TGCAGGTGAG-3′, respectively, and pFlag-CMV5/TO-BGG c-myc 3′-UTR as a template. The amplified fragments were mixed and used as a template for the second PCR conducted to generate full-length c-myc 3′-UTR fragment. The resulting fragment was digested with *Hind*III and *Pst*I and inserted into pFlag-CMV5/TO-BGG 3′-UTR(*Hind*III). The construction of pGEX6P1-Tob (1–285), pCMV-5 × Myc-Pan2 D1083A and pCMV-5 × Myc Caf1 D161A was described previously.[19]

siRNA

The sequences of siRNAs for luciferase, Tob and Tob2 were described previously.[19] CPEB siRNA consists of 5′-r (GACUCUGAAGAAACAGUUA) d(TT)-3′.

Antibodies

Antibodies used in this study were the following: anti-Flag (M2; Sigma, St Louis, MO, USA), anti-c-Myc (9E10 (Roche, Indianapolis, IN, USA); A-14, C-33 (Santa Cruz Technology, Santa Cruz, CA, USA)), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (6C5; Millipore, Bedford, MA, USA), anti-GST (Z-5, Santa Cruz Technology), anti-MBP (New England Biolaboratories). Anti-Tob was raised against His-tagged Tob (1–110 amino acids). Anti-Caf1 (for immunoprecipitation), anti-CPEB and anti-PABPC1 were raised against His-tagged full-length proteins. Anti-Caf1 was a gift from Ann-Bin Shyu.[16]

Cell culture and transfection

HeLa, T-REx HeLa and U2OS cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum. U2OS cells were purchased from ATCC. DNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described previously.[17] For siRNA transfection, Lipofectamine RNAi MAX (Invitrogen) was used. For transfection with both siRNA and plasmid DNA, cells were first transfected with siRNA using RNAi MAX. After 24 h, cells were trypsinized, and re-cultured for another 24 h before DNA transfection using Lipofectamine 2000. Following further incubation for 24 h, pulse-chase experiments were conducted.

Immunoprecipitation

For immunoprecipitations, cells were lysed with 10 μg/ml RNaseA (Sigma) or 50 U/ml RNase I (New England Biolaboratories) in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.25% Nonident P-40, 1 mM dithiothreitol, 2.5 mM ethylenediaminetetraacetic acid, 20 mM NaF, 10 mM $Na_4P_2O_7$, 0.1 mM phenylmethanesulfonylfluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. After centrifugation at 15 000*g* for 20 min, the supernatant was incubated for 2 h at 4 °C with anti-Flag IgG agarose (Sigma), or anti-Myc agarose (Sigma). The resin was then washed three times with lysis buffer, and proteins retained on the resin were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis.

GST-pull-down assay

GST-fused Tob and MBP-CPEB were produced in *E. coli* BL21 by adding 0.4 mM isopropylthio-β-galactoside and purified as described previously.[19] GST-fused Tob and MBP-CPEB were incubated with glutathione sepharose 4B (GE Healthcare) for 2 h at 4 °C in binding buffer (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% Nonident P-40, 1 mM dithiothreitol, 2.5 mM ethylenediaminetetraacetic acid). The resin was then washed three times with binding buffer. Bound proteins were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and analyzed by western blotting.

Northern blot analysis

The northern blot analysis, transcriptional pulse-chase analysis and oligo (dT)-RNase H treatment of mRNA to generate poly(A)$^-$ mRNA were performed as described previously,[19] except that the pulse transcription was induced by 10 ng/ml tetracycline for 2 h.

Real-time PCR analysis

Cells were directly harvested (steady-state level) or treated with 10 μg/ml actinomycin D (mRNA decay) and harvested at indicated times. Total RNA was isolated by the acid guanidinium–phenol–chloroform method with minor modifications and genomic DNA was removed by digestion with DNase I for 30 min. Random-primed RT of RNA (1.5 μg) was performed using SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR analysis was performed using StepOne Real-Time PCR system with PowerSYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Changes in c-myc mRNA levels were determined by the relative standard curve method using GAPDH mRNA for internal normalization. The primers for GAPDH were described previously.[19] c-myc mRNA was amplified using primers: sense, 5′-TTCGGGTAGTGGAAAACCAG-3′ and antisense, 5′-GGAACTATGACCTCGACTACGACT-3′. C-myc pre-mRNA was amplified using: sense, 5′-GCACCAAGACCCCTTTAACT-3′ and antisense, 5′-GGAAC;TATGACCTCGACTACGACT-3′. The specificity of the primer pairs and genomic DNA digestion were checked by PCR analysis using KOD FX (TOYOBO, Osaka, Japan) or PowerSYBR Green (Applied Biosystems).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

