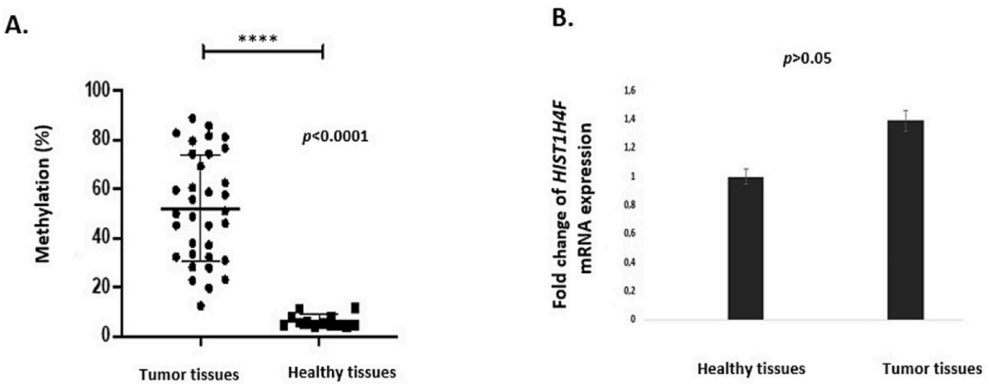


**Fig. 3.** HIST1H4F DNA methylation and gene expression. A. Mean methylation level of *HIST1H4F* gene B. *HIST1H4F* mRNA expression.

**Table 2**
*HIST1H4* gene methylation status. *HIST1H4* gene showing an increase in frequency of methylation and mean level of methylation in bladder cancer tissues relative to normal bladder tissues. Methylation positivity and negativity based on methylation level at cut-off values. Statistically significant *P*-value are displeyed in bold.

| | Methylation Frequency n (%) | **Mean** Level of Methylation (%) | *P* value |
|---|---|---|---|
| Tumor stage, | | | |
| pTa | 9/11(81,8%) | | < **0.01** |
| pT1 | 9/12 (75 %) | 41,45 | < **0.05** |
| pT2 | 13/13 (100 %) | 35,86 | < **0.001** |
| pT3 | 2/2(100 %) | 58,88 | < **0.0001** |
| | | 66,9 | |
| Lymph node | 10/10 (100 %) | | |
| Negative | 5/5 (100 %) | | |
| Positive | | 60,84 | < **0.0001** |
| | | 58,17 | < **0.0001** |
| Tumor stage, n (%) | | | |
| Low | 11/14 (78,6%) | 38,70 | < **0.001** |
| High | 22/24 (91,6%) | 51,83 | < **0.0001** |
| Muscle invasive | | | |
| positive | 15/15 (100 %) | 62,89 | < **0.0001** |
| negative | 18/23 (78,3%) | 38,66 | < **0.001** |

gene expression in T24 cells at different time intervals (0 h, 4 h, 8 h, 12 h, 16 h and 24 h). We found a difference in the expression level as time-dependent (Fig. 5B, $p < 0.05$). The expression of *HIST1H4F* gene significantly decreased at 8 h (3,12-fold) and overexpressed by 16 h (2,2-fold) and 24 h (2,7-fold) (Fig. 5A). Next, we examined the change in the methylation level of *HIST1H4F* at the time points when the expression of *HIST1H4F* was up- or down-regulated, but we could not detect any change in the methylation level of *HIST1H4F* (Fig. 5B). Similar to bladder tissues, there was no correlation between hypermethylation of *HIST1H4F* and gene expression (Spearman's rank correlation coeffeicient [$\rho$] = 0.3162, $p > 0.05$).

## 4. Discussion

Epigenetic regulation is an important mechanism in regulation of gene expression. DNA methylation and histone modifications are the best-known epigenetic regulation mechanisms. Both epigenetic modifications play important roles in the maintaining of transcriptional programs because they are dynamically reversible during cell fate determination (Begam et al., 2018; Li (a) et al., 2021). Although DNA methylation and histone modifications are two distinct epigenetic mechanisms, the influences each other. Histone modifications can mediate direct DNA methylation patterns, while DNA methylation serves as a template for maintaining histone modification patterns after DNA replication (Cedar and Bergman, 2009). Abnormal DNA methylation patterns and aberrant histone modifications are considered key feature of cancer pathogenesis and are used as biomarkers for cancer diagnosis, prognosis, and classification. Hypermethylation and region-specific histone modifications are considered as one of the cancer hallmarks that contribute significantly to the tumorigenic process by inhibiting gene activation (Esteller, 2007). However, aberrant histone modifications may be due to changes in histone protein expression at transcriptional level through DNA methylation-mediated mechanisms rather than an aberration of enzymes responsible for specific histone modifications. In particular, epigenetic regulation of histone genes is rarely studied in histone modification-dependent gene regulation. Histones are an important member of the housekeeping gene family. Individual histone proteins are expressed by multiple genes (Dong et al., 2019).

