Evaluation of TET Family Gene Expression and 5-Hydroxymethylcytosine as Potential Epigenetic Markers in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: DNA methylation is the most studied epigenetic modification in cancer. Ten-eleven translocation enzymes (TET) catalyze the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in the DNA. In the current research, we aimed to evaluate the role of 5-hmC and TET enzymes in non-small cell lung cancer (NSCLC) patients and their possible association with outcomes. Patients and Methods: ELISA was used to measure the 5-hmC levels in genomic DNA and qRT-PCR was used to evaluate TET1, TET2, and TET3 mRNAs expression levels in NSCLC tissues and their paired normal controls. Results: The levels of 5-hmC were significantly lower in NSCLC tissues than in normal tissues, with a mean ±SD of 0.28±0.37 vs. 1.84±0.58, respectively (t=22.77, p<0.0001), and this reduction was correlated with adverse clinical features. In addition, all TET genes were significantly down-regulated in NSCLC tissues in comparison to their matched normal tissues. The mean±SD level of TET1-mRNA was 38.48±16.38 in NSCLC vs.* 80.65±11.25 *in normal tissues (t=21.33, p<0.0001), TET2-mRNA level in NSCLC was 5.25±2.78 vs. 9.52±1.01 in normal tissues (t=14.48, p<0.0001), and TET3-mRNA level in NSCLC was 5.21±2.8 vs. 9.51±0.86 in normal tissues (t=14.75, p<0.0001). Downregulation of TET genes was correlated with poor clinical features. Conclusion: 5-HmC levels as well as TET1, TET2, and TET3 mRNA levels were reduced in NSCLC tissues. The reduced levels of 5-hmC and TET mRNAs were associated with adverse clinical features, suggesting that the level of 5-hmC may serve as a valuable prognostic biomarker for NSCLC.*

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Lung cancer is a main cause of cancer-related death. Non-small cell lung cancer (NSCLC) constitutes about 85% of all lung cancer types (1). In early-stage disease, the 5-year survival is about 58% compared to 15% in advanced stage (2). Therefore, early diagnosis is crucial for improving NSCLC survival rate and the identification of prognostic biomarkers is important (3, 4).

Tumor-suppressor gene (TSG)-associated epigenetic inactivation may lead to tumorigenicity and progression of malignant phenotype in many tumors including NSCLC (5-7). Aberrant DNA methylation of cancer-related gene promoters is considered a significant and early event in carcinogenesis and has gained high interest due to the reversible nature of epigenetic changes (8, 9).

DNA methylation at the fifth position of cytosine is one of the frequent epigenetic changes and has an impact on a variety of cellular functions. Generally, the progression of cancer is linked to 5-methylcytosine (5-mC) at particular genomic locations (10-12). According to the research of Shen *et al*., the conversion of 5-mC to 5-hydroxymethylcytosine (5-hmC) is essential for epigenetic plasticity (13).

Ten-eleven translocation (TET) enzymes are dioxygenases that convert 5-mC to 5-hmC in DNA by oxidation (14, 15). The oxidation products produce new epigenetic marks and promote further activation of direct or indirect de-methylation processes (16). Despite the existence of tissue- and cell-type- specific differences, approximately 5% of cytosines are classified as 5-mC and less than 1% as 5-hmC, in the mammalian cell genome (14). In mammalian genomic DNA, the TET family of dioxygenases can oxidize 5-mC to 5-hmC, 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC). The mammalian thymine DNA glycosylase (TDG) targets and binds 5-fC and 5-caC, which are then repaired to normal cytosine by base excision repair (17). Following conversion to 5-fC and 5-caC, the altered cytosine base is likely to be demethylated *via* the TDG-dependent process or other possible mechanisms (18). As a result, 5-hmC is considered the main stable epigenetic mark in the DNA de-methylation pathway, whereas 5-fC and 5-caC may serve as unstable intermediates (19). 5-hmC is recognized as the sixth base following 5-mC in the mammalian genome (20, 21).

Loss of 5-hmC may be an epigenetic signature of many cancers, with diagnostic and prognostic implications (22). The fact that reduced expression of the three TET genes has been associated with a decrease in the 5-hmC levels in various cancers suggests a plausible mechanism to explain 5-hmC loss in cancer cells (23). In addition, it has shown that reduced levels of 5-hmC are linked to tumorigenesis in genetically engineered mouse models of various types of human malignancies (23). In the current research, we aimed to evaluate the role of 5-hmC and TET enzymes in NSCLC patients and their association with the clinical characteristics of the patients.

