

The Screening of Volatile Markers for Hepatocellular Carcinoma

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Abstract

Background: Breath analysis became promising for noninvasive diagnoses of cancer with sophisticated spectrometry technology introduced. This study aimed to screen volatile markers for hepatocellular carcinoma (HCC).

Methods: Breath samples were collected from 30 HCC patients who were comorbid with type B hepatitis and cirrhosis and from 27 hepatocirrhosis patients and 36 healthy persons, both taken as controls. The volatile organic compounds in the samples were analyzed with gas chromatography/mass spectrometry and the markers were selected by comparing their levels between groups. Each of the markers was evaluated by receiver operating characteristic (ROC) curves and a discriminant function using the markers was established. The relationships of α -fetoprotein (AFP) levels and clinical stages with the concentrations of the markers were also investigated.

Results: 3-Hydroxy-2-butanone, styrene, and decane were screened as potential markers, among which 3-hydroxy-2-butanone was found to have the best diagnostic value. The diagnostic function using these markers had a sensitivity of 86.7% and a specificity of 91.7% between HCC patients and normal controls and a sensitivity of 83.3% and a specificity of 91.7% by cross-validation. No statistically significance ($P > 0.05$) was found for the concentration differences of these markers between HCC patients with AFP >400 or <400 $\mu\text{g/L}$ or between stage I-II and stage III-IV patients.

Conclusion: These volatile organic compounds could be useful as breath markers of HCC patients, independent of AFP levels or clinical stages.

Impact: Breath analysis could be useful for early diagnosis of HCC, especially for AFP-negative HCC. *Cancer Epidemiol Biomarkers Prev*; 19(9); 2247–53. ©2010 AACR.

Introduction

Most of the hepatocellular carcinoma (HCC) patients are diagnosed at advanced stages and lose the opportunity of radical resection, which is the major reason for the high mortality rate of the disease (1). Ultrasound imaging and serum α -fetoprotein (AFP) were taken as standard screening methods for early detection of primary liver cancer. However, about 30% of primary liver cancer patients are AFP negative (2, 3). Therefore, there is a dire need for new screening methods for primary liver cancer.

The detection of volatiles in human exhaled gas by Pauling et al. (4) in 1971 opened new insights into a new

scientific field: analysis of volatile organic compounds (VOC) for clinical diagnosis (5, 6). Since then, many findings have proved that breath analysis could be a promising noninvasive diagnostic method for cancer with state-of-the-art spectrometry technologies introduced, such as proton transfer reaction mass spectrometry, solid-phase microextraction (SPME), and gas sampling (7-11). In 1985, Gordon et al. applied a method for the microanalysis of gas to detect VOCs in exhaled air from lung cancer patients (12), and later in 1988, O'Neill et al. determined more exactly the specific compounds, principally alkanes and benzene derivatives, with potential as breath biomarkers of lung cancer (13). Subsequently, Phillips et al. developed a portable breath-collecting apparatus that was applied to determine volatile biomarkers in the breath of lung cancer patients, and a set of 22 VOCs were selected. A diagnostic model using these VOCs was sensitive and specific for primary lung cancer and equally accurate in the early and advanced stages (14). Other study groups, using sensor array analyses (15) or gas chromatography (GC)/mass spectrometry (MS) combined with SPME (16), also showed that a combination of VOCs rather

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than a single VOC alone successfully classified between individuals with and without lung cancer. Lately, our group discovered that 1-butanol and 3-hydroxy-2-butanone in breath could possibly be taken as useful breath biomarkers for discerning potential lung cancer patients. Our study showed that compared with lung squamous cell carcinoma, these VOC levels were significantly higher in lung adenocarcinoma (17). Breath analysis was used to detect not only lung cancer but also breast cancer (18, 19) and tumors of the head and neck (20). Regrettably, there were no related reports to date available on the diagnosis of HCC. In this study, we tried to screen the volatile markers in the breath of HCC patients, establish a diagnostic function based on these markers, and assess their possibilities for diagnosis of HCC patients.