最前線 SAIZENSEN

がん抑制遺伝子産物 Tob による mRNA 分解を介したがん抑制と学習記憶の調節

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1 はじめに

mRNA 分解の制御は，転写後における遺伝子発現の調節機構として注目を集め，近年の精力的な研究によりその分子メカニズムの解明が急速に進展した．また，高次の生命機能の制御における mRNA 分解制御の重要性，および疾患との関連性が次第に明らかとなってきている．筆者らはこれまで，ヒトを含めた真核生物の mRNA 分解開始機構を解明し，ポリ(A)鎖を有する通常の mRNA の分解制御においてがん抑制遺伝子産物 Tob(Transducer of ErbB 2)が主要な役割を果たしていることを明らかにしてきた．一方で，Tob は細胞増殖抑制や学習・記憶を含む様々な生物活性を制御することが知られている．これまで，それらの生物活性が mRNA 分解を介して制御されることが疑われてきたが，一部の例外を除く全ての mRNA に共通する一般的な mRNA 分解機構ではそのような特異的な遺伝子の発現制御を説明することができなかった．最近我々は，Tob の新規結合タンパク質として配列特異的 RNA 結合タンパク質 CPEB(cytoplasmic polyadenylation element binding protein)ファミリーを同定し，CPEB との相互作用を介した遺伝子特異的な mRNA 分解の分子メカニズムを解明した．本稿では，その研究成果を紹介するとともに，Tob の重要な機能であるがん抑制と学習・記憶の制御との関係について考察する．

2 Tob はがん抑制をはじめ多彩な生物機能を有する

Tob は，受容体型チロシンキナーゼ ErbB 2 と会合する因子として同定された分子量約 45 kDa のタンパク質であり，[1] Tob 2，BTG 1，PC 3/Tis 21/BTG 2，ANA/BTG 3，PC 3 B/BTG 4 とともに Tob/BTG ファミリーを構成している．これらはいずれも(1)N 末端のおよそ 110 アミノ酸に B cell translocation gene(BTG)ドメインと呼ばれる相同性の高い領域を有し，(2)NIH3T3 細胞に強制発現させると G 0/G 1 期から S 期への進行が抑制されるという共通した特徴を持つ．Tob の増殖抑制活性は増殖因子による細胞外からの刺激により惹起されるシグナル伝達経路の下流で制御されている．増殖刺激に伴い G 0/G 1 期から S 期へ移行する際，Tob は MAP キナーゼ(mitogen-activated protein kinase：MAP kinase)Erk 1/2 によるリン酸化を受け増殖抑制活性を失う．[2] このリン酸化は，がん遺伝子 rat sarcoma(*ras*)を介した細胞の形質転換において重要であり，細胞のがん化と関連する．さらに，後に報告された *tob* 遺伝子欠損マウスを用いた解析の結果，加齢とともに肝臓やリンパ節，肺など様々な器官において腫瘍の形成が観察されたことから，*tob* はがん抑制遺伝子であることが明らかとなった．[3]

Tob の機能異常は，ヒトにおけるがんの発症とも関連がある．例えば，肺がん手術症例における検討によると，72% において非がん部と比べ肺がん組織における Tob の発現低下が観察され，肺腺がん組織では 76% において Tob のリン酸化亢進が確認された．[4] さらに甲状腺がんの例では，甲状腺未分化がんでは全てにおいて Tob の発現低下が起こっていること，甲状腺乳頭がんでは症例の 39.7% において Tob のリン酸化亢進が見られ，腫瘍の大きさとリン酸化状態に相関があることが報告された．[5] Tob はがん・細胞増殖抑制のほかにも，骨形成[6]や学習・記憶[7]など様々な生物活性に関与することが明らかとなっている(図 1)．