Patients and Methods

The current study was conducted at the Medical Biochemistry Department, Faculty of Medicine, Zagazig University. We included 101 patients with NSCLC recruited from the Clinical Oncology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt. All patients were histopathologically confirmed to have NSCLC and underwent surgical excision. Tissue biopsies (tumor tissues and the nearby normal tissues, at least 5cm from the tumor) were obtained from all patients during surgery, flash-frozen in liquid nitrogen and were stored at –80°C until analysis. Clinical staging of all patients was done following the American Joint Committee of Cancer (AJCC) (24). The ethical committees of the Faculty of Medicine, Zagazig University, approved this research (FOMZU:317/2020). All patients had given a written informed consent.

DNA extraction and quantification of 5-hmC by ELISA. Extraction of the Genomic DNA from tumor and normal tissues was done by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) guided by the directions of the company. We used the Quest 5-hmC™ DNA ELISA Kit (Zymo Research, Irvine, CA, USA) to evaluate 5-hmC according to the company instructions. Using kit controls, we plotted a standard curve and the amount of 5-hmC was calculated as percentage.

Extraction of RNA, cDNA synthesis, and quantitative real-time polymerase chain reaction for TET mRNAs gene expression (qRT-PCR). Total RNA was extracted from the tumor and normal tissues by the miRNeasy Mini Kit (Qiagen) according to the producer's protocol. The extracted RNA was reverse transcribed into cDNA by the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) following the manufacturer's recommendations.

The mRNA expression levels of the TET genes were assessed by qRT-PCR, using the Stratagene, MX3000P quantitative PCR System with the following primers: TET1 forward (F), CCC TTG GAA ATG CCA TAG GAA; TET1 reverse (R), GAG AGC CTG CTG GAA CTG TTG; TET2 F, GGC TGT TGG CCA GAG ACT TA; TET2 R, ATA CCT GTA GGT GTT TGC CTG TTT A; TET3 F, GCC AAC TTC AAC ATA CCC TGG AC; TET3 R, CAC CTG GAT GTG GGA CTG TGTA A; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F, GCA CCG TCA AGG CTG AGA AC-3'; GAPDH R, TGG TGA AGA CGC CAG TCT CTA; as an internal control (25).

Briefly, the PCR reaction mix (total volume 20 µl) contained 5 µl cDNA, 10 µl Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 100 pmol/µl of the primers. The PCR protocol included 40 cycles of 92°C for 20 s and 60°C for 1 min.

The expression was presented as the Δ cycle threshold (ΔCt) value. The relative expression of TET mRNA was calculated using the comparative Ct method and TET values were normalized to the GAPDH mRNA (26). Data were analyzed using MxPro QPCR Software (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis. Continuous variables are presented as mean±SD. Student's *t*-test and one-way analysis of variance (ANOVA) were used to analyse differences in 5-hmC levels between different groups. Spearman's rank correlation analysis was performed to evaluate the strength and direction of the relationship between groups. For comparisons between tumor and normal tissues, receiver-operator characteristic (ROC) curve analyses were performed on patients' 5-hmC and TET mRNA levels including histopathology as a gold standard. Statistical significance was defined as a *p*-value less than 0.05. Statistical analysis was performed using version 9.4 of the SAS software package (SAS Institute, Inc., Cary, NC, USA).

Results

Levels of 5-hmC in NSCLC and their matched normal tissues. The levels of DNA 5-hmC were significantly lower in NSCLC tissues than in their matched normal tissues (0.28 ± 0.37 *vs*. 1.84 ± 0.58, respectively; $t=22.77$, $p<0.0001$) (Figure 1A). Moreover, ROC curve analysis revealed that the 5-hmC levels were highly distinguished between NSCLC tissues and normal tissues with specificity [area under the curve (AUC)=1.000, 95% CI=1.000-1.000, $p<0.0001$] (Figure 1B). The cutoff value was <1.01 with 100% sensitivity and 98.02% specificity.