Patients and Methods

Subjects

Three groups were studied: HCC patients, hepatocirrhosis patients, and healthy volunteers. The patients were recruited from the First Affiliated Hospital of Anhui Medical University, Hefei, China (42 subjects) and Anhui Provincial Hospital, Hefei, China (15 subjects). Inclusion criteria for the carcinoma group were HCC patients comorbid with type B hepatitis and cirrhosis, who were untreated and histologically or cytologically confirmed for the disease. For the hepatocirrhosis group, patients who were clinically diagnosed with hepatocirrhosis induced by chronic hepatitis B virus infection were allowed to participate in the study. Exclusion criteria for patient group were Child-Pugh class B or C, cancer history, or any other diseases such as hepatitis A or C virus infection, alcoholic hepatocirrhosis, diabetes, hyperthyrosis, chronic bronchitis, uremia, etc. The third group was composed of healthy volunteers (36 subjects) who were recruited among the patients' relatives and the hospital staff with no history of cancer or other chronic disease.

The history of smoking was obtained from all subjects, and the ex-smokers were defined as the ones who had abstained from smoking for at least 1 year before the study. The clinical stage of the HCC was determined according to the stage standard for primary liver cancer of American Joint Committee on Cancer Staging in 2002. Fourteen patients were classified as stage I-II and the others were stage III-IV. Table 1 summarizes the demographic characteristics of the studied groups.

Study design

Within this retrospective pilot study, breath samples were analyzed by means of GC/MS combined with SPME. The potential volatile markers of HCC were determined by comparing the VOCs in the breath of HCC patients and controls (healthy persons and hepatocirrhosis patients). The diagnostic values of the markers were investigated with receiver operating characteristic (ROC)

Table 1. Demographic characteristics of studied groups

	HV	HC	HCC
Subjects (n)	36	27	30
Age (mean \pm SD)	48.75 \pm 10.97	51.67 \pm 11.25	53.0 \pm 12.40
Sex (male/female)	24/12	18/9	26/4
Current smokers	7	3	8
Ex-smokers	0	2	0
AFP > 400 μ g/L (n)			14

NOTE: If subjects have abstained from smoking for no longer than a week before their breath air was collected, they were considered as current smokers.

Abbreviations: HV, healthy volunteers; HC, hepatocirrhosis; HCC, hepatocellular carcinoma.

curves and a diagnostic function, and the relationships of AFP levels and clinical stages with the concentrations of the markers were also investigated.

Breath collection

After meeting the entry criteria, a short questionnaire on personal habits, such as smoking and drinking, was answered and an appointment was arranged to collect the sample. All subjects had fasted overnight. They followed three steps in breathing into the Tedlar gas bags (4L-T2PV/L, Delin Company): take a deep breath; hold it for 4 seconds; then exhale smoothly into the bag. Simultaneously, the samples of ambient air were collected for reference. The samples were taken to the laboratory and detected immediately after collecting.

VOC extraction and analysis

The VOCs were extracted by means of SPME using a 75- μ m carboxen-polydimethylsiloxane fiber (Ampel Company), which was put into the sample bag for 30 minutes at room temperature and then thermally desorbed for 10 minutes in the GC injection port at 220°C. Analysis was done with GC/MS (GC/MS-QP 2010 Plus). The GC was coupled with a flame ionization detector. The VOCs were separated on a ZB-624 column (30.0 m length \times 0.32 μ m thickness \times 0.32 mm i.d.; Restek); the carrier gas was helium with a flow rate of 1.5 mL/min. Split mode was used with a split ratio of 4:1. The column temperature program was an initial temperature of 40°C, increase to 120°C at 5°C/min, hold for 4 minutes, then increase to a final temperature of 200°C, and hold for 5 minutes; total time was 31 minutes. The MS was in full-scan mode in the 35-400 mass to charge (m/z) range; the temperatures of interface at 280°C and electron impact ion source at 200°C were set; and the solvent cutoff time was 1.5 minutes.