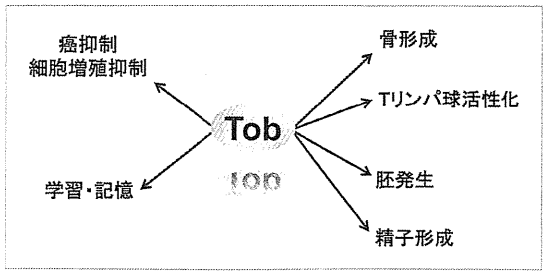

図1　がん抑制遺伝子産物 Tob の生物活性
Tob はがん抑制因子として同定されたが，細胞増殖抑制にとどまらず様々な生物過程を制御する．

3 通常の mRNA 分解における Tob の役割

増殖抑制をはじめとする様々な生物活性に加え，Tob の持つ重要な役割として筆者らは mRNA 分解の制御を明らかにした．[8,9] mRNA の分解は通常，翻訳終結を引き金として mRNA の 3'末端ポリ(A)鎖分解※より開始される(図2)．ポリ(A)鎖を約10残基程度まで短縮化された mRNA は，5'末端キャップ構造の切断とそれに続く 5'→3'エキソヌクレアーゼによる mRNA 本体の分解を受けて消失する．あるいは，その補助経路としてエキソヌクレアーゼによる 3'→5'方向の分解を受ける．ここで重要なことは，通常の mRNA 分解においてポリ(A)鎖の短縮化は，開始段階であると同時に律速段階であり，mRNA 分解ひいては遺伝子発現の最も重要な制御部位となっている．筆者らは，翻訳終結とポリ(A)鎖分解が共役して起こる mRNA 分解開始の

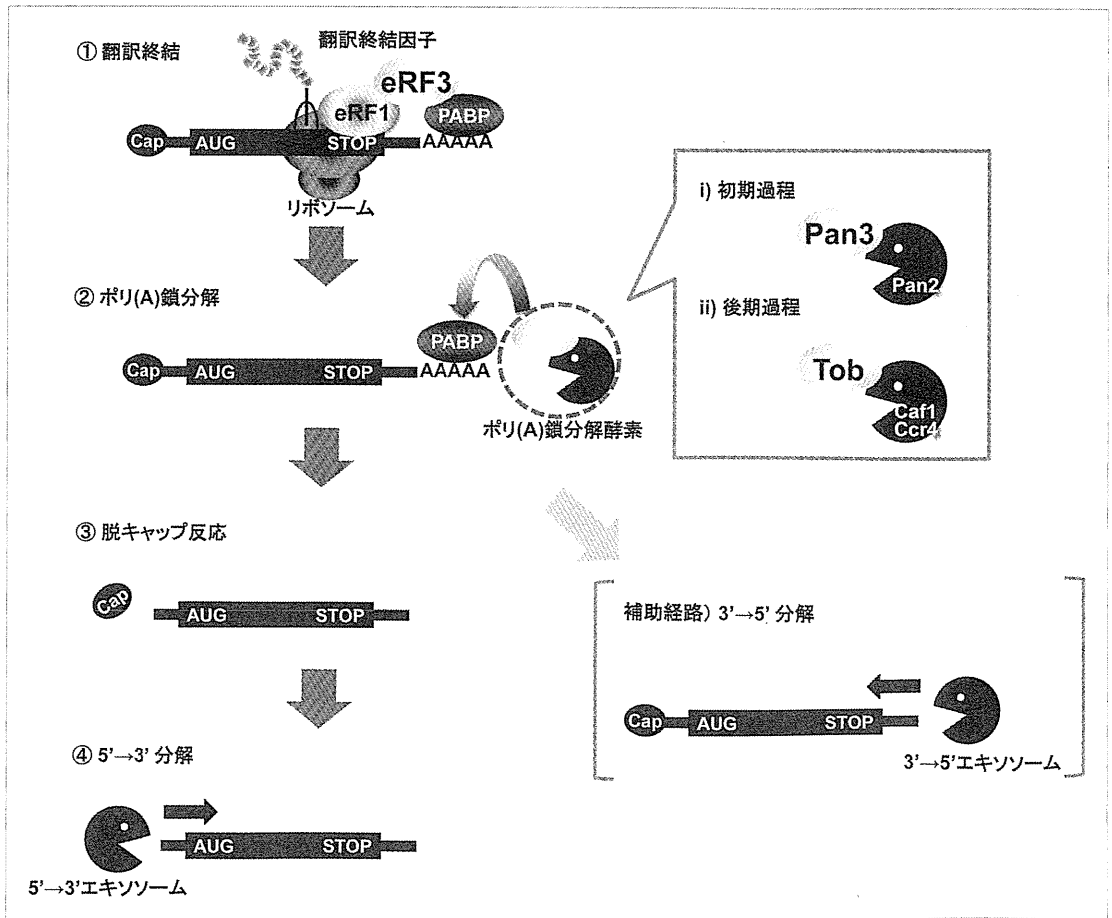

図2　通常の mRNA 分解過程
mRNA 3'末端ポリ(A)鎖の分解は mRNA 分解の開始段階かつ律速段階である．Tob は，ポリ(A)鎖分解の後期過程を制御する(本文参照)．