Histone H4 protein is one of the core octamer proteins of the nucleosome and, like other histone proteins, is expressed by multiple gene copies. The human genome contains 15 histone H4 genes that carry different codons for the same amino acid but encode the same protein. In this way, each of the individual histone H4 proteins required for replication of the entire mammalian genome is efficiently synthesized (Holmes et al., 2005; Amatori et al., 2021). Interestingly, however, histone mutations target specific genes among many that express the same histone type (Amatori et al., 2021). Many previous whole genome methylation studies have focused on hypermethylation of the *HIST1H4F* gene, one of the genes responsible for histone H4 expression, and it also being studied in other cancer types including lung, and cervical cancer (Dong et al., 2019; Li (b) et al., 2021; Sandoval et al., 2013). In one of these studies, hypermethylation of *HIST1H4F* was identified as a potential early diagnosis marker in squamous cell carcinoma (Li (c) et al., 2021). In another study, the *HIST1H4F* gene was found to be hypermethylated in patients with non-small cell lung cancer and could serve as a biomarker for classifying patients at high and low-risk stage I and with shorter relapse-free survival. Also, in another study of lung cancer, many histone genes were hypermethylated and it was also emphasized that hypermethylation of *HIST1H4F* and *HIST1H4I* could be used as an effective diagnostic marker for early-stage lung cancer with high specificity and sensitivity. Moreover, using TCGA data sets, *HIST1H4F* was shown to be hypermethylated in fifteen cancers. Therefore, it is emphasized that hypermethylation of *HIST1H4F* mat act as a universal methylation marker for cancer (Sandoval et al., 2013; Dong et al., 2019). Therefore, in this study, we mainly focused on the determining the DNA methylation profile of the *HIST1H4F* gene in bladder cancer. Kitchen et al. (2016) demonstrated that hypermethylation of *HIST1H4F* significantly increased mean methylation levels in high-grade tumors compared with low-intermediate grade tumors in non-muscle- invasive bladder cancer. In contrast to Kitchen et al., in this study, we also compared bladder cancer and healthy tissues and also both muscle-invasive and nonmuscle-invasive bladder cancer samples. Our data

