●他にいくつかの肝庇護剤があるが，使用の根拠となる科学的評価が乏しい．
●肝庇護療法の組み合わせ，回数，投与量などをテーラーメイド化する．
●肝庇護療法は病診連携の必要性の高い領域である．

表2　瀉血療法とALT値改善効果についての報告例

報告者	ALT改善率	ALT正常化率	備考
Hayashi, H. et al. (1994)	63.8%	50.0%	
Kato, J. et al. (2001)	77.0%	64.7%	
Yano, M. et al. (2002)	42.6% (1year)	46.2%	
Yano, M. et al. (2004) RCT	38.1% (3 mo)	N/A	対照と有意差あり
Kawamura, Y. et al. (2005)	65.2%	43.9%	

る過剰な鉄を赤血球造血に動員して，結果として肝臓での鉄含有量を減少させることを目的として瀉血療法が考案された[7]．具体的には1回200 m*l*/週を，約3ヵ月をめどにヘモグロビン値で11 g/d*l*，もしくはフェリチン値を10 ng/m*l*以下となるまで瀉血を行う．続いて維持瀉血期間として，ヘモグロビン値，フェリチン値を上記程度に維持するように1～3ヵ月間隔で200 m*l*の瀉血を行う．鉄欠乏状態を維持するためには鉄制限食の摂取を持続することも必要であり，鉄の一日摂取量6 mg以下を目標とした鉄制限を継続するよう指導する．瀉血療法の効果については，わが国を中心にいくつかの研究が報告されているが，RCTが行われたものは少ない(表2)．2004年にYanoらが行ったRCTでは，3ヵ月の瀉血継続によって，38.1%の患者にALT値の改善が認められ，非施行群に比べ有意差があったとしている[8]．

4.　その他の肝庇護剤

古くから，牛肝からの抽出成分を用いた製剤(プロヘパール®)や，グリチルリチン含有の経口製剤(グリチロン®)などの肝庇護剤が用いられてきたが，これらに関してはエビデンスとなる検討結果がほとんどなく，標準的な肝庇護療法としては推奨しにくい．

まとめ●

ウイルス排除を目標としたIFN療法の恩恵に預かれない患者群がいまだに存在し，さらにこれらの患者の多くは肝硬変への進展，肝発癌の危険にさらされている．本稿であげた治療法は完全ではないが，これらのリスクを軽減させる可能性がある．同一の内容を漫然と続けるのではなく，いくつかの治療法の組み合わせや，回数，投与量を調節することでできるだけALTを正常値に近づける努力をすべきであり，その方法は個々の患者によって異なるため，外来主治医がそれぞれにテーラーメイド化して処方内容を決めていく必要がある．また，肝庇護療法は安価であり，安全性も高いため，診療所レベルでの取り組みが行いやすいというメリットがある．病診連携の観点から考えても，多くのかかりつけ医にこれらの方法に習熟してもらい，積極的に行ってもらうことが重要である．肝疾患拠点病院の役割の中心の一つはインターフェロン療法の普及であるが，これとは別に肝庇護療法についてもノウハウを地域で蓄積し，公開することでこれをサポートすることができるのではないかと考えている．

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Review

Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC–ESI–MS/MS☆

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ABSTRACT

High-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is a powerful method for the microanalysis of compounds in biological samples. Compared with gas chromatography–mass spectrometry (GC–MS), this method is more broadly applicable to various compounds and usually does not require a derivatization step before analysis. However, when neutral sterols are analyzed, the sensitivities of usual HPLC–MS/MS method are not superior to those of GC–MS because the sterols are relatively resistant to ionization. In this review, we introduce the recent development of HPLC–MS/MS analysis for the quantification of non-cholesterol sterols. By adding an effective derivatization step to the conventional procedure, sterol analysis by HPLC–MS/MS surpassed that obtained by GC–MS in sensitivity. In addition, sufficient specificity of this method was achieved by selected reaction monitoring (SRM) and thorough chromatographic separation of each sterol.

Contents

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1. Introduction

Some cholesterol precursors and oxidized cholesterol (oxysterols) are important molecules in the regulation of lipid homeostasis in the body [1]. In addition, they have been used as serum biomarkers for whole body cholesterol synthesis [2,3], intestinal cholesterol absorption [4], hepatic bile acid synthesis [5,6] and the diagnosis of inherited disorders in cholesterol metabolism [7–12]. Therefore, quantification of non-cholesterol sterols in biological samples is a very important technique in studies of lipid metabolism.

Gas chromatography (GC) with flame ionization detection [2,13], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [14–16] or HPLC with refractive index (RI) detection [16] are the most generally used methods for the analyses of sterols. However, these methods cannot quantify minor components of endogenous sterols with sufficient sensitivity and specificity.

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are powerful detection methods, which are suitable for GC and HPLC systems. These detectors are not only superior in terms of sensitivity but are also highly specific compared with flame ionization, UV and RI detectors. GC–MS has been widely accepted as a reliable analytical method for the determination of sterols in biological samples [17–19]. However, during the last decade, HPLC–MS or HPLC–MS/MS has also come to be used conveniently because these methods do not always require deconjugation and derivatization steps before analysis [20,21]. In addition, while HPLC methods do not cause decomposition of some labile sterols, such as 24S,25-epoxycholesterol, the high temperatures achieved during GC methods can cause degradation of unstable sterols [22,23].

In this review, we introduce the recent development of HPLC–MS/MS methods for the quantification of sterols in biological samples. An effective derivatization step, thorough chromatographic separation and selected reaction monitoring (SRM) by MS/MS have achieved excellent sensitivity and specificity for this method. The method has become a central approach for the simultaneous quantification of sterols in small amounts of biological samples.

2. Methods to increase the sensitivity of sterols

2.1. Ionization

Advances in ionization techniques have greatly contributed to the development of LC–MS. Electron impact ionization (EI) is the most commonly used approach for GC–MS analysis of sterols. This ionization method was applied to HPLC–MS by using a particle beam (PB) interface. In 1995 Sattler et al. [24] analyzed plasma 7-dehydrocholesterol and in 1998 Careri et al. [25] quantified oxysterols by HPLC–PB-EI–MS, with detection limits of 10 ng (about 26 pmol) and 2–3 ng (about 5–7.5 pmol), respectively.

While EI is not applicable to polar or high molecular weight compounds, electrospray ionization (ESI) is broadly applicable method for polar compounds in a wide range of molecular weights (Fig. 1). In addition, this ionization source is generally exchangeable in the same mass spectrometer with atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) sources that are applicable to less polar and low molecular weight compounds. Thus, ESI and the complementary use of APCI or APPI have recently become the standard ionization methods for HPLC–MS.

Since sterols are less polar and relatively low molecular weight compounds, APCI [26–33] or APPI [20,34] have been preferentially used for analysis by HPLC–MS. The detection limits of cholesterol precursors and sitosterol by HPLC-APCI–MS were well below 1 pmol [31], that of cholesterol by HPLC-APCI–MS/MS was 2.2 pmol [32], and those of oxysterols by HPLC-APCI–MS were in the range of 0.2–0.8 ng (about 0.5–2.0 pmol) [26] or 0.1–0.75 ng (about 0.25–1.9 pmol) [27].

While it had previously been considered that ESI was not suitable for the analysis of neutral sterols, in 2007 McDonald et al. reported that sterols were sufficiently ionized when HPLC–ESI–MS/MS was employed using the Applied Biosystems 4000 QTrap triple quadrupole system [21]. According to this report, detection limits of dihydroxy- or epoxysterols were 5–60 fmol while those of monohydroxysterols were 175–2000 fmol on-column. These sensitivities are not inferior to those of APCI, but

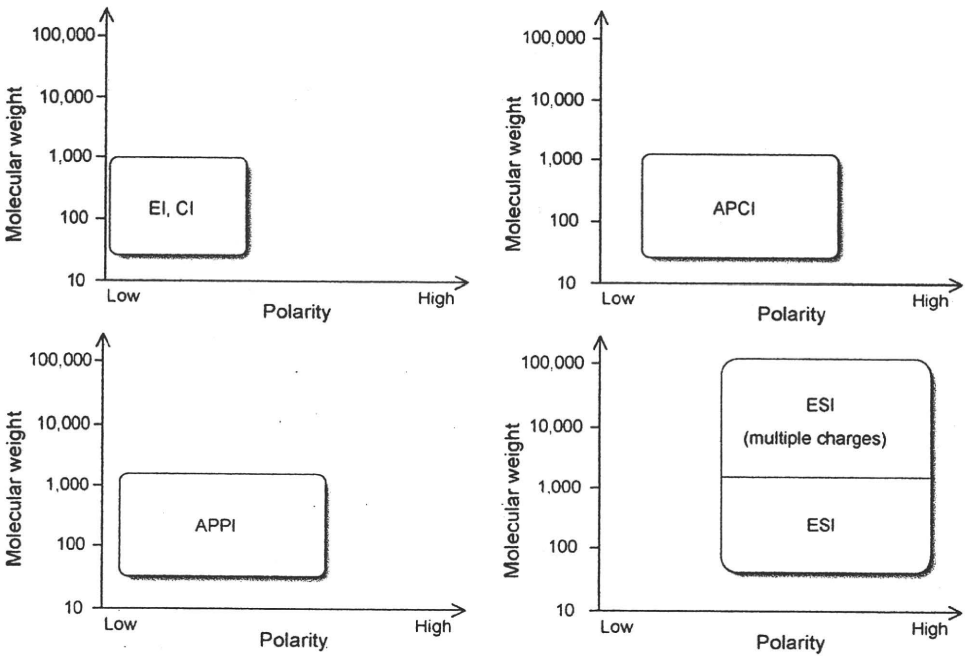

Fig. 1. Applications of various ionization methods to LC–MS. EI, electron impact ionization; CI, chemical ionization; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ESI, electrospray ionization.