※　mRNA ポリ A 鎖分解／デアデニレーションについての用語解説は，52 頁参照．

分子メカニズムを解明する過程で，Tobがポリ(A)鎖分解の制御を担う因子であることを見いだした.[8,9]

ポリ(A)鎖分解は，ゆっくりとした短縮化の起こる初期過程と，急速な短縮化とmRNA本体の分解が見られる後期過程からなり，それぞれ異なる2種類のポリ(A)鎖分解酵素複合体Pan 2-Pan 3およびCaf 1-Ccr 4により触媒される.[9~11] これら複合体と翻訳終結反応を担うeRF 1-eRF 3複合体は，いずれもmRNA 3'末端ポリ(A)鎖結合タンパク質(poly(A)-binding protein：PABP)との相互作用を介して作用する．Pan 2-Pan 3はPan 3を介して，eRF 1-eRF 3はeRF 3を介してそれぞれPABPと結合することが明らかにされていた．一方,[10,12] Caf 1-Ccr 4とPABPとの相互作用は直接的なものではなく，どのようにして結合するかは不明であった．その結合を仲介する因子を探索するにあたり，我々はTobがPABPおよびCaf 1の両者と結合できることに着目した.[13,14] リコンビナントタンパク質を用いた解析により，TobはCaf 1とPABPの結合を仲介するアダプターとして機能することが明らかとなった．Tobの機能を抑制することで，ポリ(A)鎖分解の後期過程のみが抑制されたことから，Tobのポリ(A)鎖分解制御への関与が示された．重要なことに，筆者らはTobとPan 3，eRF 3はPABPとの結合において互いに競合関係にあることを突き止め，この競合関係こそが翻訳終結からポリ(A)鎖分解に至る過程を説明できるメカニズムであることを明らかにした．すなわち，翻訳終結反応はPABPからのeRF 3の解離を伴うが，eRF 3が解離したPABPにはPan 3およびTobが順次結合できることになり，ポリ(A)鎖分解が開始されるものと考えられる．

以上のようにTobは，eRF 3およびPan 3と競合的にPABPと結合し，Caf 1-Ccr 4をmRNA上にリクルートすることでポリ(A)鎖分解を制御する役割を担っている．

4 Tobは転写産物特異的なmRNA分解も制御する

ポリ(A)鎖分解過程はmRNA分解の律速段階であり，その速度の調節はmRNAの安定性，ひいては最終的なタンパク質産生量に大きく影響することから，TobはmRNA分解を調節することによって増殖抑制をはじめとした種々の生物活性を制御しているのではないかと考えられてきた．しかしながら，上述のようにTobが一般的なmRNAの分解制御に関わるということでは，Tobが特異的な生物活性を制御することをうまく説明することはできない．筆者らは，このような特異的な制御の説明が可能なTobの新規結合タンパク質としてCPEBファミリーを同定した．CPEBファミリーは，細胞質ポリアデニル化因子として知られるCPEBおよびCPEB 2-4の4種より構成され，これらはいずれもC末端に構造的に類似したRNA結合ドメインを介してmRNAの3'非翻訳領域(3'untranslated region：3'UTR)に結合する.[15] Tobは，これらCPEBファミリーと直接的に結合する．

CPEBとCPEB 3を対象として複合体形成様式を解析した結果，CPEBsはTobを介してCaf 1と結合することを確認した．このことは，CPEB/CPEB 3がTob-Caf 1との結合を介したポリ(A)鎖分解に働くことを示唆している．実際に，ファージ由来のRNA結合タンパク質MS 2を融合してCPEBあるいはCPEB 3を，3'UTRにMS 2結合配列を持つレポーターmRNAに強制的に結合させたとき，レポーターmRNAのポリ(A)鎖の急速な短縮化とそれに伴うmRNAの不安定化が観察された．この効果は酵素活性を持たないCaf 1変異体の発現により強力に抑制されたことから，Tob-Caf 1のリクルートが分解促進に関与していることが示された．では，TobはCPEBファミリーとの相互作用を介して，具体的にどのようなmRNAの分解を制御するのだろうか．以下では，CPEBとCPEB 3に着目した研究結果について述べる．