The VOCs were identified by means of their mass spectrums and confirmed by comparing their main

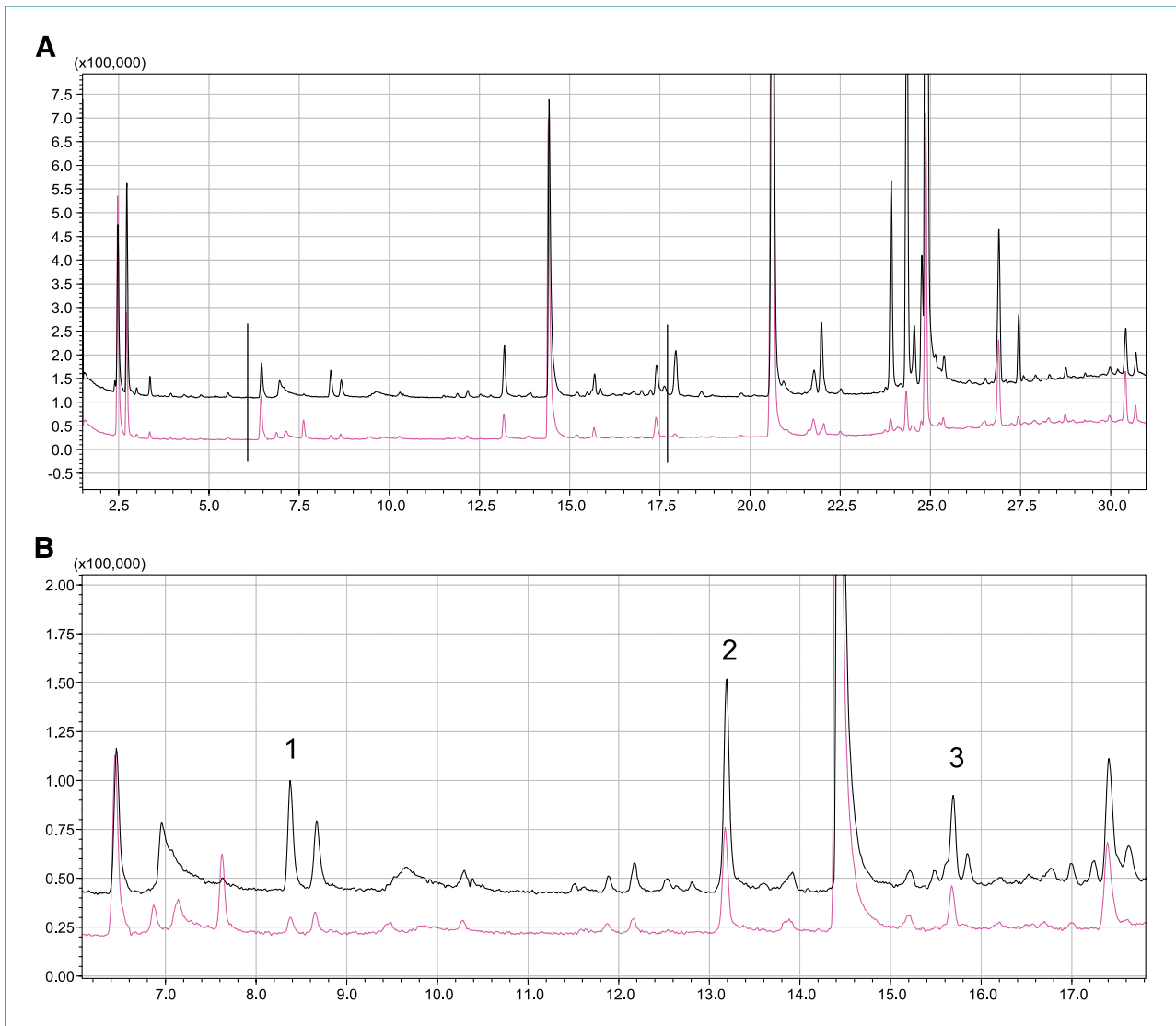


Figure 1. Comparison of chromatography profiles between a normal control and a HCC patient. A, the graph of scanning in 31 min. B, the graph of the enlarged part in A between the two vertical lines. Red graph, a normal control, male, 52 y old, nonsmoker. Black graph, a HCC patient, male, 50 y old, stage IIIA, AFP = 219.2 µg/L, nonsmoker. 1, 3-hydroxy-2-butanone; 2, styrene; 3, decane. Y-axis, ion abundance; X-axis, retention time (in minutes).

fragment ions with those of the standards. The potential volatile markers of the cancer were selected by comparing the chromatography peak profiles between healthy persons and HCC patients. The markers were quantified with their standard curves.