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Table 1
Detection limits of cholesterol and representative oxysterols by different analytical methods.

Author	Year	Reference	Method (ionization mode)	Derivatization	Lower limit of detection	
					Cholesterol	Oxysterol[a]
Sanghvi et al.	1981	[36]	GC–MS (P-EI)	TMS ether	NA	120 fmol (7αOH)
Hylemon et al.	1989	[14]	HPLC-UV	C4	NA	20 pmol (7αOH)
Honda et al.	1991	[35]	GC–HR-MS (P-EI)	DMES ether	NA	4 fmol (7αOH)
Careri et al.	1998	[25]	HPLC–PB-MS (P-EI)	–[b]	5 pmol	5 pmol (7βOH)
Manini et al.	1998	[26]	HPLC–MS (P-APCI)	–	NA	500 fmol (7βOH)
Van Berkel et al.	1998	[38]	HPLC–MS/MS (P-ESI)	FC ester	41 amol	NA
Razzazi-Fazeli et al.	2000	[27]	HPLC–MS (P-APCI)	–	NA	1.2 pmol (7αOH)
Nagy et al.	2006	[31]	HPLC–MS (P-APCI)	–	<1 pmol	NA
Tian et al.	2006	[32]	HPLC–MS/MS (P-APCI)	–	2.2 pmol	NA
Griffiths et al.	2006	[46]	HPLC–MS/MS (P-ESI)	Girard P hydrazone	NA	<2.5 fmol
McDonald et al.	2007	[21]	HPLC–MS/MS (P-ESI)	–	1 pmol	60 fmol (7αOH)
Honda et al.	2008	[43]	HPLC–MS/MS (P-ESI)	picolinyl ester	260 amol	NA
Honda et al.	2009	[44]	HPLC–MS/MS (P-ESI)	picolinyl ester	NA	10 amol (7αOH)

Abbreviations: P-EI, positive electron impact ionization; TMS, trimethylsilyl; NA, not available; 7αOH, 7α-hydroxycholesterol; HPLC-UV, HPLC equipped with an ultraviolet detector; C4, 7β-hydroxy-4-cholesten-3-one; GC–HR-MS, high-resolution GC–MS; DMES, dimethylethylsilyl; HPLC–PB-MS, HPLC–MS with particle beam interface; 7βOH, 7β-hydroxycholesterol; P-APCI, positive atmospheric pressure chemical ionization; P-ESI, positive electrospray ionization; FC, ferrocenecarbamate.

[a] Data of 7α-Hydroxycholesterol or other oxysterols with the structures similar to 7α-Hydroxycholesterol.

[b] Without derivatization.

one weak point is that the sensitivity depends greatly on the instruments.

2.2. Derivatization

As shown in Table 1, conventional HPLC-APCI–MS for the detection of one of the representative oxysterols, 7α-hydroxycholesterol (1.2 pmol) [27], is not as sensitive as GC–MS (4–120 fmol) [35,36] because sterols are relatively resistant to ionization. On the other hand, this oxysterol may be quantified by HPLC–ESI–MS/MS [21] with sensitivity (60 fmol) equivalent to GC–MS, but not all HPLC–ESI–MS/MS instruments are applicable to this sensitive analysis of sterols.

To overcome these problems, sterols have been derivatized to more polar structures. The charged moieties were introduced into the hydroxyl group of the sterols as an *N*-methylpyridyl ether [37], a ferrocenecarbamate ester [38], a sulfate [39], a mono-(dimethylaminoethyl) succinyl ester [40], a dimethylglycine ester [41], and a picolinyl ester [42–44]. Furthermore, the native carbonyl group of oxysterols or the 3-oxo structure, converted from 3β-hydroxysterols by cholesterol oxidase, was derivatized to Girard P hydrazone [45–47]. Each of these derivatizations enhanced the ionization efficiency of the sterols in the ESI process and markedly increased the sensitivity.

As for ionization polarity, the sulfate derivatives are easily deprotonated and exhibit a high ionization efficiency in the negative ESI mode [39]. In contrast, the other derivatives are positively charged permanently or easily protonated, so that they are suitable for the positive ESI mode. Generally speaking, the negative mode exhibits lower background noise compared with that in positive mode. However, the positive mode provides much abundant ions than negative one [48].

It may be noted here that derivatizations are useful to increase the ionization of steroids, not only in ESI, but also in the APCI processes [49,50]. However, derivatized sterols have been preferably analyzed by ESI because ESI is broadly applicable to various derivatives. In the positive APCI mode, the introduction of moieties with proton affinity increases ionization, while those with highly polar functional groups inhibit ionization and decrease the sensitivity [50]. Thus, the selection of effective derivatives for positive APCI is not as easy as that for ESI. Negative APCI is also used after the addition of electron affinity moieties to sterols. This electron-capturing derivatization in negative APCI mode was first reported by Singh et al. [51] and has been applied to the determination of tissue cholesterol by Kuo et al. [52].

3. Methods to increase the selectivity of each sterol

3.1. Use of appropriate internal standards

Deuterium-labeled sterols are ideal internal standards for quantification by HPLC–MS. The addition of internal standards compensates for the loss of target sterols during clean-up procedures and for the variation in injection volume onto the HPLC column. Thus, internal standards are necessary for accurate quantification by chromatographic methods. In addition, internal standards are used to determine the variation in the retention time of each sterol among samples. When peaks of target sterols are very small or they are not completely separated from interfering peaks, the retention time of the internal standard gives additional information to identify the target peaks. Several deuterated standards are commercially available, as reported by McDonald et al. [21]. Although deuterated analogs are not available for all sterols, deuterated sterol with a structure similar to the target sterol can be used as a surrogate [21,44]. Alternatively, we can use coprostanol as a convenient internal standard for monohydroxysterols in human serum [43]. Coprostanol is synthesized from cholesterol by intestinal bacteria but is not absorbed from the intestine and is not detected in human serum.

3.2. Sample clean-up

The structures of non-cholesterol sterols are similar to native cholesterol, which usually exists at least 100–10,000 times greater than the target sterols in bulk-lipid extracts from biological samples. Therefore, good separation from cholesterol is necessary for reliable quantification of the non-cholesterol sterols. Because oxysterols and epoxysterols are more polar than cholesterol, most of them can be separated from cholesterol by a solid-phase extraction cartridge [18,53]. However, the complete separation of some less polar oxysterols and non-cholesterol monohydroxysterols from cholesterol by using such a cartridge is difficult. Thus, for the analysis of whole sterol profiles in biological samples, the role of solid-phase extraction is limited to the elimination of nonpolar compounds, such as fatty acyl esters of cholesterol [53], that are strongly retained on reversed-phase HPLC columns.

3.3. Separation by HPLC

Since we have not achieved selective elimination of cholesterol from bulk-lipid extracts using solid-phase extraction cartridges,

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non-cholesterol sterols must be separated from cholesterol by the final HPLC–MS or HPLC–MS/MS analyses. In addition, the separation between non-cholesterol sterols is also important to quantify each sterol. However, isobaric sterol isomers, e.g. cholesterol and lathosterol [43] or 24S-hydroxycholesterol and 25-hydroxycholesterol [21,44], often exhibit similar precursor to product ion fragmentations, so that even SRM cannot always differentiate these sterols. Therefore, careful HPLC separation of each sterol is crucially important to quantify these isomers by selected ion monitoring (SIM) or SRM [33,43].

Although normal phase columns can be used for the separation of sterols by HPLC-PB–MS [24,25] and HPLC–APCI-MS [16], reversed-phase columns are preferably used in HPLC–PB-MS [25], most of the HPLC–APCI-MS [26–31,33], HPLC–APPI-MS [20,34], and virtually all HPLC–ESI–MS methods with [42–44,47] and without derivatization [21]. Normal phase HPLC sometimes achieves better separation of each sterol compared with reversed-phase HPLC [16], but the latter is preferred for HPLC–MS because it displays higher reproducibility than normal phase and the polar mobile phase favors ionization.

Our experiences show that there are many minor unidentified sterols in biological samples and complete chromatographic or mass spectrometric separation of all sterols by a single analysis is impossible at present. We need to select the best column and mobile phase according to the target sterols in which we are interested.

3.4. Selection by MS/MS

Although MS/MS is not an almighty method for the differentiation of each sterol, it is much more specific and sensitive than UV and RI detectors [16]. The triple quadrupole mass spectrometer is the most suitable instrument for the highly sensitive quantification of sterols. SRM obtained by MS/MS can eliminate interfering peaks with different precursor to product ion fragmentations at specific collision energies. In addition, the monitoring of multiple SRM pairs for a single sterol adds confidence to the identification of the compound and provides further information regarding compound identification based on their relative intensities [21].