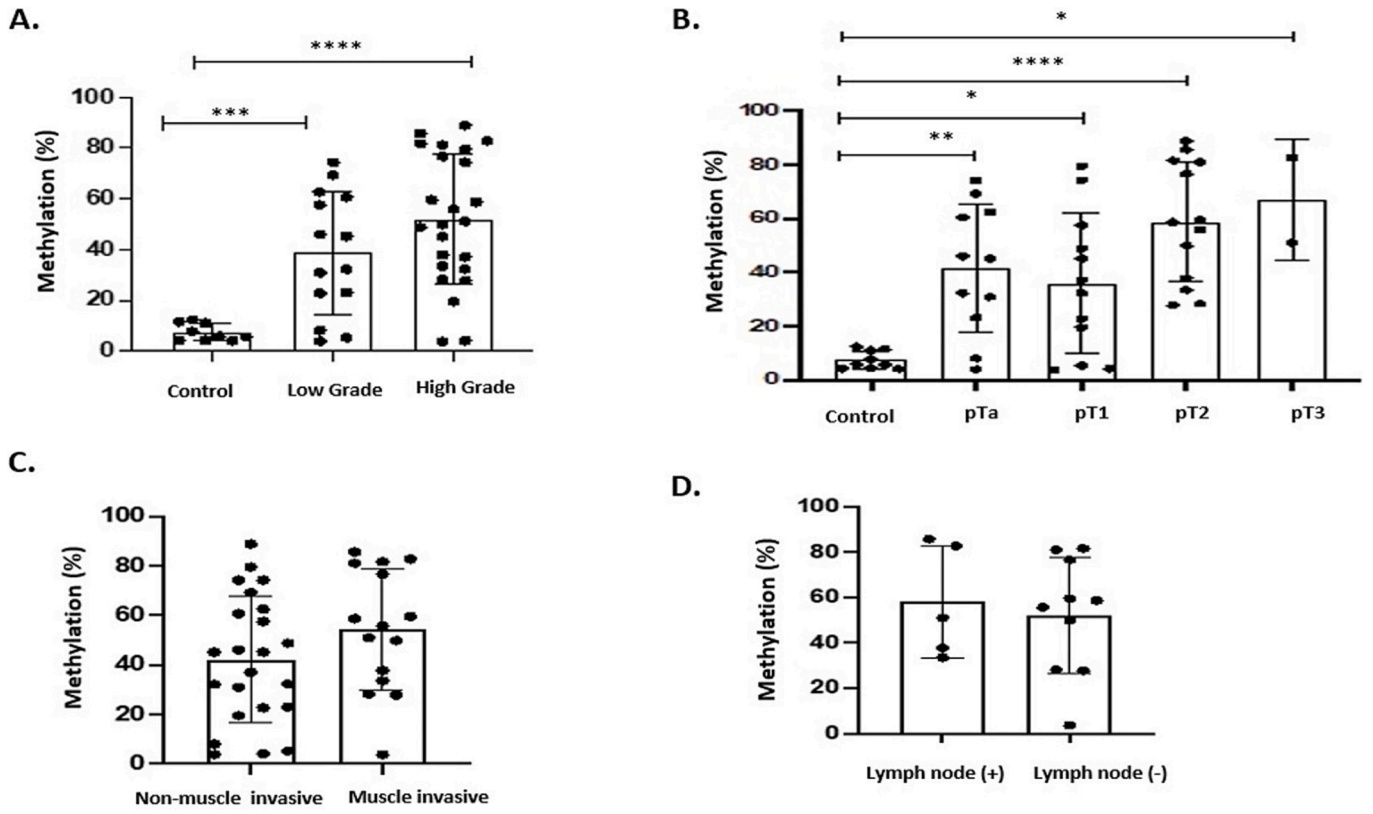


**Fig. 4.** Mean methylation level of *HIST1H4F* according to the clinicopathological parameters of patients and controls **A.** High-grade tumors vs low-grade tumors, **B.** between tumor stages, and **C.** Non-muscle invasive vs muscle invasive **D.** lymph node metastasis negative vs positive according to ROC curve. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, ****; $p < 0.0001$.