1. Tobによるがん抑制は，CPEBとの結合を介したがん遺伝子 *c-myc* の発現抑制によって説明できる

CPEBはmRNA 3'UTRに存在するCPEと呼ばれるウリジンに富む配列に結合する．ほ乳動物細胞におけるCPEBの標的mRNAとしては当時，がん遺伝子 *c-myc*，*c-jun* およびがん抑制遺伝子 *p53* が同定されていた．これら遺伝子の3'UTRを付加し

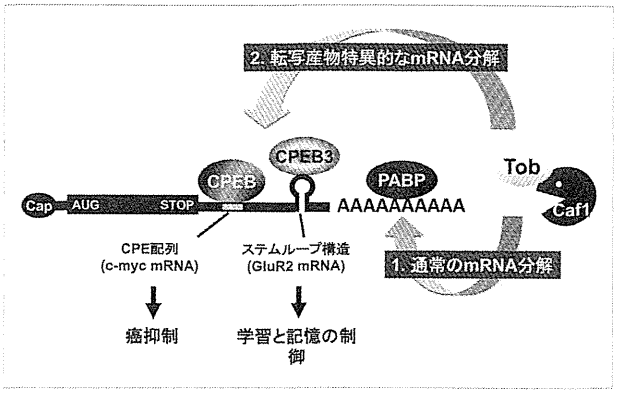

図3　Tobによるmrna分解制御

Tobはポリ(A)鎖結合タンパク質PABPとの結合を介して通常のmRNA分解を制御している．一方で，Tobは配列特異的なRNA結合タンパク質CPEBsとの結合を介して，3'UTRにCPEBs特異的結合配列を持つmRNAのみを対象として，転写産物特異的にmRNA分解を制御する機能も有している．

たレポーターmRNAに対するCPEBの影響を解析したところ，*c-myc* 3'UTRレポーターのみにおいてCPEB過剰発現によるmRNA量の低下が見られた．レポーターmRNAの分解過程を詳細に調べた結果，CPEBはポリ(A)鎖の短縮化を大幅に加速することでmRNAを不安定化していることが確認された．この分解促進はTobのノックダウンあるいは酵素活性を欠くCaf1変異体の発現により抑制されたことから，CPEBはTob-Caf1との結合を介して*c-myc* mRNAの分解を制御していることが示された(図3)．[16]

*c-myc*は，血清刺激に応答して直ちに発現上昇する初期応答遺伝子として知られる．血清刺激後のc-myc mRNAの急激な蓄積は，転写活性化とmRNAの安定化が同時に起こることで達成される．血清刺激の前後における複合体解析を試みたところ，TobとCPEBは血清飢餓時において安定に結合するが，血清刺激後には直ちに解離することが分かった．一方で，TobとCaf1との結合は血清刺激による影響を受けなかったことから，血清刺激後にTobはCaf1と共にCPEBから解離することが明らかとなった．TobあるいはCPEBをノックダウンした細胞では血清飢餓時においてmRNA安定化に伴う*c-myc* mRNA量の増加が見られ，血清刺激後の転写活性化には影響を与えないにも関わらず*c-myc* mRNA発現上昇の度合いが減弱していた．これらの結果から，血清飢餓時においてCPEB-Tob-Caf1複合体は*c-myc* mRNAの分解促進により発現量を最低限に保っているが，血清刺激後にはCPEBからのTob-Caf1の解離により*c-myc* mRNAが安定化し，c-Mycの急速な蓄積が起こることが示された．