Another way to improve the selectivity of SRM is to increase the resolution of the triple quadrupole mass spectrometer. Although the resolution depends on the capacity of the mass spectrometer, analysis with higher resolution reduces interfering peaks and improves S/N ratio of the chromatogram. Furthermore, Griffiths et al. have reported high-resolution MS by a hybrid quadrupole/time of flight (TOF) mass spectrometer [46] or high-resolution MS^n by a hybrid linear ion-trap/Fourier transform mass spectrometer [54]. These mass spectrometers exhibit excellent selectivity, but sensitivity and dynamic range for quantification do not reach those achieved by the triple quadrupole mass spectrometer.

4. Characteristics of picolinyl ester derivative of sterols

4.1. Sensitivity

We have successfully introduced a picolinyl moiety into the hydroxyl group of various sterols and have demonstrated that the picolinyl ester derivatization is a simple and versatile method for sensitive and specific quantification using positive HPLC–ESI–MS/MS [42–44]. The idea originated from a report by Yamashita et al. [55] in which they compared HPLC–ESI–MS/MS behaviors among the picolinyl, 6-methylpicolinyl, nicotinyl, 2-methyoxynicotinyl and isonicotinyl derivatives of estrone, estradiol, dehydroepiandrosterone and testosterone. The picolinyl derivatives showed the best HPLC–ESI–MS/MS behavior and 100-fold higher detection response by SRM compared with underivatized steroid molecules [55,56]. In addition, they have successfully applied the picolinyl derivatization to corticosteroids [57,58] and aldosterone [59,60].

As for sterols, the detection limits (S/N = 3) of cholesterol picolinate and oxysterol dipicolinates by HPLC–ESI–MS/MS (SRM) analysis were about 260 amol and 5–25 amol on-column, respectively [43,44], which was about 3860-fold and 1000-fold, respectively, more sensitive than those with underivatized HPLC–ESI–MS/MS analysis [21]. On the other hand, the detection limits of native cholesterol and oxysterols by HPLC-APCI–MS/MS analysis were about 100 fmol and 10 fmol, respectively [43,44].

4.2. Mass spectra

All picolinyl ester derivatives of monohydroxysterols exhibited adduct ions of $[M + Na + CH_3CN]^+$ as the base peaks [43], while those of di-, tri- and tetra-hydroxysterols, ~~epoxysterols~~ and ketosterols showed $[M + Na]^+$ ions as the base peaks under our HPLC–ESI–MS conditions [44]. However, it should be noted here that the base peaks would change depending on the composition of mobile phase. In contrast to other derived moieties, the picolinyl group is not permanently charged, so that even in the case of oxysterols with multiple hydroxyl groups, a single charged ion was predominant in the positive ESI mass spectra.

Collision of $[M + Na + CH_3CN]^+$ of the picolinyl derivatives of monohydroxysterols at a relatively low collision energy (10–15 V) resulted in the predominant formation of $[M + Na]^+$ as product ions, while the use of higher collision energies (25–30 V) resulted in the [picolinic acid + Na]$^+$ ($m/z = 146$) ion as the most abundant product ion. In contrast, collision of $[M + Na]^+$ of the picolinyl derivatives of di-, tri- and tetra-hydroxysterols, ~~epoxysterols~~ and ketosterols resulted in the formation of [M + Na–picolinic acid]$^+$ or [picolinic acid + Na]$^+$ ions at any specific collision energy depending on the sterols (10–30 V). Representative MS/MS fragmentation patterns of the picolinyl derivatives are shown in Fig. 2, and the most suitable collision energies and precursor to product ions of each sterol for SRM are listed in Table 2.

4.3. Synthesis of derivatives

The derivatization and purification steps are very simple [44]. As shown in Fig. 3, the reagent mixture, consisting of 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, picolinic acid, pyridine and triethylamine, is added to the sterol extract, and incubated at 80 °C for 60 min. Excess reagents are then precipitated by the addition of *n*-hexane, and the clear supernatant containing picolinyl ester derivatives is collected and evaporated at 80 °C under nitrogen. The residue is redissolved in 50 μl of acetonitrile and an aliquot is used for HPLC–ESI–MS/MS analysis. The derivatives are stable for at least 6 months in the acetonitrile solution.

In general, this esterification progresses easily at room temperature, but the hydroxyl groups at the C-5α, C-20α and C-25 positions of oxysterols are resistant to picolinyl ester formation at room temperature. In these resistant positions, C-25 is completely esterified by heating at 80 °C for 60 min, but the C-5α and C-20α positions are not esterified at all even if the reaction mixture is heated at 80 °C.

It has been pointed out that cholesterol can be autoxidized during sample preparation [61]. However, to analyze whole sterol profiles in biological samples, it is difficult to remove cholesterol selectively before derivatization. Therefore, we determined the formation of oxysterols from pure cholesterol in the derivatizing conditions, and no significant amounts of oxysterols were detected. The results suggest that the autoxidation of cholesterol during the derivatization step is negligible.

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Table 2
Positive ESI-SRM and HPLC data of the picolinyl ester derivative of each sterol[a]

Picolinyl ester derivatives	SRM condition			HPLC data (RRT[c])	
	Precursor to product (*m/z*)	Collision energy (*V*)	Pattern[b]	C18[d]	C18 aQ[e]
24S-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.34	0.30
25-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.36	0.37
27-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.40	0.42
7α-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.42	0.33
7β-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.44	0.36
5α-Cholesta-8(9),14,24-trien-3β-ol	551 → 510	12	B	0.71	0.71
Cholesta-5,7,24-trien-3β-ol	551 → 510	12	B	0.73	0.79
Cholesta-5,8,24-trien-3β-ol	551 → 510	12	B	0.75	0.78
5α-Cholesta-7,24-dien-3β-ol	553 → 512	12	B	0.81	0.87
Zymosterol	553 → 512	12	B	0.82	0.86
Desmosterol	553 → 512	12	B	0.83	0.88
5α-Cholesta-8(9),14-dien-3β-ol	553 → 512	12	B	0.84	0.87
5α-Cholesta-6,8(9)-dien-3β-ol	553 → 512	12	B	0.85	0.83
7-Dehydrocholesterol	553 → 512	12	B	0.87	0.92
8-Dehydrocholesterol	553 → 512	12	B	0.89	0.91
Lathosterol	555 → 514	15	B	0.97	0.98
8-Lathosterol	555 → 514	15	B	0.98	0.98
Cholesterol	555 → 514	15	B	1.00	1.00
Coprostanol	557 → 516	14	B	1.05	0.91
Cholestanol	557 → 516	14	B	1.10	1.04
4-Methyl-5α-cholesta-8(9),24-dien-3β-ol	567 → 526	12	B	0.89	0.89
4-Methyl-5α-cholesta-8(9),14-dien-3β-ol	567 → 526	12	B	0.90	0.92
24S,25-Epoxycholesterol	569 → 528	12	B	0.42	0.53
7-Ketocholesterol	569 → 528	12	B	0.53	0.48
4-Methyl-5α-cholest-8(9)-en-3β-ol	569 → 528	12	B	1.07	1.01
Campesterol	569 → 528	12	B	1.10	1.03
20α-Hydroxycholesterol[f]	571 → 530	14	B	0.40	0.43
5β,6β-Epoxycholestanol	571 → 530	14	B	0.68	0.64
5α,6α-Epoxycholestanol	571 → 530	14	B	0.70	0.68
4,4′-Dimethyl-5α-cholesta-8(9),14,24-trien-3β-ol	579 → 538	14	B	0.84	0.78
4,4′-Dimethyl-5α-cholesta-8(9),24-dien-3β-ol	581 → 540	14	B	0.97	0.93
4,4′-Dimethyl-5α-cholesta-8(9),14-dien-3β-ol	581 → 540	14	B	1.01	0.99
4,4′-Dimethyl-5α-cholest-8(9)-en-3β-ol	583 → 542	14	B	1.19	1.04
Sitosterol	583 → 542	14	B	1.22	1.07
Sitostanol	585 → 544	14	B	1.36	1.11
Lanosterol	595 → 554	12	B	1.01	0.90
Dihydrolanosterol	597 → 556	15	B	1.24	1.01
27-Hydroxy-7-dehydrocholesterol	633 → 510	22	C	0.49	0.62
7β-Hydroxycholesterol	635 → 146	22	A	0.61	0.53
7α-Hydroxycholesterol	635 → 146	22	A	0.62	0.51
6-Hydroxycholesterol	635 → 146	22	A	0.69	0.63
4β-Hydroxycholesterol	635 → 146	22	A	0.78	0.76
22R-Hydroxycholesterol	635 → 512	22	C	0.47	0.55
22S-Hydroxycholesterol	635 → 512	22	C	0.50	0.48
24R-Hydroxycholesterol	635 → 512	22	C	0.50	0.56
24S-Hydroxycholesterol	635 → 512	22	C	0.50	0.57
25-Hydroxycholesterol	635 → 512	22	C	0.53	0.66
27-Hydroxycholesterol	635 → 512	22	C	0.58	0.71
5β-Cholestane-3α,7α-diol	637 → 514	22	C	0.64	0.49
7α,27-Dihydroxy-4-cholesten-3-one	649 → 146	28	A	0.18	0.17
7α,12α-Dihydroxy-4-cholesten-3-one	649 → 146	28	A	0.19	0.14
Cholestan-3β,5α,6β-triol[f]	653 → 146	28	A	0.60	0.50
7α,27-Dihydroxycholesterol	756 → 510	20	D	0.33	0.31
5β-Cholestane-3α,7α,12α-triol	758 → 635	28	C	0.32	0.24
5β-Cholestane-3α,7α,12α,25-tetrol	879 → 756	20	C	0.15	0.12

Abbreviations: ESI, electrospray ionization; SRM, selected reaction monitoring; RRT, relative retention time.