The method was validated by investigating its precision and linearity. The precision was assessed by relative standard deviation (RSD). The standard gases were directly prepared in the bag filled with helium. Six replicate measurements of standard gases in

Table 2. The alveolar gradients and statistical differences of the markers between groups

	HV (ng/L)	HC (ng/L)	HCC (ng/L)	HV vs HCC	HV vs HC	HC vs HCC
3-Hydroxy-2-butanone	0.04 (0-1.48)	2.51 (0.80-5.66)	7.91 (3.49-16.65)	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.002
Styrene	1.91 (0-7.70)	5.05 (0-18.38)	17.76 (6.53-23.75)	<i>P</i> < 0.001	<i>P</i> = 0.146	<i>P</i> = 0.015
Decane	1.12 (0.19-2.73)	3.19 (0.83-8.58)	5.74 (2.11-14.82)	<i>P</i> < 0.001	<i>P</i> = 0.028	<i>P</i> = 0.076

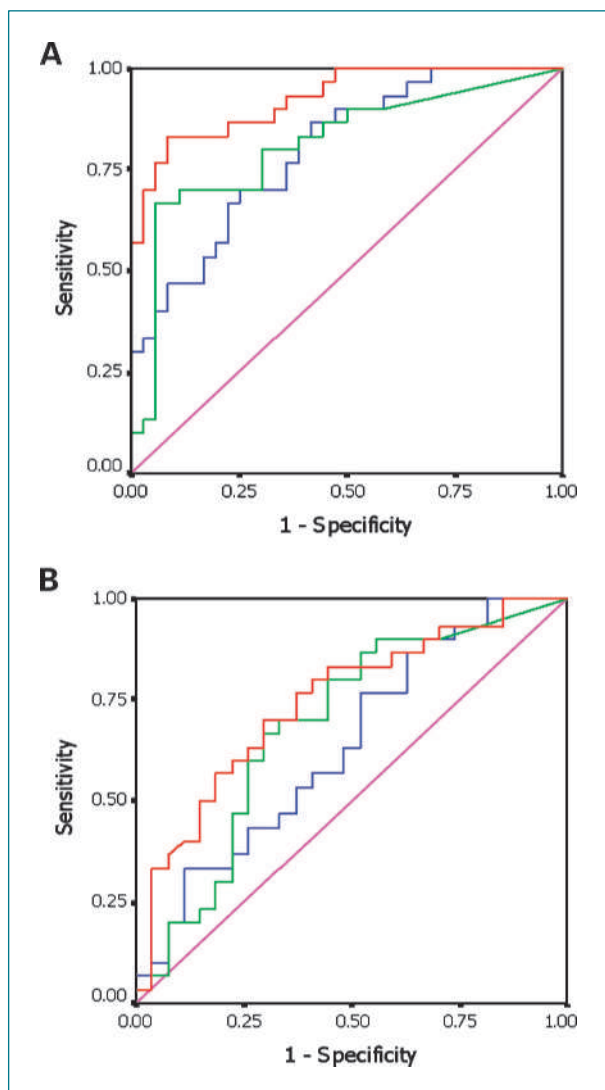


Figure 2. The ROC curves of 3-hydroxy-2-butanone (red curve), styrene (green curve), and decane (blue curve). A, ROC curves between healthy volunteers and HCC patients. B, ROC curves between hepatocirrhosis patients and HCC patients. The discriminant power of the markers was assessed by the area under the ROC curve (AUC). If AUC > 0.5, the marker was taken as possessing diagnostic value ($P < 0.05$).

constant concentration were done to obtain the peak area values. Precision was calculated as RSD of the values. The linearity (regression equation) was established by analyzing the relationship between the con-

centrations of standard gases and peak areas. Five different concentrations of the selected VOCs were measured to obtain standard curves. Two analyses were repeated for each concentration.

Statistical analysis

The alveolar gradient of each VOC was determined as its abundance in breath minus its abundance in ambient air. SPSS 10.0 was used for the statistical analyses. The filtration and comparison of the volatile markers were statistically analyzed with the Mann-Whitney U test, and $P < 0.05$ was considered significant. The concentrations were expressed as median values (25th-75th percentile) in nanograms per liter for each of the selected VOCs. For the markers, ROC curves were used to assess their discriminative power for the subjects with and without HCC; then, a diagnostic model was established with Fisher's linear discriminant functions. For the model, all subjects from the HCC and healthy volunteer groups were used to establish the model. The accuracy of the model was tested by cross-validation with a leave-one-out classification procedure, in which each subject was classified by an equation derived from the other subjects who were used in the derivation of the model except the validated one. The model was further tested in the hepatocirrhosis group.