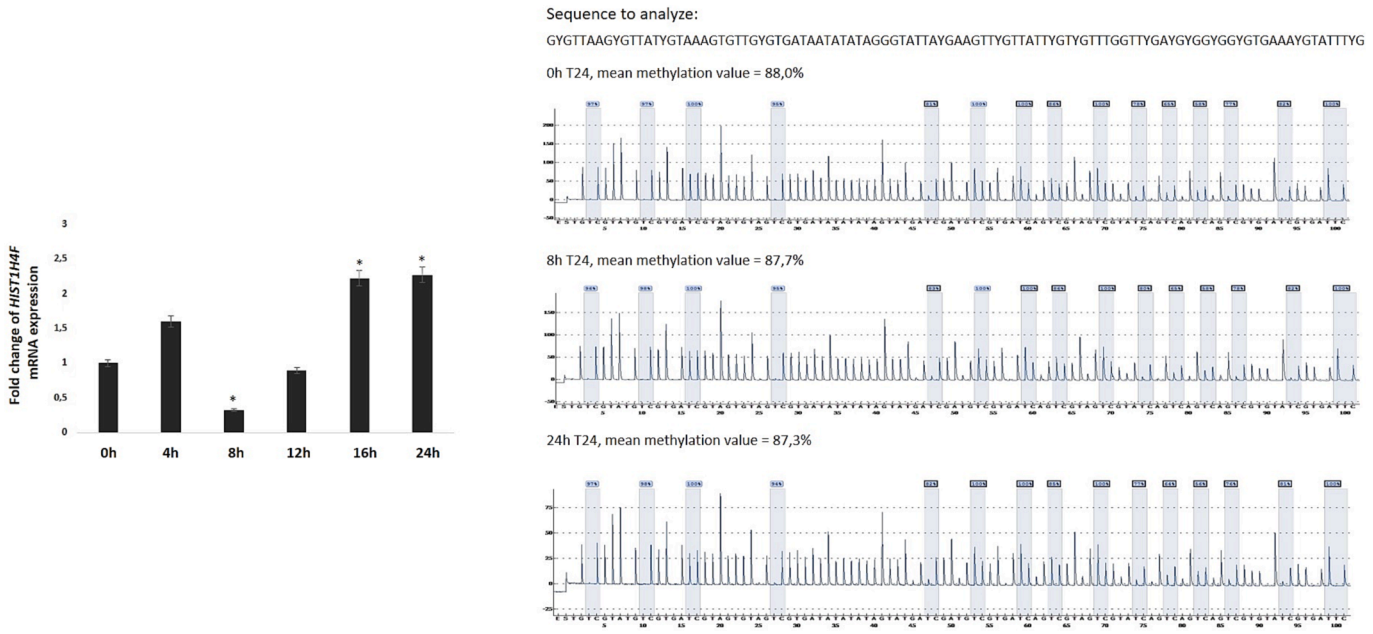


**Fig. 5.** Gene expression and DNA methylation analysis of *HIST1H4F* gene in T24 cells. **A.** *HIST1H4F* mRNA expression **B.** DNA methylation analysis by pyrosequencing. A pyrogram of the exon region of the *HIST1H4F* gene.

shown that the *HIST1H4F* gene was hypermethylated in bladder cancer patients compared with healthy control. However, there were no significant differences in the methylation patterns of *HIST1H4F* between tumor samples for tumor grade, lymph node metastasis (non-muscle/muscle invasive) status according to methylation frequency, and mean methylation levels in invasive bladder cancer patients. Our results indicate that hypermethylation of the *HIST1H4F* gene is particularly common in bladder cancer. Hypermethylation of the *HIST1H4F* gene has high diagnostic value according to analysis of ROC.

Theoretically, each gene copy responsible for histone H4 is expected

to contribute 7 % to the histone 4 pool (expression of 15 genes is 100 %) because it encodes the same protein. However, in reality, this is not the case, Holmes et al. (2005) showed differences in the expression of H4 RNA species. They found that five highly expressed genes (H4/d, H4/e, H4/j, H4/n, and H4/o) in three normal (fetal liver, fetal colon, and IMR90) and four tumor-derived cell types (HCT116, T98G, SaOS, HeLa) make up the majority of the total pool (>76 %). Another difference is that these highly expressed genes account for a disproportionate amount of total H4 mRNA in tumor cells compared to normal cells. For example, five highly expressed genes contribute 55 % in normal cells, whereas this proportion is 80 % in tumor cells. Thus, this study showed that a subset of individual H4 genes is expressed differentially distinct from the theoretically expected contribution to the total H4 mRNA pool in cancer cells compared with the expression of histone H4 genes in normal cells. This is not due to mRNA stability but to the diversity of promoter elements and/or overall chromatin organization of histone gene clusters. However, they demonstrated that the overall expression of histone H4 in normal and cancer cells is the same despite suppression of some histone H4 genes. They found that this is compensated by the expression of another histone H4 gene. Therefore, in next step, we investigated how aberrant hypermethylation of *HIST1H4F* affects gene expression at transcriptional levels. Due to low RNA integrity, only about 36 % of primary bladder cancer tissues could be analyzed. We demonstrated that the *HIST1H4F* gene is expressed in bladder cancer tissues despite hypermethylation and found no significant differences in mRNA expression compared with control tissues. Analyses of a larger number of bladder cancer samples may provide better information.