[a] Some data in this table have been reported in our previous paper [43,44].

[b] Patterns of precursor to product ions. A: $[M+Na]^+ \rightarrow$ [picolinic acid + Na]$^+$; B: $[M+Na+CH_3CN]^+ \rightarrow [M+Na]^+$; C: $[M+Na]^+ \rightarrow$ [M + Na–picolinic acid]$^+$; D: $[M+Na]^+ \rightarrow$ [M + Na–2 picolinic acids]. A, B, C and D correspond to those in Fig. 2.

[c] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

[d] A reversed-phase C18 column, Hypersil GOLD (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific) was employed. Initially, the mobile phase was comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid, then it was programmed in a linear manner to acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min. The flow rate was 300 μl/min, and the column was maintained at 40 °C using a column oven. The retention time of cholesterol 3β-picolinate by this condition was around 28.5 min.

[e] Polar endcapped C18 column, Hypersil GOLD aQ (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific) was used. Initially, the mobile phase was comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid; then it is programmed in a linear manner to acetonitrile–methanol (50:50, v/v) containing 0.1% acetic acid over 40 min. The final mobile phase was kept constant for an additional 2 min. The flow rate was 300 μl/min, and the column was maintained at 40 °C. The retention time of cholesterol 3β-picolinate by this condition was around 36.5 min.

[f] Hydroxyl groups at the C-5α and C-20α positions of oxysterols are not derivatized.

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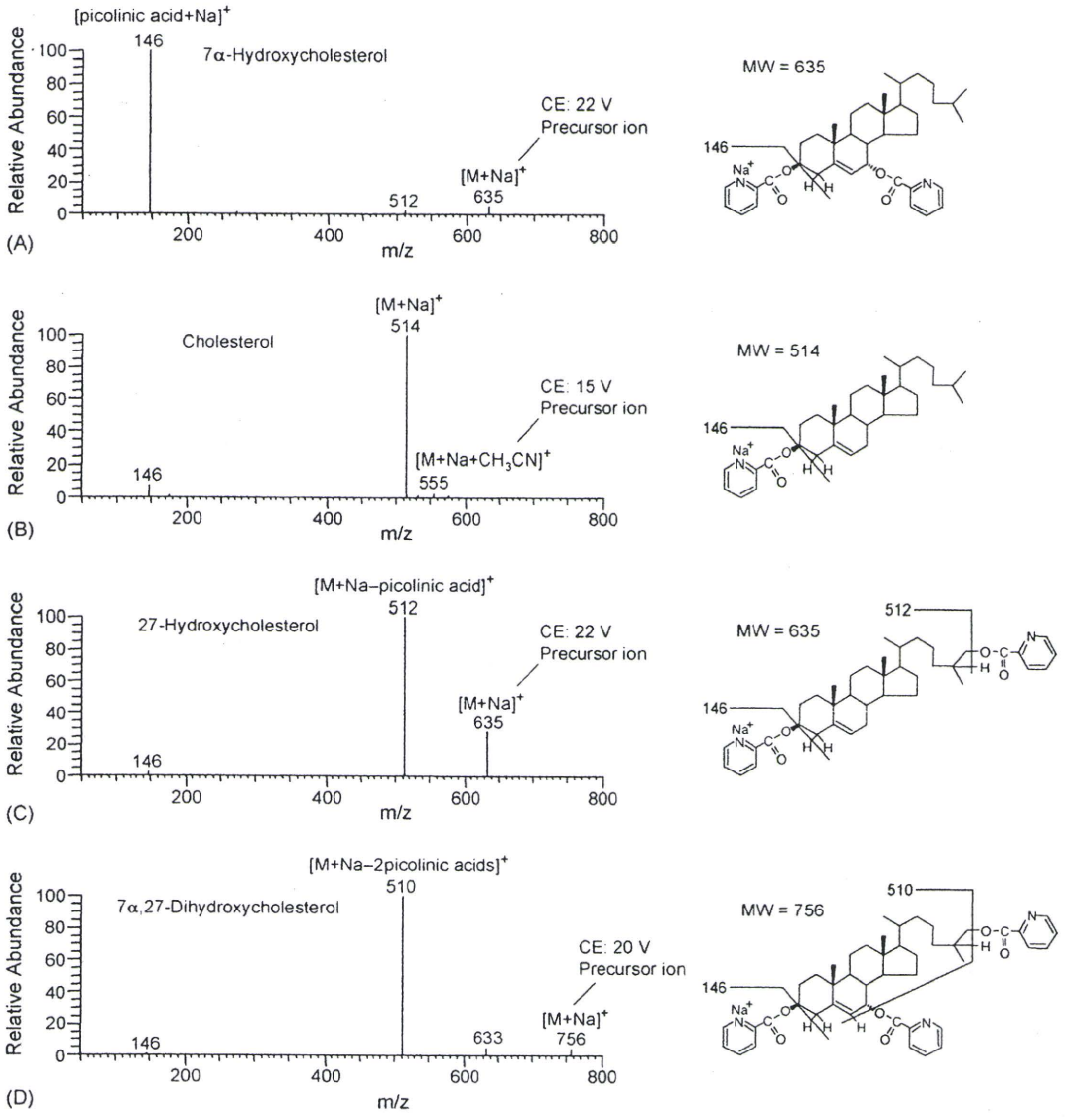

Fig. 2. Representative positive ESI–MS/MS fragmentation patterns of the picolinyl ester derivatives of sterols. (A) 7α-hydroxycholesterol, (B) cholesterol, (C) 27-hydroxycholesterol, (D) 7α,27-dihydroxycholesterol. $[M+Na]^+$ was used as precursor ions for A, C and D, while $[M+Na+CH_3CN]^+$ was used as a precursor ion for B. Fragmentation patterns of A, B, C and D correspond to those in Table 2. The general LC–MS/MS conditions were as follows: introducing solvent, acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid; flow rate, 300 μl/min; spray voltage, 1000 V. CE, collision energy. In the case of oxysterols with multiple hydroxyl groups (A, C and D), the position of sodium in the picolinyl derivatives has not been determined. In structural formulae, sodium ion was tentatively added to picolinyl group at the C-3β position.

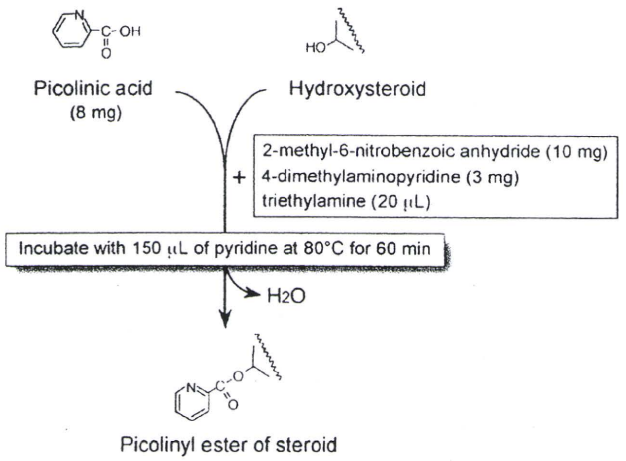

Fig. 3. The formation of picolinyl ester derivative and the conditions of the reaction.

Transesterification of fatty acyl esters during the formation of picolinyl esters is another possibility for the overestimation of sterols. However, the incubation of pure cholesteryl stearate in the reaction mixture showed that the transesterification was not probable.

4.4. Chromatographic separation

HPLC is performed using a reversed-phase Hypersil GOLD column (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific, San Jose, CA, USA). In our previous reports, monohydroxysterols [43] and oxysterols [44] were measured separately, but both sterols can be analyzed simultaneously because the HPLC column and gradient conditions are the same. Initially, the mobile phase is comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid, and it is then programmed in a linear manner to acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase is

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maintained constant for an additional 20 min. The flow rate is 300 μl/min, and the column is maintained at 40 °C using a column oven.

