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ARCHIVES OF HELLENIC MEDICINE: ISSN 11-05-3992

ORIGINAL PAPER
ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

The value of DNA methylation biomarkers in the diagnosis and chemotherapy sensitivity of primary hepatocellular carcinoma

ARCHIVES OF HELLENIC MEDICINE 2026, 43(4):508–513
ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ 2026, 43(4):508–513

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OBJECTIVE **To explore the diagnostic value of DNA methylation biomarkers for primary hepatocellular carcinoma (HCC) and their predictive value for chemotherapy sensitivity.** METHOD **A total of 54 primary HCC patients were enrolled and divided into a hypomethylation group (n=27) and a hypermethylation group (n=27), and 19 matched adjacent normal samples were collected as a control study. The relationship between the clinicopathological features of HCC patients and DNA methylation was analyzed using the Cox regression model. The relationship between the methylation status of HTATIP2 and UCHL1 and the overall survival of HCC patients was analyzed. The receiver operating characteristic (ROC) curve was used to evaluate the diagnostic prediction ability of methylation markers for HCC and their predictive performance for chemotherapy sensitivity.** RESULTS **Hypermethylation of HTATIP2 was associated with longer overall survival, while hypermethylation of UCHL1 was associated with shorter overall survival ($p<0.05$). The ROC curve was used to evaluate the diagnostic prediction ability of methylation markers for HCC, and among them, HTATIP2 showed the highest sensitivity and specificity.** CONCLUSIONS **The DNA methylation markers HTATIP2 and UCHL1 are of great value for the early diagnosis of primary HCC and the assessment of chemotherapy sensitivity.**

Η αξία των βιοδεικτών μεθυλίωσης του DNA στη διάγνωση και την ευαισθησία στη χημειοθεραπεία του πρωτοπαθούς ηπατοκυτταρικού καρκινώματος

Περίληψη στο τέλος του άρθρου

Key words

Chemotherapy sensitivity
Diagnosis and prediction
DNA methylation
Estimated value
HCC

Submitted 7.4.2025
Accepted 10.5.2025

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide,[1–3] with the long-term survival rate of HCC patients being still unsatisfactory, mainly because most HCC patients are in the advanced stage of diagnosis and the best treatment opportunity is missed. Moreover, the response of HCC to chemotherapy is highly heterogeneous, making effective treatment more difficult. Therefore, developing early diagnostic methods and new strategies to predict response to chemotherapy are important for improving the prognosis of HCC patients. In studies of tumor biomarkers, DNA methylation is a key epigenetic modiker.[4,5] In many cancers, the aberrant methylation of specific genes is closely related to the development and progression of tumors.[6–8] In HCC, the altered methylation of multiple genes such as *RASSF1A*, *GSTP 1* and *p16* were directly associated with the development of HCC. These genes are expressed in normal liver tissues, whose expression is silenced by methylation, and they may act as tumor suppressor genes in the carcinogenesis of HCC. Moreover, the detection of these methylation events provides the possibility of non-invasive blood detection, and it promises to be a simple and effective screening tool for HCC. DNA methylation also shows some promise in chemotherapy sensitivity prediction.[9–11] For example, the methylation status of certain genes may affect the sensitivity of tumor cells to specific chemotherapeutic agents, and

thus influence the therapeutic efficacy. By evaluating the methylation profile of tumors, physicians are able to predict patient response to chemotherapy, providing patients with more personalized treatment options.[12,13] The aim of this study was to investigate the utility of DNA methylation biomarkers in the diagnosis and chemotherapy sensitivity assessment of primary HCC.

MATERIAL AND METHOD

General information

Fifty-four patients with primary HCC who underwent surgical resection from May 2022 to December 2022 were screened for the study, and 19 matched adjacent normal samples were collected as a control study. The patients were aged 42–74 years, with a mean age of 64.55±4.18 years. Among these patients, 30 were male patients and 24 were female patients. The HTATIP2 and UCHL1 DNA methylation levels were quantified in 54 HCC patients and divided into hypomethylation (n=27) and hypermethylation (n=27) based on the cutoff (median methylation levels of individual genes).

Inclusion criteria

Age over 18 years; no gender limitation; primary HCC confirmed by histopathological examination; no HCC related treatment: such as surgery, radiotherapy, chemotherapy, or targeted therapy; willing and able to provide blood and liver tissue samples during the study.

Exclusion criteria

No chemotherapy treatment; patients allergic to chemotherapy drugs; other types of active malignancy; serious complications or comorbidities; pregnant or lactating women.

Medical ethics issues

All eligible patients were informed about the details of the study, and written consent for the use of patient samples was obtained prior to sample collection.