TET expression in NSCLC tissues and their matched normal tissues. We studied the expression levels of *TET1*, *TET2*, and *TET3* mRNAs in both NSCLC tissues and matched normal tissues. All *TET* genes were significantly downregulated in
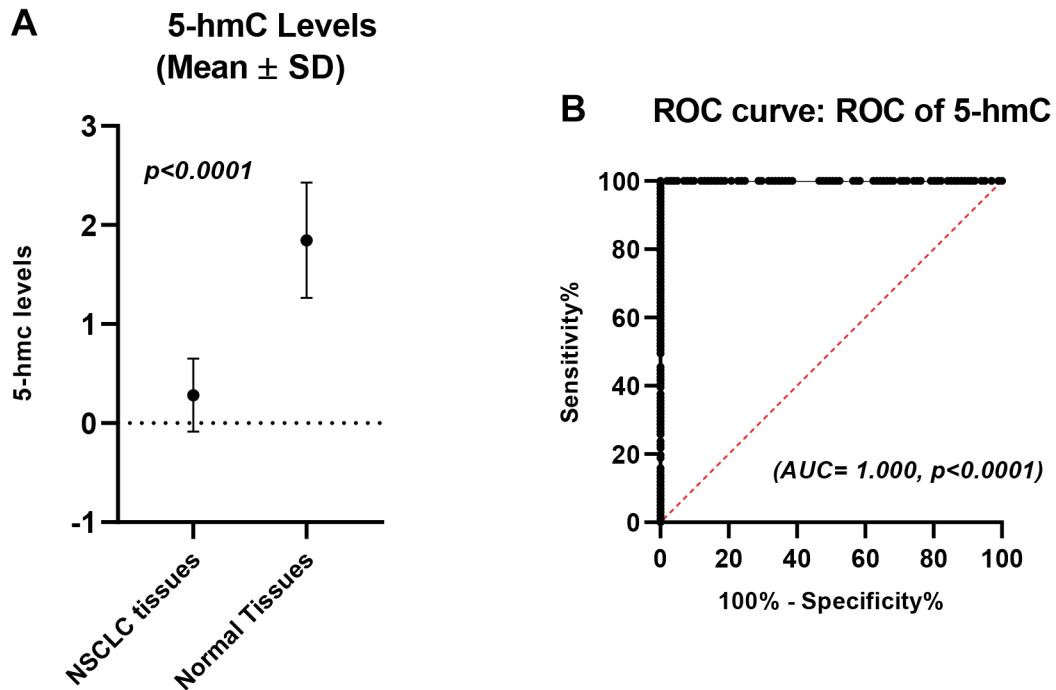
Figure 1. *Levels of 5-hydroxymethylcytosine (5-hmC). (A) ELISA was used to evaluate the levels of 5-hmC (%) in 101 matched pairs of non-small cell lung cancer (NSCLC) and normal tissue specimens (p<0.0001). (B) The area under the receiver operating characteristic (ROC) curve (AUC) of 5-hmC was 1, p<0.0001.*

NSCLC tissues in comparison to their matched normal tissues. TET1-mRNA level was 38.48±16.38 in NSCLC *vs*. 80.65±11.25 in normal tissues (t=21.33, $p<0.0001$), TET2-mRNA level in NSCLC was 5.25±2.78 *vs*. 9.52±1.01 in normal tissues (t=14.48, $p<0.0001$), and TET3-mRNA level was 5.21±2.8 in NSCLC *vs*. 9.51±0.86 in normal tissues (t=14.75, $p<0.0001$) (Figure 2A, Figure 3A, and Figure 4A).

ROC curves were plotted to evaluate the specificity of *TET1*, *TET2*, and *TET3* relative mRNA levels in discrimination between NSCLC tissues and normal tissues. The analysis revealed a high specificity of TET1, TET2, and TET3 for discrimination between NSCLC and normal tissues. The AUC for *TET1* was 0.97 ($p<0.0001$, cutoff value >62.31, sensitivity 98.02% and specificity 94.06%), for *TET2* was 0.95 ($p<0.0001$, cutoff value >8.122, sensitivity 95.05%, specificity 84.16%), and for *TET3* was 0.95 ($p<0.0001$, cutoff value 8.195, sensitivity 98.02%, specificity 85.15% specificity) (Figure 2B, Figure 3B, and Figure 4B).