Relative retention times (RRTs), expressed relative to the retention time of cholesterol, are listed in Table 2. The RRTs show that the separation of sterols by the Hypersil GOLD column is excellent, but several weak points are also indicated. First, 7β-hydroxycholesterol gives a peak just before 7α-hydroxycholesterol, and reliable quantification of each hydroxycholesterol can occasionally be difficult. Second, the retention times of 7α-hydroxy-4-cholesten-3-one and 24S,25-epoxycholesterol are very close to each other, and ~~therefore, both peaks are not differentiated~~. However, because 7α-hydroxy-4-cholesten-3-one does not survive alkaline hydrolysis, the peak detected after alkaline hydrolysis is 24S,25-epoxycholesterol alone. Third, lanosterol gives a peak just after cholesterol. Although the monitoring ion for lanosterol is different from that for cholesterol, a huge cholesterol peak in biological samples can sometimes interfere with the lanosterol peak.

These problems are resolved by using another reversed-phase column, Hypersil GOLD aQ (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific). This column is usually used for separations employing highly aqueous mobile phases, but we use it as follows: initially, the mobile phase is comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid; it is then programmed in a linear manner to acetonitrile–methanol (50:50, v/v) containing 0.1% acetic acid over 40 min. The final mobile phase is maintained constant for an additional 2 min. The flow rate is 300 μl/min, and the column is maintained at 40 °C using a column oven.

The RRTs by the Hypersil GOLD aQ column are also shown in Table 2. Compared with the Hypersil GOLD column, the width of each peak tends to be wide, and the order of elution from the column is very different. Good chromatographic separations are achieved between 7α-hydroxycholesterol and 7β-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one and 24S,25-epoxycholesterol, and cholesterol and lanosterol. However, the lathosterol and 8-lathosterol peaks are not differentiated.

The separations of picolinylated sterols by these reversed-phase columns are not at all inferior to the separation of free sterols by reversed-phase HPLC [15,21]. For example, the separation of 24-hydroxycholesterol and 25-hydroxycholesterol was difficult, but DeBarber et al. achieved the chromatographic separation and quantification by using a mobile phase consisted of acetonitrile–methanol–water and APCI-MS/MS detector [33]. In contrast, McDonald et al. failed to quantify these sterol isomers separately by using an eluent of methanol–water and ESI–MS/MS detector [21]. They did not use acetonitrile because the presence of acetonitrile significantly reduced signal intensity of sterols analyzed by this detector. As for picolinylated 24- and 25-hydroxycholesterols, they were well separated each other by using acetonitrile–methanol–water as a mobile phase, and excellent sensitivities were achieved by ESI–MS/MS detector.

4.5. Sample preparation

Long term storage or repeated freeze and thaw of biological samples should be avoided because it stimulates cholesterol autoxidation [61]. Addition of the antioxidant, butylated hydroxytoluene, to the sample before sample preparation produced only a modest decrease in oxidation. Therefore, minimizing oxidation by using good lab practices is important [21].

To analyze the unesterified fraction of sterols, serum (1–5 μl), subcellular fraction of tissue (0.1–1.0 mg protein), or cell homogenate (1×10^4–1×10^5 cells) is dried with the added internal standards, and directly derivatized to the picolinyl esters [43]. To analyze the total (unesterified + esterified) fraction, saponification is carried out in 1 N ethanolic KOH at 37 °C for 1 h, and sterols are extracted with *n*-hexane before derivatization [44]. It may be mentioned here that some sterols occur as conjugates with sulfuric or glucuronic acid [62–64]. Negative ESI mode without derivatization is suitable for the analyses of these conjugated sterols, and the conjugated sterols are much more polar than picolinyl esters of unconjugated sterols.

Because this assay method is very sensitive, we can minimize the loading of derivatized sample on the HPLC column. Although the solid-phase extraction/purification step is omitted, target sterols are successfully separated by the HPLC–MS/MS step. In case of human serum analysis, less than 1 ng of picolinyl esters of non-cholesterol sterols are injected onto the column with approximately 200 ng of cholesterol picolinate. Under our HPLC conditions, this amount of cholesterol picolinate is easily trapped in the Hypersil GOLD and Hypersil GOLD aQ columns and eluted at around 28.5 min and 36.5 min, respectively, which is well separated from the picolinyl esters of most non-cholesterol sterols.

While picolinyl esters of sterols are very soluble in acetonitrile, nonpolar compounds, such as fatty acyl esters of cholesterol, remain underivatized and do not dissolve in the final acetonitrile solution. Nonpolar compounds are strongly retained on reversed-phase HPLC columns, but in this method, loading of the nonpolar compounds on the column is minimized.

4.6. Precision and accuracy

The linearity of the standard curves, as determined by simple linear regression, was excellent, as reported in our previous papers [43,44]. Reproducibilities and recoveries of some sterols were validated according to a one-way layout and polynomial equation, respectively [43,44]. The variances between sample preparations and between measurements by this method were calculated to be 1.6–12.7% and 2.5–16.5%, respectively. In these results, higher values of the variances (over 10%) were obtained by the quantification of sterols that showed extremely low concentrations in the samples. To test matrix effects, the recovery experiments were performed using human serum or rat liver microsomes spiked with 0.05–12 ng of sterols. Recoveries of the sterols ranged from 86.7% to 107.3% with a mean recovery of 99.3%, which suggests that matrix effects are not significant in this assay. This method provides reproducible and reliable results for the quantification of sterols in small amounts of biological samples.

5. Perspectives

HPLC–MS or HPLC–MS/MS does not require a derivatization step before the analysis of sterols, which is advantageous for a high-throughput assay. However, the addition of the derivatization step has markedly improved the sensitivities of the neutral sterols. Thus, simple and rapid procedures do not always produce good results for the microanalysis of biological samples. In addition, since many sterols have the same molecular weight and similar structures, a thorough chromatographic separation is essential to maintain the selectivity even if the latest model of mass spectrometer is operated in a high-resolution mode.

The recent development of steroid biochemistry has demonstrated that there are considerable bioactive or biomarker sterols among intermediates and their derivatives in the biosynthetic pathways of cholesterol, bile acids and steroid hormones. Moreover, there are still many unidentified sterols in biological samples. Therefore, not only sensitive and specific quantification of targeted sterols but also metabolomic analysis of whole sterol profiles will become an important methodology for steroid biochemistry and its clinical applications.

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Expression levels of heat shock protein 20 decrease in parallel with tumor progression in patients with hepatocellular carcinoma

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Abstract. Heat shock protein (HSP) 20, a low-molecular-weight HSP, is constitutively expressed in various tissues, such as smooth muscle, skeletal muscle, and liver. However, the characteristics and function of HSP20 have not been precisely understood. In the present study, we investigated correlations of expression levels of HSP20 in hepatocellular carcinoma (HCC) tissues and the surrounding tissues with clinical and pathologic characteristics in 53 resected HCC specimens. Although HSP20 was detected in all 53 HCC tissues, the expression levels were reduced compared with those in the adjacent non-tumor tissues. The expression levels of HSP20 were inversely correlated with tumor stage by TNM classification ($p<0.01$), presence of microvascular invasion ($p<0.05$), and tumor size ($p<0.05$). Our findings strongly suggest that HSP20 may play a role against the progression of human HCC.

Introduction

Cells produce heat shock proteins (HSPs), when exposed to various kinds of biological stress such as heat and chemicals (1). HSPs are classified into high-molecular-weight HSPs such as HSP70, HSP90 and HSP110, and low-molecular-weight HSPs with molecular masses from 10-30 kDa such as HSP20, HSP27 and αB-crystallin according to apparent molecular sizes. It is well recognized that high-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization and translocation (1). Though the functions of low-molecular-weight HSPs are not as well characterized as those of the high-molecular-weight HSPs, it is recognized that they may also have chaperone functions (1). The human genome codes for 10 low-molecular-weight HSPs (2). In their C-terminal half, these proteins share a sequence element of ~100 amino acid residues called the α-crystallin domain, and toward their N-terminal end, they share a less conserved but nevertheless similar domain (3). HSP20 was co-purified with HSP27 and αB-crystallin from skeletal muscle, and it was identified as a member of the crystallin family (4). Although HSP20 is not induced by heat or chemical stress, it is highly expressed in normal skeletal and smooth muscle, heart and liver tissues where it may be essential, but the exact role of HSP20 remains to be clarified (4).

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and it causes more than one million deaths annually (5,6). Factors that indicate tumor progression in association with patient outcome reportedly include tumor size, number of tumors, vascular invasion that can be evaluated pathologically and imaging diagnosis (7-10). Tumor markers for HCC such as α-fetoprotein levels and *des*-γ-carboxy prothrombin levels are reported to be additional indicators of tumor progression associated with patient survival (10-13). However, these factors are not sufficient to accurately discriminate the tumor progression of HCC patients towards the accurate prediction of patient survival. It is, therefore, necessary to further investigate other indicators for the evaluation of tumor progression and for the prediction of patient outcome.

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Key words: heat shock protein 20, hepatocellular carcinoma, tumor stage

To date, it has been reported that expression of certain HSPs can be correlated with the carcinogenic process as well as with the degree of differentiation and cell proliferation, and moreover, they have been implicated in the regulation of apoptosis (14,15). In addition, evidence is accumulating about the usefulness of the prognostic implications of HSPs in certain cancer types, especially high-molecular-weight HSPs (14,15). We have recently shown that attenuated phosphorylation of HSP27 correlates with tumor progression in patients with HCC (16). Among low-molecular-weight HSPs, HSP27 has been the most extensively studied, but to the best of our knowledge there has been no report about the relationship of HSP20 and tumor progression. Therefore, in the present study, we tried to investigate the relationship between HSP20 and HCC in 53 resected HCC specimens.