In addition to hypermethylation of *HIST1H4F,* Dong et al (2019) also investigated how aberrant hypermethylation of *HIST1H4F* affects gene expression. They analyzed the mRNA expression of *HIST1H4F* in fifteen tumor types, including bladder cancer, from the TCGA database and surprisingly found that in most types of tumors, *HIST1H4F* has no or very low gene expression in most tumor types, both in normal controls and tumor samples. At the same time, they verified their findings in MRC5 cell line (normal lung fibroblast) and A549 cell line (lung cancer) and observed that there was no expression in either cell line, although *HIST1H4F* was hypermethylated in A549 cells and hypomethylated in MRC5 cells. Consistent with these results, they concluded that hypermethylation of HIST1H4F does not affect gene expression and may not be involved in tumorigenesis. Although there is conflicting information, hypermethylation in gene bodies controls gene expression, either positively or negatively (Watanabe et al., 2004; Yang et al., 2014). We then examined the *HIST1H4F* gene expression in T24 cells (the *HIST1H4F* gene is hypermethylated) at different time intervals, as histone H4 genes are regulated in a replication-dependent manner. We found significantly changes in gene expression as time dependent. However, we did not detect any change in the methylation level of *HIST1H4F* at the times when *HIST1H4F* expression is up- or down-regulated. These results indicate that there is no significant relationship between methylation and gene expression for the *HIST1H4F* gene in bladder cancer. Thus, our results indicate that expression of histone *HIST1H4F* gene may be controlled by 5′regulatory sequences, as noted by Stein et al. (1992), rather than by DNA methylation in the exon region. Fifteen CpG dinucleotides that we analyzed are located in the exon region of the *HIST1H4F* gene, and this region overlaps with the most of *N*-terminal tail and part of the globular domain of histone H4 proteins (Fig. 1). This information and our results indicate that the hypermethylation of *HIST1H4F* may not affect gene expression but may play a role in bladder cancer tumorigenesis by chromatin information.

To our knowledge, this is the first report to investigate the effect of *HIST1H4F* gene methylation on *HIST1H4F* mRNA in primary bladder cancer tissues. As is well known, CpGs are hot-shot regions of the genome, and despite the presence of a sophisticated repair system, approximately-one-third of all point mutations causing genetic diseases in human result from C-to-T or G-to-A transitions at a CpG site (Tomatsu et al., 2006). Fifteen CpG dinucleotides that we analyzed are located in the exon region of the *HIST1H4F* gene, and this region overlaps with the most of the *N*-terminal tail and part of the globular domain of histone H4 proteins (Fig. 1). Histone H4 is subject to several posttranslational modifications that supports epigenetic control of transcription (Holmes et al., 2005; Ghule et al., 2014). In particular, histone H4 lysine methylation is dynamic and reversible but a stable modification (Sadakierska-Chudy and Filip, 2015; van Nuland and Gozani, 2016; Corvalan and Coller, 2021). Such as, H4K20 methylation regulates vital biological processes including gene expression, silencing, DNA replication, cell cycle regulation, and DNA damage response. Alteration of H4K20 methylation has been associated with numerous diseases, including cancer and developmental disorders (van Nuland and Gozani, 2016). We found that the *HIST1H4F* gene is hypermethylated at high frequency but could not determine the relationship between hypermethylation and expression of *HIST1H4F*. Our data, together with the results of Dong's group, suggest that hypermethylation of HIST1H4F in exon region does not affect mRNA expression, but that the epigenetic status of HIST1H4F loci may affect the chromatin information.

## 5. Conclusion

According to our findings, hypermethylation of *HIST1H4F* is cancer-specific in the light of information in the literature, and can be used as a potential diagnostic biomarker for bladder cancer, considering that this gene exhibits hypermethylation in all stages of bladder cancer. However, hypermethylation of *HIST1H4F* has been observed not only in bladder cancer but also in many other cancers. However, our results that are in accordance with literature suggest that the hypermethylation of *HIST1H4F* is not associated with expression at the transcriptional level. Hypermethylation of *HIST1H4F* may play a role in tumorigenesis by affecting the chromatin information. The role of hypermethylation of *HIST1H4F* in tumorigenesis remains unclear. Therefore, further studies are needed not only determine the effects of hypermethylation of *HIST1H4F* on tumorigenesis but also co-evaluation of expression, and methylation levels of other genes responsible for histone H4 expression.

## CRediT authorship contribution statement

**Nuray Varol:** Project administration, Conceptualization, Investigation, Methodology, Data curation, Validation, Writing – review & editing, Funding acquisition. **İbrahim Keles:** Methodology, Data curation. **Handan Yildiz:** Conceptualization, Investigation, Methodology. **Cem Karaosmanoglu:** Methodology, Formal analysis. **Mustafa Karalar:** Methodology, Data curation. **Kursad Zengin:** Methodology, Data curation. **Hasmet Sarici:** Methodology, Data curation. **Cigdem Tokyol:** Methodology, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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