Correlation between 5-hmC levels and TET expression in NSCLC tissues. Spearman correlation analysis revealed a statistically significant positive correlation between 5-hmC levels and mRNA expression levels of *TET1* (r_s=0.900, $p<0.001$), *TET2* (r_s=0.888, $p<0.001$), and *TET3* (r_s=0.887, $p<0.001$), in NSCLC tissues (Figure 5).

Correlation of 5-hmC levels and TET expression with clinical characteristics. Levels of 5-hmC were correlated with clinical characteristics of the patients. Specifically, low levels of 5-hmC were significantly correlated with older age and female sex, as well as with adverse clinical features, such as SCC, high-grade tumors, large tumor size, lymph node involvement, and advanced tumor stage (Table I). Similarly, low mRNA expression levels of *TET1*, *TET2*, and *TET3* were also significantly associated with all the above-mentioned clinical features (Table II).

Discussion

Cancer is now widely recognized as a genetic and epigenetic disorder, as certain cancer types often exhibit genetic mutations and repeated changes in the epigenetic environment. In many malignancies, genes encode enzymes, and protein complexes that regulate epigenetic patterns are the most commonly mutated (2). Lung cancer is driven by the accumulation of genetic and epigenetic changes in lung tissue (27). More specifically, genes including *p16INK4a, RASSF1A,* and *FHIT* have been found to be usually hypermethylated in NSCLC (28).

DNA methylation is a complicated process and stable epigenetic alteration that allows information to be transmitted from parent to daughter cells. The TET enzymes (TET1, 2,
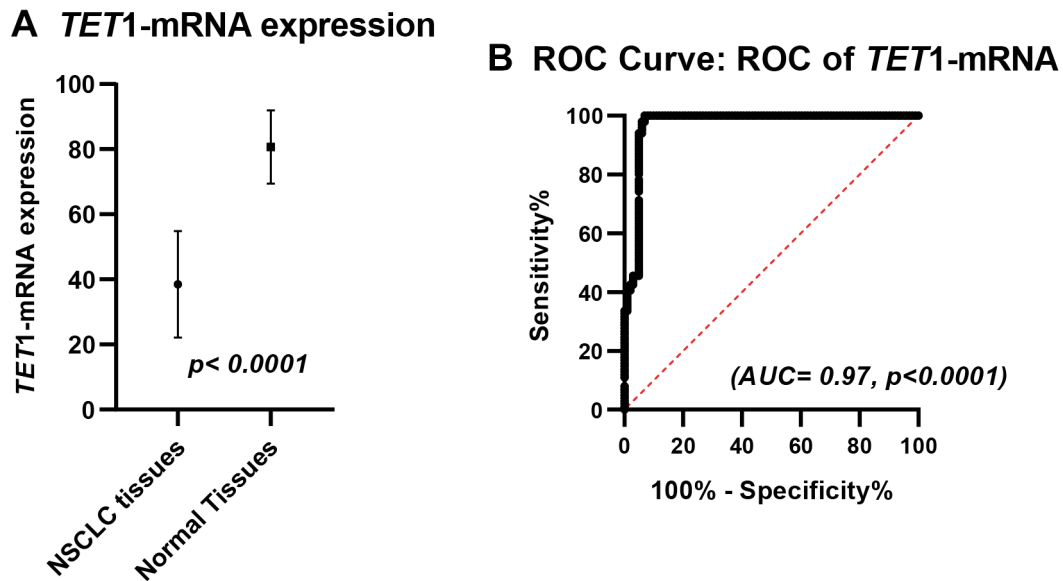
A *TET*1-mRNA expression

B ROC Curve: ROC of *TET*1-mRNA

Figure 2. *mRNA expression levels of TET1. (A) TET1 mRNA expression levels in 101 matched pairs of non-small cell lung cancer (NSCLC) and normal tissue specimens (p<0.001). (B) The area under the receiver operating characteristic (ROC) curve (AUC) of TET1 was 0.97, p<0.0001.*