Materials and methods

Patients. Fifty-three patients (46 men, 7 women, mean age: 66.9±8.4 years), having been diagnosed with HCC at the Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan underwent hepatic resection between September 2002 and August 2005. Liver cirrhosis was present in 24 patients, and chronic hepatitis was present in 29. Fourteen patients were infected with hepatitis B virus, and 34 were infected with hepatitis C virus. The remaining 5 patients had evidence of alcoholic cirrhosis. No patient had previously undergone preoperative chemotherapy.

The resected HCC specimens were obtained according to protocol approved by the Committee for the Conduct of Human Research at Ogaki Municipal Hospital. Informed consent was obtained from all patients.

Surgical specimens. Primary HCC tissues were obtained from all patients by surgical resection at the Department of Surgery, Ogaki Municipal Hospital. The excised tissue was divided into two parts, and one part was fixed with 20% neutral formalin overnight. The fixed tissue was then dehydrated with 100% methanol and xylene and embedded in paraffin wax. A three-micron-thickness of this tissue was used for immunohistochemical staining. The other part of the resected tissue was snap-frozen in liquid nitrogen and stored at -80°C until used for Western blot analysis.

Pathological evaluations. The pathological features of HCC were evaluated by two of the authors (N.Y. and Y.K.) without knowledge of the HSP20 status of the tumor. The specimen was stained with hematoxylin and eosin, and the entire specimen was examined. Differentiation of HCC was classified as well-, moderately, or poorly differentiated HCC on the basis of the classification by the International Working Party (17). Vascular invasion and infiltration to the tumor capsule were evaluated macroscopically.

Western blot analysis. Snap-frozen samples were homogenized and sonicated in lysis buffer containing 62.5 mM Tris-HCl

Table I. Comparison of the protein levels of HSP20 with the clinical and pathological characteristics of 53 patients with HCC.

	p value	
	Tumor tissue	Non-tumor tissue
Gender male (n=46), female (n=7)	0.896	0.627
Underlying disease liver cirrhosis (n=25), chronic hepatitis (n=28)	0.957	0.010[a]
Etiology of liver disease HBV (n=13), HCV (n=35), alcoholic (n=5)	0.662	0.482
Number of tumors solitary (n=40), multiple (n=13)	0.374	0.718
Tumor size (mm) <20 (n=12), 20-50 (n=32), >50 (n=9)	0.048[a]	0.697
Vascular invasion negative (n=35), positive (n=18)	0.040[a]	0.669
Infiltration to capsule negative (n=28), positive (n=25)	0.203	0.673
Tumor stage I (n=9), II (n=25), III (n=11), IV (n=8)	0.003[a]	0.449
Histological classification (differentiation) well- (n=11), moderately (n=35), poorly (n=7)	0.858	0.636

HBV, hepatitis B virus; HCV, hepatitis C virus; [a]$p<0.05$.

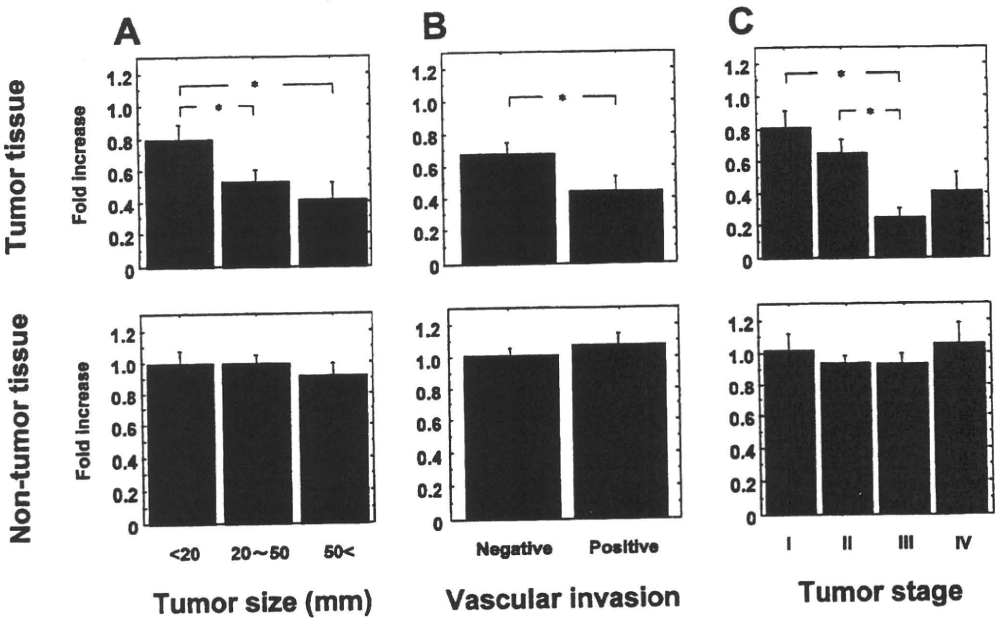

Figure 1. HSP20 levels in patients with HCC. Protein extracts from 53 HCC specimens (tumor and adjacent non-tumor tissue, respectively) were analyzed with antibodies against HSP20 and ß-actin. Signal intensities on X-ray film were quantified with NIH image software. The histograms show quantitative representations of the levels of HSP20 after normalization to levels of ß-actin. Values on the vertical axis represent the mean ± SE of independent experiments. The values were calculated as the average values with those of small (tumor size <20 mm) HCC (left column), negative vascular invasion (center column) and tumor stage I HCC (right column) equal to 1.0. *$p<0.05$.

(pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. Western blot analysis was performed as described previously (18) with polyclonal antibodies against HSP20 and HSP27 (Stressgen Biotechnologies, Victoria, British Columbia, Canada). Peroxidase-conjugated antibodies against rabbit IgG were used as secondary antibodies against the above-mentioned primary antibodies. Primary antibodies against ß-actin (Sigma-Aldrich Co, St. Louis, MO) were detected with peroxidase-conjugated antibodies against mouse IgG as secondary antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film with the ECL Western blotting detection system (GE Healthcare UK Ltd, Buckinghamshire, UK). Protein band intensities were determined by integrating the optical density over the band area (band volume) with NIH image software. HSP20 levels were normalized to those of ß-actin.

Immunohistochemical analysis. Immunohistochemical staining of some specimens was performed with the streptavidin-biotin complex method to investigate expression and localization of HSP20. Primary antibodies were anti-HSP20 rabbit polyclonal antibodies (Stressgen Biotechnologies, Golden, CO). Briefly, deparaffinized sections were treated with 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxidase activity. Sections were immersed in 0.05 M citrate buffer (pH 6.0), heated in a microwave oven for 15 min, and then incubated with primary antibodies for 2 h at room temperature. Each section was treated sequentially with biotinylated secondary antibodies (anti-rabbit-IgG) and streptavidin-peroxidase complex (Dako Chem Mate, Kyoto, Japan). Finally, immune complexes were visualized with 3,3'-diamino-benzidine tetrahydrochloride as a chromogen. Mayer's hematoxylin was used as a counterstain.

Statistical analysis. Patient clinical data were expressed as mean ± SD. The data were analyzed with the SPSS software program (Release 11.5.1J standard version; SPSS Japan, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to determine the significance of differences between protein expression and grade of tumor differentiation or tumor stage. Nonparametric data were analyzed with the Mann-Whitney U test, Kruskal-Wallis test, or Spearman's correlation coefficient (r). All p values were derived from two-tailed tests and $p<0.05$ was accepted as statistically significant. A Spearman's correlation coefficient of $r\geq0.400$ was accepted as a positive correlation.

Results

Correlations of HSP20 levels according to characteristics of HCC. The levels of HSP20 were compared with the clinical and pathological characteristics of 53 patients with HCC, including gender, underlying liver disease, etiology, number of tumors, tumor size, vascular invasion, infiltration to the tumor capsule, and tumor stage (evaluated according to the TNM classification of the International Union Against Cancer) (19), and histological classification (Table I). Comparisons of the levels of HSP20 revealed significant differences with respect to tumor size ($p=0.048$), vascular invasion ($p=0.040$) and tumor stage ($p=0.003$) in tumor tissues, while there were no significant differences in HSP20 levels in adjacent non-tumor tissues, except in those tissues with underlying liver disease (Table I). In the non-tumor tissues, the levels of HSP20 in liver cirrhotic tissue were significantly higher than those in chronic hepatitis patient tissue (Table I).