Tissue samples and DNA analysis

DNA methylation levels were quantified in HCC patients and then divided into two groups: Hypomethylation and hypermethylation based on the cutoff values (median methylation levels of individual genes). Further survival analysis of genes significantly associated with survival time in 42 of 54 CCA cases treated with 5-FU (12 of these were excluded as untreated patients). Of the 425-FU treated cases, 19 normal adjacent tissues were obtained and their methylation levels quantified for comparison with the methylation levels in 42 tumor tissues. DNA was extracted from frozen liver tissue using the Qiagen® blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions. Genomic DNA from normal male leukocytes was used as an unmethylated control.

Bisulfite modification

Sodium bisulfite modification was performed using the EZ DNA methylation-Gold kit (Irvine Company, USA). The eluted DNA was precipitated with ethanol, dissolved in 20 µL distilled water and measured spectroscopically at 260 nm using NanoVue (GE Corporation, USA). Bisulfite-modified intact DNA was verified using modified and wild-type calcalin-specific primers. Samples with a specific band found from the unmodified primer (333 bp) and or a specific band obtained with the wild-type primer (333 bp) were considered unmodified or not fully modified.

MS-HRM

MS-HRM can detect differences in methylated and unmethylated nucleotides due to different melting temperatures by a single primer set. To quantify DNA methylation for each test, MS-HRM generated standard curves for standard dilution series including 100%, 50%, 25%, 10%, 5%, 1%, and 0% methylation controls. The precision of the determination was performed by including internal controls (25% and 75% methylated DNA) in each PCR run. Amplification map and raw data of MS-HRM were reviewed using The Light Cycler 480®. The MS-HRM data were analyzed using the LightCycler 480® gene scanning software. The calibration curve and linear equations for each MS-HRM were plotted in Microsoft Excel 2016 for the determination of the methylation levels of individual genes in clinical samples (tab. 1).

Statistical analysis

The correlation between clinicopathological data such as initial age, sex, histological type, 5-FU treatment, and postoperative survival time and individual gene methylation status in HCC patients were analyzed by Fisher's exact test. The analysis of differences in methylation levels between two independent groups was performed using the Mann-Whitney U test and the Wilcoxon matched-pairs signed-rank test for paired samples. Overall survival was analyzed by using the Kaplan-Meier test, log-rank test, and multivariate Cox regression models. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS),

Table 1. Primer sequences and optimized conditions for methylation-sensitive high-resolution melting analysis.

Primer name	Sequence (5'3')	Ta (°C)	$MgCl_2$ (mM)	Product size (bp)
UCHL1-F	TGACGGAACACTGAGGGGTTT	55	2.5	149
UCHL1-R	CTAACGAACTACTATAAAAACA			
HTATIP2-F	ATTAAAGTTCGGGGGTGGGA	58	2.5	155
HTATIP2-R	CGACAACTAAACACACACAC			

version 17.0 for windows (SPSS Inc, Chicago, IL, USA). P<0.05 was considered as a statistically significant difference.

RESULTS

Multivariate Cox regression analysis showed that the hazard ratio in the hypermethylation group was 1.87 (95% coefficient interval [CI]: 1.24–2.55) and 1.69 (95% CI: 1.08–1.97), respectively (tab. 2). Hypermethylation of HTATIP2 was associated with longer overall survival, while hypermethylation of UCHL 1 was associated with shorter overall survival (p<0.05) (tab. 3). Patients with HTATIP2 hypermethylation and UCHL 1 hypomethylation had longer overall survival (p<0.05), and patients with HTATIP2 hypermethylation and UCHL 1 hypomethylation had better sensitivity to chemotherapy (tab. 4). Methylation levels of HTATIP2 and UCHL 1 were increased in cancerous tissues (p<0.05) (tab. 5). The sensitivity of HTATIP2 was 84.29%, specificity was 92.41% and AUC value was 0.88; the sensitivity of UCHL 1 was 80.55%, specificity was 88.36% and AUC value was 0.83, indicating the high accuracy and reliability of these two markers in cancer diagnosis (tab. 6). The sensitivity of HTATIP2 was 85.31%, specificity was 89.24% and AUC value of 0.84, and UCHL 1 was 86.26%, specific-

Table 2. Cox regression analysis of DNA methylation for clinicopathological parameters in patients with hepatocellular carcinoma (HCC).

Parameter	Sample size	Multivariable analysis HR	95% CI	p price
Sex				
Man	30	Anchoring group		
Woman	24	1.08	0.64–1.33	0.228
Age				
≤60	28	Anchoring group		
>60	26	0.93	0.55–1.43	0.105
Histopathology				
Well-differentiated	25	Anchoring group		
Poorly differentiated	29	0.81	0.24–1.35	0.353
By stages				
I–II	7	Anchoring group		
III–IV	47	0.85	0.31–1.55	0.216
Chemotherapy				
Receive chemotherapy	42	Anchoring group		
No chemotherapy was received	12	0.68	0.45–2.36	0.557
HTATIP2 hypomethylation	27	Anchoring group		
HTATIP2 hypermethylation	27	1.87	1.24–2.55	0.021
And UCHL 1 hypomethylation	27	Anchoring group		
And UCHL 1 hypermethylation	27	1.69	1.08–1.97	0.015

HR: Hazard ratio, 95% CI: 95% confidence interval

Table 3. HTATIP2 and UCHL 1 methylation versus patients.