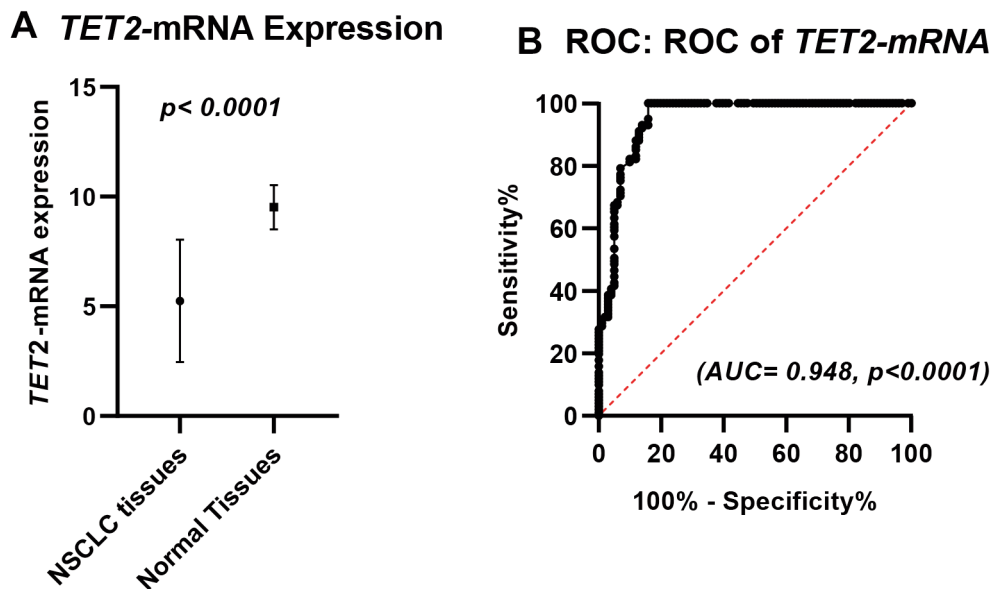## A *TET*2-mRNA Expression

B ROC: ROC of *TET2-mRNA*

Figure 3. *mRNA expression levels of TET2. (A) TET2 mRNA expression levels in 101 matched pairs of non-small cell lung cancer (NSCLC) and normal tissue specimens (p<0.001). (B) The area under the receiver operating characteristic (ROC) curve (AUC) of TET2 was 0.95, p<0.0001.*

and 3), which are responsible for fine-tuning of demethylation, can reverse DNA methylation by converting 5-mC bases to 5-hydroxymethyl marked bases (29-31). Growing evidence has confirmed the vital role of TET enzymes in controlling gene expression, enhancing cell differentiation, and preventing malignant transformation (32).

In the current research, we aimed to evaluate the role of 5-hmC and TET enzymes in NSCLC.

We studied 5-hmC in NSCLC and normal tissues using ELISA and observed a significant suppression of the 5-hmC level in NSCLC tissues compared to the normal controls. Moreover, we plotted a ROC curve for 5-hmC levels, which
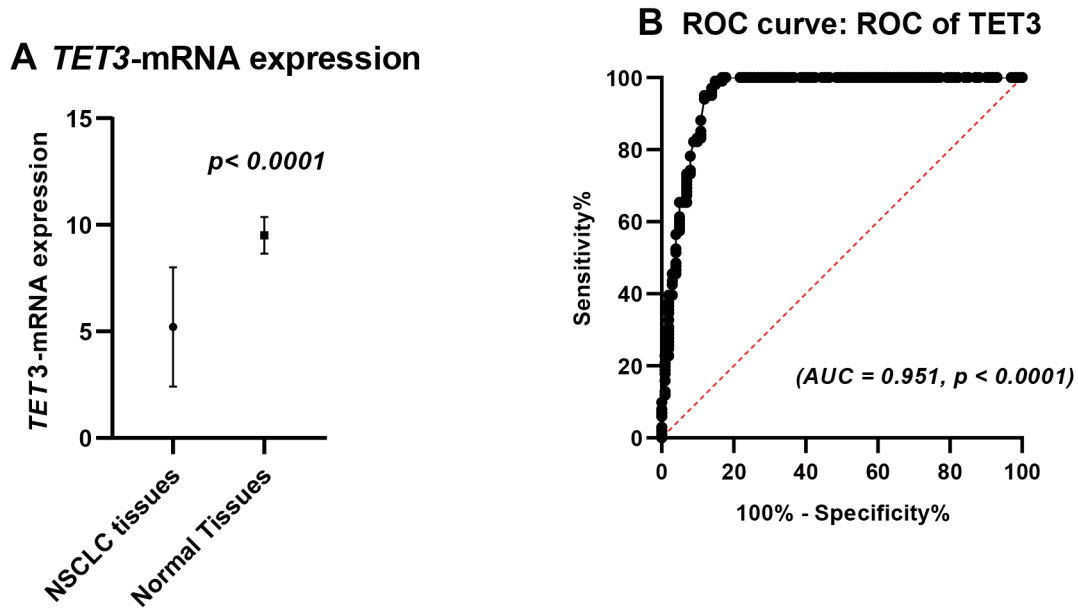
A *TET3*-mRNA expression