HSP20 levels according to tumor size, vascular invasion and tumor stage are shown in Fig. 1. A trend toward decreased expression levels of HSP20 in tumor tissues was

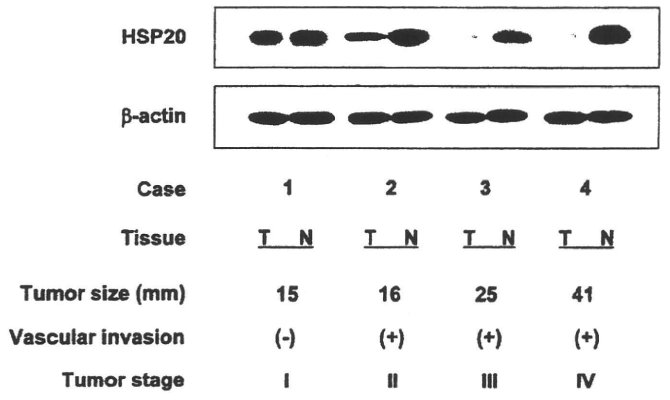

Figure 2. HSP20 levels in four representative patients with HCC according to tumor size, vascular invasion and tumor stage. Comparison with those in non-tumor tissue. Protein extracts were analyzed with antibodies against HSP20 and ß-actin. T, tumor tissue; N, adjacent non-tumor tissue.

observed as tumor size, vascular invasion and tumor stage increased, suggesting that the levels of HSP20 in the adjacent non-tumor tissues were higher than those in the tumor tissues (Fig. 1A, B and C; upper panel). On the other hand, HSP20 levels in the adjacent non-tumor tissues were not correlated with these factors, suggesting that the levels of HSP20 in the tumor tissues were attenuated in parallel with HCC progression (Fig. 1A, B and C; lower panel). Western blot images of HSP20 expression in 4 representative patients with HCC according to tumor size, vascular invasion and tumor stage are shown in Fig. 2.

Immunohistochemical analysis of HSP20 in HCC specimens. To confirm our results from Western blot analysis, we performed immunohistochemical analysis of HSP20 in HCC tumor and non-tumor tissues. Immunohistochemical staining of HSP20 in stage-IV-HCC specimens containing tumor and non-tumor tissue is shown in Fig. 3. Immunoreactivity for HSP20 in tumor tissue was markedly lower than that in non-tumor tissue.

Comparisons between the levels of HSP20 and the levels of phosphorylated HSP27 in HCC tumor tissues. HSP27, a low-molecular-weight HSP, is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser-82) (1). We previously reported that attenuation of phosphorylated HSP27 (Ser-15, Ser-78 and Ser-82) in tumor tissue correlates with HCC progression (16). Therefore, we investigated the correlation between the levels of HSP20 and the levels of phosphorylated HSP27 that had been determined in the previous study. The levels of phosphorylated HSP27 (Ser-15) were significantly correlated with the levels of HSP20 ($r=0.505$, $p<0.001$; Fig. 4A). On the contrary, the levels of phosphorylated HSP27 (Ser-78), phosphorylated HSP27 (Ser-82) or total HSP27 were not correlated with those of HSP20 (Fig. 4B, C and D, respectively).

Discussion

In the present study, we showed that attenuation of HSP20 levels correlated with tumor progression in tumor tissues of patients with HCC. In addition, the HSP20 levels correlated inversely with tumor size and vascular invasion of HCC, both of which are indications of an advanced tumor. To the best of our knowledge, this is the first report of a significant relation between HSP20 levels and progression of HCC.

Recently, we reported that attenuation of phosphorylated HSP27 is correlated with HCC progression (16). It is recognized that HSP27, HSP20 and αB-crystallin form one type of complex (3,20). It has been shown that phosphorylation of HSP27 is associated with the disassembly of HSP27 complexes (21,22). In the present study, we found significant correlation between the levels of HSP20 and that of phosphorylated HSP27 (Ser-15), but not Ser-78 and Ser-82. Although the differential role of the three phosphorylation sites are not known, our findings suggest that HSP20 and phosphorylated HSP27 (Ser-15) may have suppressive effects on HCC progression. In addition, these results suggest that phosphorylated HSP27 (Ser-78) and phosphorylated HSP27 (Ser-82) may have different roles in HCC progression. Further investigations are required not only to clarify the exact role of HSP20, but also to determine whether these HSPs can be prognostic factors in HCC. Moreover, HSPs not only have prognostic implications but also have therapeutic implications for cancer (14). Among HSPs, the use of the HSP90 inhibitor,

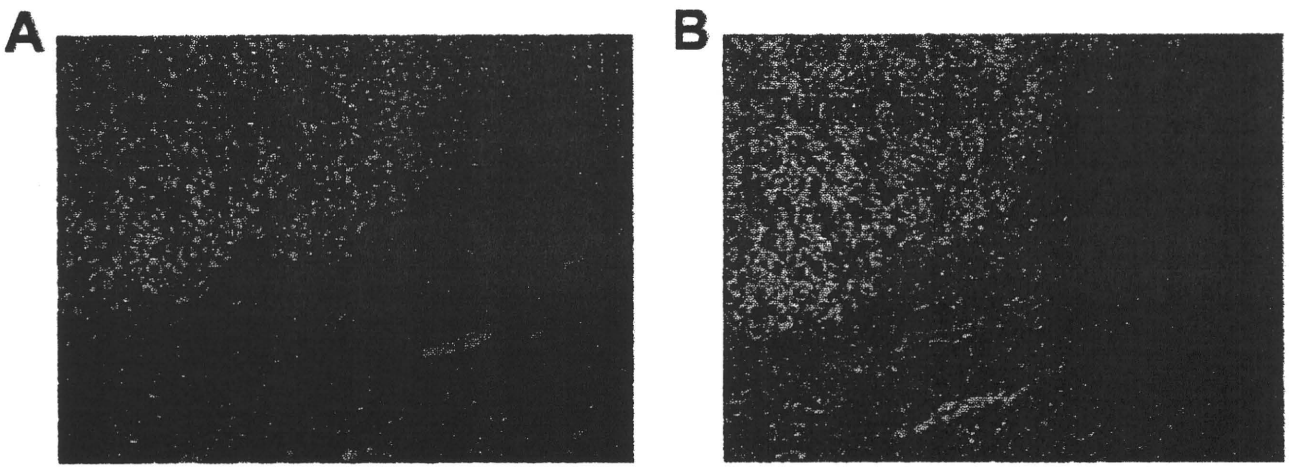

Figure 3. Immunohistochemical analysis of HSP20 in a patient with HCC (tumor stage IV) and adjacent non-tumor tissue (chronic hepatitis) (A). The same patient specimen stained with hematoxylin and eosin. (B).

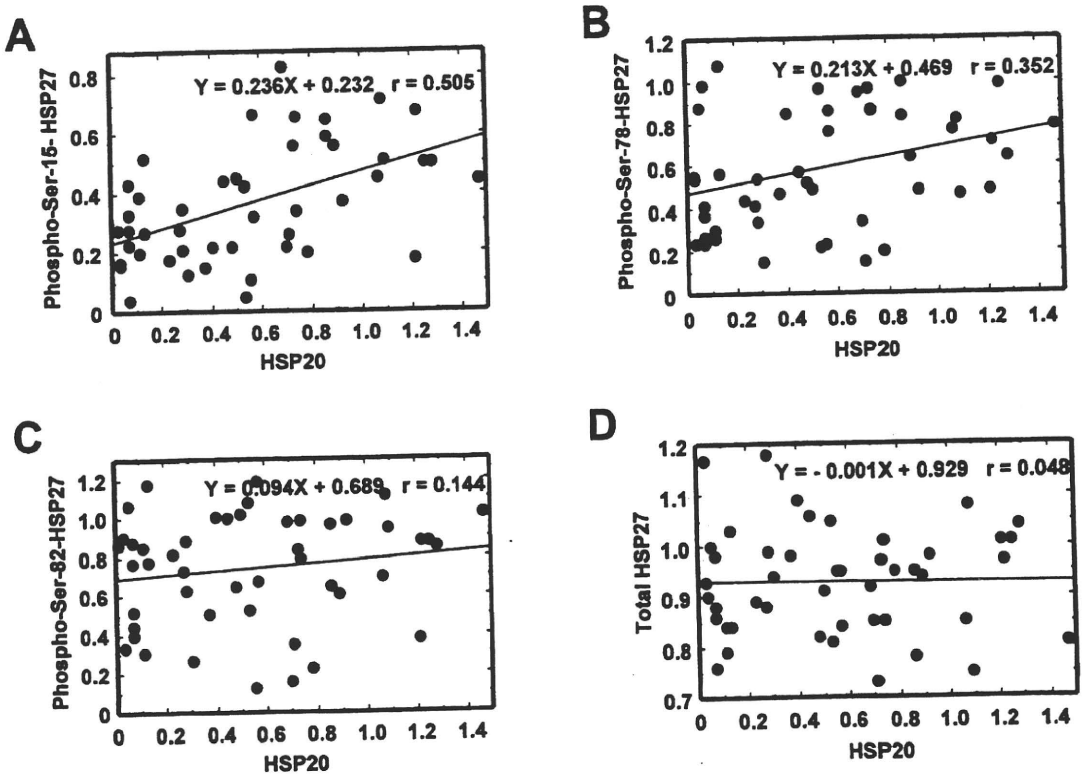

Figure 4. Correlations between the levels of HSP20 and the levels of (A) Ser-15-phosphorylated HSP27, (B) Ser-78-phosphorylated HSP27, (C) Ser-82-phosphorylated HSP27, and (D) total HSP27. The expression levels of HSP20, phosphorylated HSP27, and total HSP27 were determined by the band intensities obtained from Western blot analysis, and then normalized to those of ß-actin.

which is under phase I trial has been extensively studied (14,15). Although the role of HSP20 in HCC is not precisely known, further investigations would help us to use HSP20 as a target for cancer therapy.