*c-myc*遺伝子の脱制御は，ヒトやその他の動物における細胞のがん化と深く関連している．Tobとがんとの関係は第1章で詳しく述べたが，Tob-Caf1のポリ(A)鎖分解活性はTobによる細胞増殖の抑制に必須であることが近年報告された．[17,18] さらに，CPEBの発現異常とがんの間にも関連性が指摘されている．卵巣がん患者の腫瘍組織では，CPEBの発現が健常者と比べ大きく減少しており，[19] 非悪性のヒト乳腺上皮細胞株MCF-10Aの形質転換による初期の乳がん形成，および上皮間葉転換の過程を観察したモデル実験では，がんの進行に伴うCPEB発現の減少が観察された．[20] さらに，CPEBを欠損したマウスは腫瘍を形成しやすく，そのマウスから単離された胎児繊維芽細胞MEFは，分裂を繰り返しても細胞老化に至らず不死化している．このことはCPEBにがん抑制的な役割があることを強く示唆しており，細胞の不死化は*c-myc* 遺伝子の発現亢進によるものと考えられている．[21]

以上のことから，CPEBとの結合を介した*c-myc* mRNA分解の制御は，Tobのがん抑制遺伝子としての機能を説明する分子メカニズムの1つであると考えられる．

2. TobはCPEB3との結合を介したAMPA型グルタミン酸受容体GluR2の発現制御により学習と記憶を制御する

当初CPEB3は，AMPA型グルタミン酸受容体(α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor：AMPA receptor)サブユニットGluR2 mRNAの3'UTRに存在する特徴的なステムループ構造に結合して，そのタンパク質発現を抑制することのみが報告されていた．[15]

GluR2 3'UTRを付加したレポーター遺伝子を作製しCPEB3がレポーターの発現に与える影響を解析したところ，CPEB3によるレポーターmRNAの減少が確認され，それはやはりポリ(A)鎖分解の

促進に伴うmRNAの不安定化によることが判明した．さらに，TobあるいはCPEB 3をノックダウンした神経芽細胞腫SK-N-SH細胞では内在性GluR 2 mRNAの半減期がコントロールと比べ有意に延長しており，細胞内における実際のGluR 2 mRNAの安定性制御に対するCPEB 3とTobの重要性が示された．これらのことから，TobはCPEB 3との結合を介してGluR 2の発現をmRNA分解の過程で制御することが示された(図3)．[22]

グルタミン酸受容体は中枢神経系のシナプス後膜に多く発現して興奮性神経伝達を司り，学習や記憶といった高次の脳機能に深く関わる．GluR 2を欠くマウスでは，海馬CA 1領域における長期増強(long-term potentiation：LTP)の亢進が観察されることから，[23] TobとCPEB 3を介したGluR 2発現の抑制はLTPを増強させることが予想される．逆に，この機能の破綻はLTPの減弱をもたらすと考えられるが，実際にTobを欠損したラットでは，学習・記憶の障害とともにLTPの抑制が観察された．[7] さらに，CPEB 3の発現に影響する一塩基多型の解析によると，CPEB 3はヒトにおけるエピソード記憶の形成と関わるようである．[24] これらのことから，TobとCPEB 3との結合を介した*GluR 2* mRNA分解制御の，学習・記憶への関与が伺える．

5 おわりに

上述のように，TobはPABPとの結合を介した通常のmRNA分解だけでなく，CPEBファミリーとの結合を介した転写産物特異的なmRNA分解も制御している(図3)．最近になって，骨形成に関与する転写因子*Osterix* mRNAも，Tobのパラログ Tob 2とCPEB 2-4との相互作用による制御を受ける可能性が示唆された．[25] Tobによる特異的な制御を受けるmRNAは今後も更に同定されていくことが予想され，将来的にはTobの生物活性が種々の特異的mRNAの分解制御で説明できるようになると考えられる．

さらに，上述の*c-myc* mRNA分解の例で見られたように，Tobを介したmRNA分解は細胞外部からの刺激によりオンとオフが切り替えられる．CPEB 3との結合を介した*GluR* 2 mRNA分解においても，*N*-methyl-D-aspartate(NMDA)などによる受容体刺激によって同様の制御がなされている可能性が高い．今後の研究により，Tobを介したmRNA分解がどのようなシグナル伝達の下流で制御され，どのようなメカニズムで遺伝子発現スイッチのオンオフが制御されるのか，その詳細な解明が期待される．

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Negative regulation of hepatitis B virus replication by forkhead box protein A in human hepatoma cells

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ABSTRACT

Hepatitis B virus (HBV) replication is controlled by liver-enriched transcriptional factors, including forkhead box protein A (FOXA) members. Here, we found that FOXA members are directly and indirectly involved in HBV replication in human hepatic cells. HBV replication was elevated in HuH-7 treated with individual FOXA members-specific siRNA. Reciprocally, the downregulation of HBV replication was observed in FOXA-induced HuH-7. However, the mechanism of downregulation is different among FOXA members at the level of HBV RNA transcription, such as precore/pg RNA and 2.1 kb RNA. In addition, FOXA1 and FOXA2 suppressed nuclear hormone receptors, such as HNF4α, that are related to HBV replication.