B ROC curve: ROC of TET3

Figure 4. *mRNA expression levels of TET3. (A) TET3 mRNA expression levels in 101 matched pairs of non-small cell lung cancer (NSCLC) and normal tissue specimens (p<0.001). (B) The area under the receiver operating characteristic (ROC) curve (AUC) of TET3 was 0.95, p<0.0001.*

showed a high specificity of 5-hmC for the discrimination between NSCLC and normal tissues. Several studies have also reported reduced levels of 5-hmC in various malignant tumors (33-35). In a study conducted by Murata *et al*. in patients with esophageal cancer squamous cell carcinoma (ESCC), 5-hmC was found at lower levels in ESCC tissues compared to normal mucosa (36). Moreover, Wang *et al*. (37) have revealed global hypomethylation of 5-hmC in lung squamous cell carcinoma tissues compared to paired normal controls, suggesting 5-hmC as a potential biomarker for the early detection of lung cancer.

In the present study, we observed that mRNA expression of *TET* genes was reduced in NSCLC tissues compared to normal tissues. Our observation was in consistency with the study by Murata *et al*. in a series of patients with oesophageal cancer, in which only the *TET2* level was statistically related to the 5-hmC level while *TET1* and *TET3* were not correlated to 5-hmC (36). Kudo *et al*. used immunostaining to examine 5-hmC levels in human cancer tissues (colonic, hepatic, brain, renal, rhabdomyosarcoma, and lung) and normal tissues and discovered that fluorescence patterns denoting 5-hmC were considerably lower in cancerous tissue compared to healthy tissues. The results demonstrated that 5-hmC levels were diminished in colon cancer (72.7%) and stomach cancer (75.0%), confirming the presence of a process that can inhibit the activity of 5-hmC levels in solid malignancies (38). Also, in a series of 546 prostate cancer patients who underwent radical prostatectomy, Storebjerg *et al*. investigated- 5-hmC

by immunohistochemistry of tissue microarray as a possible epigenetic marker. They reported that 5-hmC was significantly reduced in prostatic cancer tissue specimens compared to the normal prostatic tissues (39).

The current study also showed a positive correlation between 5-hmC levels and *TET1*, *TET2*, and *TET3* mRNA expression in NSCLC tissues. Our observation is in consistency with previous reports in colon adenocarcinoma, lung small cell carcinoma, and other malignancies (33, 34). Putiri *et al*. have previously reported that the depletion of TET1 in human embryonic carcinoma cells resulted in an increase of 5-mC in promoter elements and a decrease of 5-hmC. At the same time, loss of TET2 and TET3 reduced 5-hmC in a subset of TET1 targets, indicating a functional co-dependence (40). These findings are in line with the known function of TET proteins to catalyze DNA CpG demethylation, allowing normal cells to maintain an appropriate balance between CpG methylation and demethylation (41, 42). Interestingly, it has been shown that CpG methylation-mediated inactivation of the *TET1* gene elevated 5-mC levels in many cancer cells of multiple tissue types, suggesting the existence of a DNA methylation feedback loop including CpG methylation and TET1 during tumorigenesis (42).