In conclusion, our present results strongly suggest that expression levels of HSP20 decrease with progression in tumor stages in patients with HCC and that HSP20 may have a suppressive effect on the advancement of human HCC.

Acknowledgements

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Letter to the Editor

Simultaneous multicentric occurrence of early hepatocellular carcinoma in a patient with persistent alpha-fetoprotein elevation

Dear Editor

Alpha-fetoprotein (AFP) is a tumor marker for hepatocellular carcinoma (HCC).[1,2] However, we sometimes encounter patients with persistently elevated serum AFP in whom HCC is not detected. Whether the elevation of AFP is an indicator of the presence of HCC or an indicator of a high potential for hepatocarcinogenesis is not clear. Here, we describe the clinical course of a patient with persistently elevated AFP.

A 65-year-old woman had been diagnosed with chronic hepatitis C virus infection in 1991 and has since been followed up every 2 months at the Department of Gastroenterology, Ogaki Municipal Hospital. Her serum AFP remained normal (<20 ng/dL) between 1991 and 2004, but it was elevated to >400 ng/dL on December 2004. The elevation persisted thereafter, fluctuating between 400 ng/dL and 1000 ng/dL despite the constant mild elevation of serum alanine aminotransferase (ALT) activity and the lack of elevation of *Lens culinaris* agglutinin A-reactive fraction of AFP and des-gamma-carboxy prothrombin. In January 2005, she underwent angiography and angiography-assisted computed tomography (CT) (i.e. CT during arterial portography [CTAP] and CT during hepatic arteriography [CTHA]), which are one of the most sensitive modalities for detecting hepatic tumor.[3] No liver tumor, however, was detected during this examination.

A hepatic mass lesion was detected during a routine outpatient ultrasonography in February 2006. The patient's serum AFP at that time was 970.4 ng/dL. She again underwent angiographic examination of the liver. Four small liver nodules (<2.0 cm in diameter) were found as attenuated areas during CTAP, and partially increased attenuation was detected within three of the four attenuated areas (arrows, Fig. 1a1–a3). In contrast, during CTHA, three minute enhanced areas were found that corresponded to three of the areas of increased attenuation found during CTAP (arrows, Fig. 1b1–b3). One tumor lacked an enhanced area on the CTHA. Ultrasonography-guided fine-needle biopsy of the tumors revealed four well-differentiated HCC, within one of which the component of moderate differentiation was observed. Her remnant liver function was Child–Pugh class B at diagnosis and each of the four tumors was treated by radiofrequency thermal ablation. Her serum AFP decreased to 290.8 ng/dL 1 month after the treatment.

A decrease in portal venous flow and an increase in arterial blood flow are characteristics of typical HCC.[4] However, an increase in arterial blood flow is absent in early-stage HCC, whereas a decrease in portal venous flow is present. In the nodule-in-nodule-type HCC,[5] early-stage HCC contains a part with features of typical HCC within a tumor. Three of the four HCC we found in the present case were of this nodule-in-nodule-type HCC, and the other was an early-stage HCC. These HCC are unlikely to produce intrahepatic metastases[6] and were therefore believed to develop simultaneously and independently and were considered a multicentric occurrence.[7]

Although AFP is the tumor marker that is used most widely for monitoring the development of HCC, AFP also increases in association with hepatocyte regeneration.[8] AFP therefore does not always reflect the development of HCC. Because AFP elevation indicates enhanced liver regeneration as well as advanced liver fibrosis,[9] however, it could also be a marker for a high risk of developing HCC[10] and further multicentric ocurrence of HCC. Patients with persistent AFP elevation should therefore be monitored intensively for HCC, and careful imaging studies with sensitive methods for HCC should be required in order not to miss the development of HCC only, but also the multicentric occurrence of HCC.

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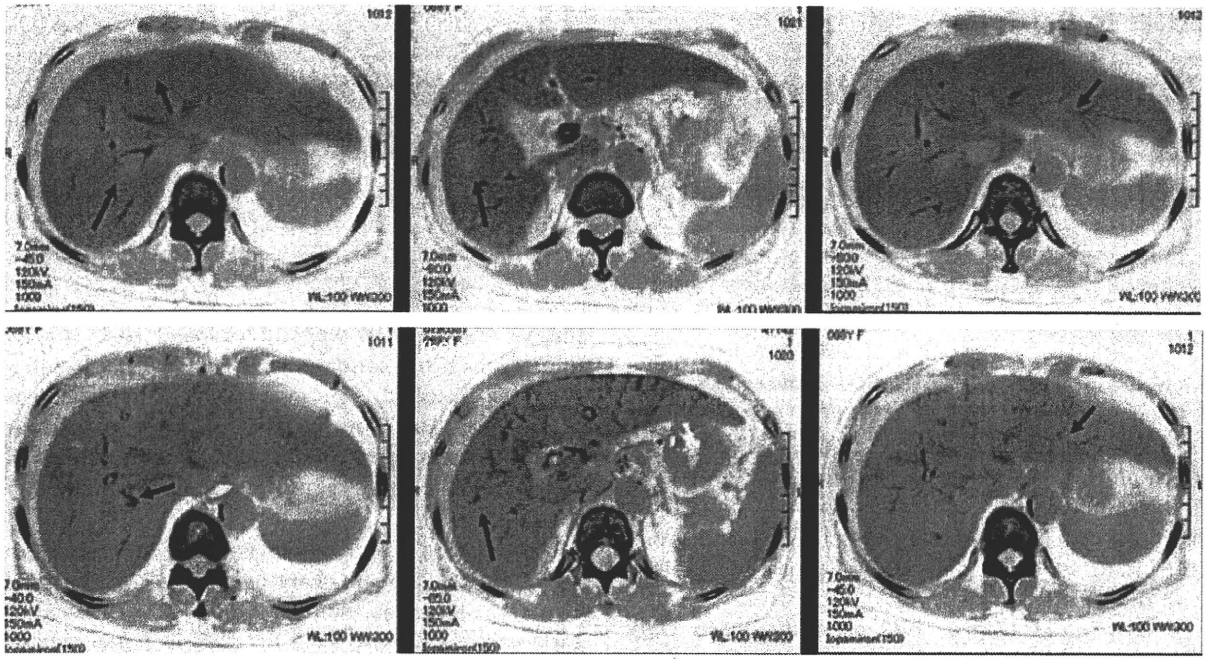

Figure 1 Findings of (a1–a3) computed tomography (CT) during arterial portography (CTAP) and (b1–b3) CT during hepatic arteriography (CTHA) in February 2006. (a1–a3) Four small liver nodules (all < 2.0 cm in diameter) were observed as attenuated nodules (low-density areas), three of which contained a small area of increased attenuation (arrows). (b1–b3) Minute high-density areas were observed that corresponded to the areas of increased attenuation on CTAP images, except in one tumor (arrows).

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その他 ウイルス肝炎診療において知っておくべきこと

A型肝炎について知っておくべきこと

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ポイント

- A型肝炎はA型肝炎ウイルス(HAV)の経口感染により伝播する感染症である.
- 初発症状としては,全身倦怠感・尿濃染などのほかに心窩部痛や右上腹部痛などがあり,時として胆道系疾患との鑑別を要する.
- 診断はIgM型HAV抗体の検出による血清学的診断で行う.
- 急性肝炎を生ずるが,慢性化はしない.
- わが国で以前は局地的な流行がみられたが,現在は稀である.

A型肝炎ウイルス(HAV)の経口感染で生ずるA型肝炎は,急性肝炎として典型的なものであり,診断が比較的容易でありかつ劇症化しなければ経過も良好であるため,新しく消化器内科を研修する医師が最初に受け持つことの多い疾患の1つかもしれない.しかし,診断にはしっかりした知識に基づいた鑑別が必要であり,また非定型的な経過をとる症例もあることを知っておく必要がある.本稿ではA型肝炎について最低限知っておくべきことを述べる.

疫 学

A型肝炎は経口感染で伝播するため,居住地域の衛生環境が感染に影響する.わが国は以前,幼少児期に感染することの多い高侵淫地域であったが,衛生環境の整備に伴い低侵淫地域となった.現在では中高年においてもHAV抗体の保有率は50%以下である[1].このことは逆に言えば,わが国では中高年以降の発症例が増加していることを意味する[2].そして高年齢での発症例には重症例が多いとする報告もある.

以前はA型肝炎の局地的な流行が散発的に認められたが,最近は報告がされていない.一方,最近の特徴として,男性同性愛者間の性感染症としてのA型肝炎の蔓延[3],海外旅行における感染,および輸入食品を介した輸入感染症としてのA型肝炎がある.

臨床症状

たまたま検診などの採血でトランスアミナーゼの高値を指摘され受診することも稀にはあるが,ほとんどの場合は症状があって受診し採血でトランスアミナーゼの高値が判明,もしくは近医での採血でトランスアミナーゼの高値が判明して紹介され受診することが多い.症状としては,急激な肝障害による全身倦怠感・食欲不振・嘔気や血清総ビリルビン値の上昇に伴う尿

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