Results

Volatile markers of HCC patients

The levels of the VOCs were assessed initially according to the values of their peak areas. 3-Hydroxy-2-butanone, styrene, and decane were selected as the markers in the breath of HCC patients (Fig. 1). The concentration values were calculated with their regression equations, with linear correlations ranging from 0.988 to 0.998 and the RSD values of the markers ranging from 2.40% to 6.72%. The alveolar gradient and the statistical significances of the between-group differences of the markers are shown in Table 2.

There was a statistically significant difference for 3-hydroxy-2-butanone between all three groups; no statistically significant difference for styrene between the healthy volunteer and hepatocirrhosis groups; and no statistically significant difference for decane between the hepatocirrhosis and HCC groups.

Table 3. The diagnostic values of the markers between healthy volunteers and HCC patients

	Cutoff point	Sensitivity (%)	Specificity (%)	AUC (95% CI)	P
3-Hydroxy-2-butanone	2.44	83.3	91.7	0.926 (0.865-0.986)	0.000
Styrene	14.92	66.7	94.4	0.812 (0.702-0.92)	0.000
Decane	1.64	86.7	58.3	0.798 (0.694-0.902)	0.000

Table 4. The diagnostical values of the markers between hepatocirrhosis patients and HCC patients

	Cutoff point	Sensitivity (%)	Specificity (%)	AUC (95% CI)	P
3-Hydroxy-2-butanone	4.32	70.0	70.4	0.745 (0.616-0.873)	0.002
Styrene	13.73	66.7	70.4	0.686 (0.544-0.829)	0.016
Decane	2.20	76.7	48.1	0.637 (0.492-0.782)	0.076

The discriminant power assessment

The discriminant power of the markers between groups was assessed by the ROC curves (Fig. 2; Tables 3 and 4). Taking 2.44 ng/L as a cutoff value for 3-hydroxy-2-butanone, with a sensitivity of 83.3% and a specificity of 91.7%, the sum of sensitivity plus specificity was maximal. Taking 4.32 ng/L as a cutoff value for 3-hydroxy-2-butanone, with a sensitivity of 70.0% and a specificity of 70.4%, the sum of sensitivity plus specificity was maximal.

The diagnostical function of HCC patients

The model was established using 3-hydroxy-2-butanone, styrene and decane.

Diagnostical function:

$$Y_1 = 0.039x_1 + 0.05x_2 + 0.108x_3 - 0.977$$

$$Y_2 = 0.302x_1 + 0.183x_2 + 0.401x_3 - 5.665$$

x_1 is 3-hydroxy-2-butanone in alveolar gradient, x_2 is styrene in alveolar gradient, and x_3 is decane in alveolar gradient. If $Y_1 > Y_2$, we consider the person as cancer negative; if $Y_1 < Y_2$, we consider the person as cancer positive.

The model was assessed with the persons from the HCC and healthy volunteer groups, with a sensitivity of 86.7% (26 of 30 patients) and a specificity of 91.7% (33 of 36 persons). Only 2 of 16 HCC patients with AFP <400 µg/L were diagnosed as cancer negative. Cross-validation with the leave-one-out classification method yielded a sensitivity of 83.3% (25 of 30 patients) and a specificity of 91.7% (33 of 36 persons). The model was

further tested with hepatocirrhosis patients; 18 of 27 (66.7%) persons were diagnosed as cancer negative and the other 9 (33.3%) were cancer positive.

The relationships of AFP levels and clinical stages with the levels of the markers

The concentration differences of 3-hydroxy-2-butanone, styrene, and decane had no statistical significance ($P > 0.05$) between HCC patients with AFP >400 or <400 µg/L or between patients of clinical stage I-II or stage III-IV (Table 5).