We noted that reduced 5-hmC levels were associated with adverse clinical and prognostic features in NSCLC, such as older age, female sex, SCC, high-grade tumors, the large size of tumors, lymph node infiltration, and advanced

A

Spearman rank correlations between 5-hmC and TET1 expression in NSCLC

r_s=0.900, p<0.0001
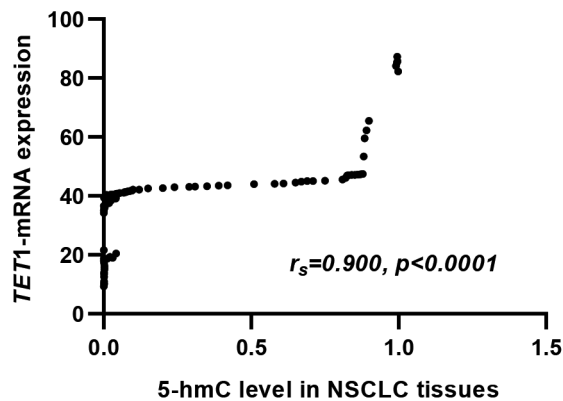
B

Spearman rank correlations between 5-hmC and TET2 expression in NSCLC tissues

r_s= 0.888, p<0.0001
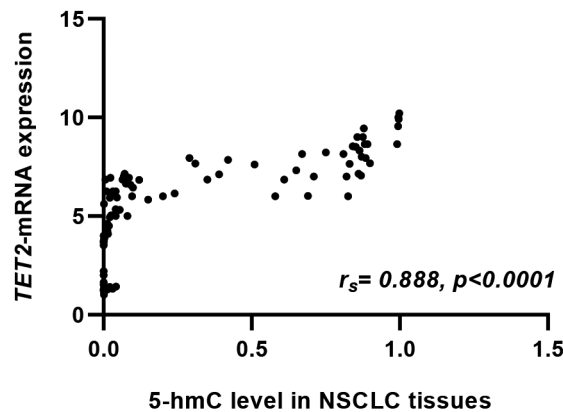
C

Spearman rank correlations between 5-hmC and TET3 expression in NSCLC tissues
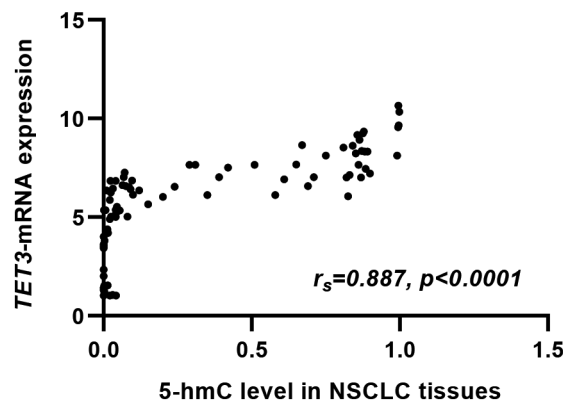

r_s=0.887, p<0.0001

Figure 5. *Spearman's correlation between 5-hmC and TET1, TET2, and TET3 mRNA levels in non-small cell lung cancer (NSCLC). Correlation between (A) 5-hmC levels and TET1 expression (r_S=0.900, p<0.001), (B) 5-hmC levels and TET2 expression (r_S=0.888, p<0.001), and (C) 5-hmC levels and TET3 expression (r_S=0.887, p<0.001).*

Table I. *5-hmC levels and patients' clinical features.*

		5-hmC		*p*-Value
		Mean	SD	
Age				
<50 years	48	0.57	0.35	<0.0001
≥50 years	53	0.01	0.01	
Sex				
Male	66	0.43	0.38	<0.0001
Female	35	0.0046	0.0093	
Pathology				
Adeno	62	0.45	0.38	<0.0001
SCC	39	0.005	0.0094	
Grade				
I	27	0.85	0.097	<0.0001
II	23	0.22	0.18	
III	26	0.015	0.015	
IV	25	0.005	0.011	
T				
T1	36	0.74	0.22	<0.0001
T2	46	0.039	0.044	
T3	19	0.002	0.003	
N				
N0	29	0.83	0.11	<0.0001
N1	44	0.095	0.13	
N2	28	0.005	0.01	
Stage				
I	29	0.84	0.115	<0.0001
II	44	0.095	0.128	
III	28	0.005	0.01	

SCC: Squamous cell carcinoma; SD: standard deviation.