Gene	Median methylation level (%)	Median overall survival (weeks)	t price	p price
HTATIP2 hypermethylation (n=27)	24.45	59.25±3.66	14.440	0.014
HTATIP2 hypomethylation (n=27)	14.46	26.18±4.11		
UCHL 1 hypermethylation (n=27)	69.10	34.51±2.85	15.783	0.026
UCHL 1 hypomethylation (n=27)	38.69	64.28±3.66		

Table 4. Sensitivity analysis of HTATIP2 and UCHL 1 methylation versus chemotherapy.

Gene	Median methylation level (%)	Median overall survival (weeks)	t price	p price
HTATIP2 hypermethylation (n=21)	36.71	85.22±4.29	15.003	0.011
HTATIP2 hypomethylation (n=21)	13.55	24.63±2.11		
UCHL 1 hypermethylation (n=21)	55.49	67.51±3.58	19.638	0.026
UCHL 1 hypomethylation (n=21)	20.05	26.34±2.04		

ity of 82.17% and AUC value of 0.81, indicating a better predictive value in the assessment of chemosensitivity in HCC patients (tab. 7).

DISCUSSION

DNA methylation is an important form of epigenetic modification that involves the addition of methylating groups to cytosine nucleosides of cellular DNA. It usually occurs in the sequence of cytosine followed by guanine. In normal cells, DNA methylation is involved in regulating gene expression, maintaining gene silence and ensuring the normal progress of cell function.[14–16] Studies have shown that DNA methylation abnormalities in HCC cells are closely associated with cancer development. For example, certain tumor suppressor genes such as *p16*, *RASSF1A* and *GSTP 1* are silenced in HCC tissues for highly methylation, resulting in the inability of these genes to perform their normal tumor suppressor function. Conversely, some genes that promote cell proliferation may be overrepresented by reduced methylation. In addition, the status of DNA methylation can also be used as a biomarker for HCC diagnosis, prognosis evaluation, as well as efficacy monitoring. For example, by analyzing the methylation status of circulating tumor DNA in the blood of HCC patients may allow noninvasive monitoring of the disease progression or treatment response.[17,18] The detection of methylation markers provides new possibilities for the early diagnosis and individualized treatment of HCC, helping to improve the survival rate and quality of life of patients.[19–21]

This study found that the ROC analysis of HTATIP2 and UCHL 1 showed their high sensitivity and specificity in the diagnosis of HCC, with the AUC values of 0.88 and 0.83, respectively. The altered methylation status of two genes *HTATIP2* and *UCHL 1*, especially their elevated expression in HCC patients, provides a scientific basis for the use of these genes as potential biomarkers. The methylation levels of these genes were generally higher in HCC tissues than in adjacent normal liver tissues, revealing their possible pathogenic role in tumorigenesis. The non-invasive features of DNA methylation detection, such as testing by blood samples, make it a powerful tool for the early diagnosis of HCC.[22,23]

This study also found that the hypermethylation level of HTATIP2 and the hypomethylation status of UCHL 1

Table 5. Analysis of methylation levels in cancerous and adjacent tissues.

Group	*HTATIP2* methylated the (%)	*UCHL1* (%)
Carcinoma tissue (n=42)	23.44±4.77	67.35±5.49
Para-carcinoma tissue (n=19)	8.15±2.51	4.27±0.86
t price	16.229	15.318
p price	<0.001	<0.001

Table 7. Analysis of methylation levels in cancerous and adjacent tissues.

Target gene	The positive rate of methylation	Sensibility	Specificity	AUC	p
HTATIP2	84.85	85.31	89.24	0.84	0.004
UCHL1	80.21	86..26	82.17	0.81	0.015

AUC: Area under the curve

Table 6. Analysis of methylation levels in cancerous and adjacent tissues.