Discussion

In the present study, we investigated HCC patients comorbid with type B hepatitis and cirrhosis, the healthy volunteers, and hepatocirrhosis patients induced by chronic hepatitis B virus infection. The two latter groups were used as controls, as most of the HCC cases developed from cirrhotic liver (21) and chronic hepatitis B virus infection was the major cause of cirrhosis in China (2, 22). About 30% of HCC patients were AFP negative (AFP <20 µg/L; refs. 3, 23) and serum AFP levels were significantly different among HCC patients with different cancer size and tumor-node-metastasis (TNM) stages (3). Because the sample size was relatively small, the level differences of the markers were investigated between HCC patients with AFP >400 µg/L and AFP <400 µg/L; simultaneously, the levels between patients at stage I-II and stage III-IV were also assessed.

Three potential VOCs, 3-hydroxy-2-butanone, styrene, and decane, were selected. The correlation coefficients for

Table 5. The levels of the markers in HCC patients with different AFP levels and clinical stages

	3-Hydroxy-2-butanone	Styrene	Decane
AFP (µg/L)			
<400	5.81 (2.06-16.20)	19.88 (8.95-28.17)	4.43 (1.82-15.84)
>400	10.31 (4.63-18.79)	15.92 (3.59-20.70)	6.84 (2.11-14.58)
<400 vs >400	$P = 0.240$	$P = 0.101$	$P = 0.608$
Stage			
I-II	14.08 (4.42-18.88)	19.88 (15.02-24.65)	4.43 (1.77-8.46)
III-IV	5.47 (2.87-10.90)	15.99 (4.63-24.07)	10.46 (2.25-15.84)
I-II vs III-IV	$P = 0.088$	$P = 0.212$	$P = 0.280$

NOTE: The data are presented as median values (25th-75th percentile)

linear regression ranged from 0.988 to 0.998 and the RSDs were no more than 6.72%, which indicated that the method was reliable. The levels of the selected VOCs in the breath of HCC patients were significantly higher than those in healthy volunteers; therefore, we believe they were produced during cancer metabolism and could be markers of HCC patients in breath.

Breath analysis was reported mainly in studies of lung cancer, and only in a handful of reports on breast cancer (18, 19) and carcinomas of the head and neck (20). Styrene and decane were also found as volatile markers of lung cancer by other research groups (14, 24), but 3-hydroxy-2-butanone was not reported to date as a marker of cancer in breath. The levels of styrene between healthy volunteers and hepatocirrhosis patients showed no statistically significant difference ($P = 0.146$), indicating that the marker could be used to discriminate between the HCC and cancer-negative patients. Decane could possibly predict high-risk patients with hepatocirrhosis because it showed no statistically significant difference between the hepatocirrhosis and HCC groups ($P = 0.076$).

In the present study, we investigated the diagnostic values of the VOCs with ROC curves, and 3-hydroxy-2-butanone was found to be the best marker for HCC, with a sensitivity of 83.3% and a specificity of 91.7% between healthy volunteers and HCC patients and with a sensitivity of 70.0% and a specificity of 70.4% between hepatocirrhosis patients and HCC patients. The diagnostic model using the three VOCs may be a better choice in diagnosis, which showed a sensitivity and a specificity of 86.7% and 91.7%, respectively, with a sensitivity of 83.3% and a specificity of 91.7% by cross-validation with the leave-one-out classification method. Fourteen of 16 HCC patients with AFP <400 $\mu\text{g/L}$ were diagnosed as cancer positive, indicating that the model had high accuracy and the diagnos-

tic ability would not decrease in patients with lower AFP levels. The further testing with hepatocirrhosis patients showed that 9 of 27 patients were cancer positive. To determine whether these patients had a higher risk to develop HCC needed a long-term follow-up study.

The differences in the levels of 3-hydroxy-2-butanone, styrene, and decane were not statistically significant ($P > 0.05$) between HCC patients with AFP >400 $\mu\text{g/L}$ or AFP <400 $\mu\text{g/L}$ or between patients at stage I-II or stage III-IV; thus, we could suppose that the diagnostic ability of the markers for early-stage HCC would not decrease, which was consistent with the reports of Phillips et al. that the diagnostic accuracy was similar in all TNM stages of lung and breast cancers (18, 25). Because the diagnostic function and these markers had no relationship with AFP levels and clinical stages, we could deduce that they could possibly be helpful for early diagnosis of HCC, especially for AFP-negative HCC.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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