tumor stage. In addition, decreased levels of *TET1*, *TET2*, and *TET3* in NSCLC were also associated with poor prognostic features, in agreement with previous findings in esophageal cancer (36), renal cell carcinoma (43), brain astrocytoma (44), and urinary bladder cancer (45). Chen *et al.* showed that 5-hmC was lost in the renal cell carcinoma tissues compared to the matched normal tissues and, moreover, the lower 5-hmC levels were associated with short overall survival (43). In addition, Zhang *et al.* reported a correlation of reduced 5-hmC with higher pathological grades and shorter survival in a series of patients with WHO grade II diffuse astrocytomas (44). Moreover, Peng *et al.* conducted a study in patients with urinary bladder cancer. They noted a partial or complete loss of immunoreactivity of 5-hmC malignant tissues compared to normal bladder tissues, while patients with higher 5-hmC immunoreactivity had longer survival than those with lower immunoreactivity. In addition, low 5-hmC immunoreactivity was associated with higher tumor stage and metastasis (45). Misawa *et al.* (25) have revealed that the levels of 5-hmC were related to the stage of the tumor,

Table II. *TET mRNAs expression and patients' clinical features.*

		TET1		*p*-Value	TET2		*p*-Value	TET3		*p*-Value
		Mean	SD		Mean	SD		Mean	SD	
Age										
<50 years	48	49.7	13.01	<0.0001	7.52	1.22	<0.0001	7.51	1.3	<0.0001
≥50 years	53	27.9	11.5		3.11	2.03		3.1	1.9	
Sex										
Male	66	47.1	12.1	<0.0001	7.03	1.42	<0.0001	6.99	1.5	<0.0001
Female	35	22.3	9.97		1.9	1.12		1.9	1.2	
Pathology										
Adeno	62	47.5	12.2	<0.0001	7.15	1.33	<0.0001	7.1	1.4	<0.0001
SCC	39	23.5	10.4		2.1	1.28		2.1	1.3	
Grade										
I	27	55.6	15.2	<0.0001	8.22	1.11	<0.0001	8.25	1.14	<0.0001
II	23	42.4	1.09		6.64	0.77		6.54	0.75	
III	26	37.9	2.16		4.78	1.3		4.71	1.27	
IV	25	17.02	6.18		1.26	0.24		1.23	0.25	
T										
T1	36	52.6	14.1	<0.0001	7.9	1.12	<0.0001	5.6	3.4	<0.0001
T2	46	39.7	2.11		4.83	1.74		4.7	1.8	
T3	19	14.6	3.1		1.2	0.15		1.2	0.19	
N										
N0	29	54.8	14.9	<0.0001	8.1	1.17	<0.0001	8.1	1.2	<0.0001
N1	44	40.2	2.5		5.83	1.23		5.7	1.21	
N2	28	18.9	8.03		1.4	0.53		0.18	0.1	
Stage										
I	29	54.8	14.9	<0.0001	8.1	1.17	<0.0001	8.13	1.19	<0.0001
II	44	40.2	2.2		5.83	1.23		5.73	1.208	
III	28	18.9	8.03		1.4	0.53		1.37	0.53	

SCC: Squamous cell carcinoma; SD: standard deviation.

and that lower levels of 5-hmC were associated with decreased disease-free survival and local recurrence in head and neck squamous cell carcinoma (HNSCC).

The present study has some limitations. First, our findings were limited to patients having surgery for clinically localized tumors; hence our observations cannot be applied to NSCLC patients with advanced or metastatic disease. Nonetheless, one of the strengths of the current study is that our findings are based on a relatively large consecutive and representative cohort from a single clinical institution, with clinical annotation and follow-up information accessible for all patients. Our study was limited to a relatively large NSCLC cohort from Egypt, and additional independent confirmation is needed. Future validation trials should involve various large NSCLC patient cohorts with long clinical follow-up and more ethnicities.

Conclusion

The levels of 5-hmC were reduced in NSCLC tissues compared to the normal controls and the decreased levels were related to adverse clinical features. In addition, 5-hmC levels were significantly correlated with *TET1*, *TET2*, and *TET3* mRNA expression. *TET* genes were downregulated in NSCLC patients and this downregulation was associated with poor prognostic features. Our data imply that 5-hmC is significantly reduced in NSCLC and suggest that loss of 5-hmC may represent a valuable prognostic biomarker for NSCLC.

Conflicts of Interest

The Authors declare no conflicts of interest

Authors' Contributions

Acknowledgements

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