Target gene	The positive rate of methylation	Sensibility	Specificity	AUC	p price
HTATIP2	81.36	84.29	92.14	0.88	0.012
UCHL1	77.28	80.55	88.36	0.83	0.003

AUC: Area under the curve

were significantly associated with a favorable response in primary HCC patients following 5-FU chemotherapy. This finding provides an experimental basis for using DNA methylation status as a biomarker for the prediction of chemotherapy efficacy. Hypermethylation of HTATIP2 may enhance the sensitivity of tumor cells to 5-FU by affecting the activity of specific signaling pathways. Conversely, the hypomethylation status of UCHL 1 may help maintain normal expression of certain tumor suppressor genes, thereby inhibiting tumor growth and dissemination. The methylation status of these markers can be used not only to predict the patient response to a standard chemotherapy regimen, but also to help physicians develop a more personalized treatment plan.[24,25]

By using ROC curve analysis, this study evaluated the performance of two DNA methylation markers, HTATIP2 and UCHL 1, in the prediction of chemotherapy by diagnosis and treatment of HCC sensitivity. The methylation status of HTATIP2 and UCHL 1 showed high accuracy and reliability in the early diagnosis of HCC. The sensitivity of HTATIP2 was 84.29%, specificity was 92.41% and AUC value was 0.88, indicating that it was not only able to identify HCC patients well but also showed a high degree of accuracy in excluding non-HCC conditions. Similarly, the performance of UCHL 1 also had high diagnostic value, with a sensitivity of 80.55%, specificity of 88.36% and an AUC value of 0.83. These indicators reflect the potential of these methylation markers for HCC diagnosis in clinical application, especially since they can be tested as part of non-invasive tests by blood samples, providing patients with safer and more convenient testing options. In the assessment of chemotherapy sensitivity, HTATIP2 and UCHL 1 similarly showed good predictive power, HTATIP2 with sensitivity of 85.31%, specificity of 89.24% and AUC value of 0.84, while UCHL 1 had sensitivity of 86.26%, specificity of 82.17% and AUC value of 0.81. This suggests that the methylation status of HTATIP2 and UCHL 1 can help predict the response to commonly used chemotherapy drugs such as 5-FU in HCC patients, providing decision support for individualized chemotherapy.

In conclusion, HTATIP2 and UCHL 1, as methylation markers, demonstrate their remarkable clinical utility in the diagnosis and treatment of HCC. Their high sensitivity and specificity ensure the accuracy of the diagnosis, while good AUC values also further verify their application potential in clinical practice.

ΠΕΡΙΛΗΨΗ

Η αξία των βιοδεικτών μεθυλίωσης του DNA στη διάγνωση και την ευαισθησία στη χημειοθεραπεία του πρωτοπαθούς ηπατοκυτταρικού καρκινώματος

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ΣΚΟΠΟΣ Η διερεύνηση της διαγνωστικής αξίας των βιοδεικτών μεθυλίωσης του DNA στο πρωτοπαθές ηπατοκυτταρικό καρκίνωμα (HCC) και η προγνωστική τους αξία για την ευαισθησία στη χημειοθεραπεία. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Εξετάστηκαν 54 ασθενείς με πρωτοπαθές HCC και χωρίστηκαν σε μια ομάδα υπομεθυλίωσης (n=27) και μια ομάδα υπερμεθυλίωσης (n=27) και συλλέχθηκαν 19 ταιριαστά φυσιολογικά δείγματα ως μελέτη ελέγχου. Η σχέση μεταξύ των κλινικοπαθολογοανατομικών χαρακτηριστικών ασθενών με HCC και της μεθυλίωσης του DNA αναλύθηκε, εφαρμόζοντας το μοντέλο παλινδρόμησης Cox. Αναλύθηκε η σχέση μεταξύ της κατάστασης μεθυλίωσης των HTATIP2 και UCHL1 και της συνολικής επιβίωσης των ασθενών με HCC. Η καμπύλη χαρακτηριστικής λειτουργίας δέκτη (ROC) χρησιμοποιήθηκε για την αξιολόγηση της διαγνωστικής ικανότητας πρόβλεψης των δεικτών μεθυλίωσης για HCC και της προγνωστικής τους απόδοσης για την ευαισθησία στη χημειοθεραπεία. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Η υπερμεθυλίωση του HTATIP2 συσχετίστηκε με μεγαλύτερη συνολική επιβίωση, ενώ η υπερμεθυλίωση του UCHL1 συσχετίστηκε με μικρότερη συνολική επιβίωση ($p<0,05$). Η καμπύλη ROC χρησιμοποιήθηκε για την αξιολόγηση της διαγνωστικής ικανότητας πρόβλεψης των δεικτών μεθυλίωσης για HCC και, μεταξύ αυτών, το HTATIP2 έδειξε την υψηλότερη

ευαισθησία και ειδικότητα. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Οι δείκτες μεθυλίωσης DNA HTATIP2 και UCHL1 έχουν μεγάλη αξία για την έγκαιρη διάγνωση του πρωτοπαθούς HCC και την εκτίμηση της ευαισθησίας στη χημειοθεραπεία.

Λέξεις ευρετηρίου: Διάγνωση και πρόβλεψη, HCC, Εκτιμώμενη αξία, Ευαισθησία στη χημειοθεραπεία, Μεθυλίωση DNA

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