1. Introduction

Hepatitis B virus (HBV) is one of the major causes of acute and chronic hepatitis leading to liver cirrhosis and to hepatocellular carcinoma (HCC). HBV has a partially double-stranded circular 3.2 kb genome which carries four viral genes, C (for core and e antigen), P (for DNA polymerase), S (for surface antigens), and X (for X protein). The expression of viral transcripts is regulated by four promoters (Cp, S1p, S2p, and Xp) and two enhancers (Enhancer I and II) [1]. The binding of liver-specific transcriptional factors such as hepatocyte nuclear factors (HNFs) and CCAAT/enhancer-binding protein family (C/EBP) members to those promoters and enhancers is thought to determine the liver tropism of HBV [2].

There are no cell culture systems that reflect the HBV life cycle because differentiated phenotypes of the liver are partially diminished or changed in the culture. For example, the lack of Na^+/taurocholate cotransporting polypeptide (NTCP), which was characterized as a functional HBV receptor, was reported in HuH-7 and HepG2 cells [3]. It has also been reported that C/EBPα is involved in the terminal differentiation of the liver and its upregulation in some HCC cell lines contributes to cell growth [4]. These results suggested that the intracellular environment of HCC-derived cell lines, including the expression of liver-specific transcriptional factors, was not suitable for HBV replication.

Forkhead box protein A (FOXA), also known as hepatic nuclear factor 3 (HNF3), consists of three members, FOXA1 (HNF3α), FOXA2 (HNF3β) and FOXA3 (HNF3γ). FOXA is one of the liver-enriched transcriptional factors and plays important roles in both liver development and liver metabolism [5,6]. FOXA is also thought to be a key regulator of HBV replication, because all HBV promoters and enhancers contain a FOXA-binding motif. In fact, FOXA has been shown to activate the transcriptional activity of HBV promoters and enhancers in a reporter assay [7–11]. However, pregenomic

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RNA (pgRNA) expression was repressed by FOXA2 in NIH3T3 cells that stimulate HBV replication by transfecting both HBV- and HNF4α-encoded plasmids [12]. Pervious studies were performed using non-hepatic cells. Therefore, further studies using genome-length HBV and human hepatic-derived cells will be needed to understand the roles of FOXA members in HBV replication. There are several reports indicating that HBV is regulated by FOXA2 in vivo. For instance, HBV replication was decreased in HBV transgenic mice transfected with rat FOXA2 [13]. Moreover, the distribution of HBV replication was negatively correlated with FOXA2 expression in the liver of patients with chronic hepatitis B [14]. These results suggested that, at the very least, FOXA2 negatively regulated HBV replication. To further elucidate the role of FOXA in HBV replication, studies describing other FOXA members are required. In this report, we investigated the role of all FOXA members in HBV replication using human hepatic-derived cell culture systems.

2. Materials and methods

2.1. HBV plasmid, antibodies, and siRNAs

HBV plasmid (pUC19/C_JPNAT) was kindly provided by Dr. Tanaka (Nagoya City University). Anti-FOXA1 antibody (Ab) (Anti-FOXA1 (ab2)) was obtained from Sigma (St. Louis, MO). Anti-FOXA2 Ab (D56D6) was obtained from Cell Signaling Technology (Beverly, MA). Anti-FOXA3 Ab (ab108454) and anti-HNF4α Ab were obtained from Abcam (Cambridge, MA). Anti-HBsAg (bs-1557G) Ab was obtained from Bioss (Boston, MA). siRNAs were obtained as siGENOME SMARTpool siRNA (human FOXA1: M-010319-01; human FOXA2: M-010089-01; human FOXA3: M-010319001; and Non-Targeting siRNA Control pool: D-001206-13) from Thermo Fisher Scientific (Waltham, MA).

2.2. Silencing of FOXA gene expression by RNA interference

HuH-7 cells were plated on a collagen-coated plate at a density of 2×10^4 cells/cm^2 and precultured in 10% FBS/DMEM for 24 h. The precultured HuH-7 cells were transfected with control, FOXA1-, FOXA2-, or FOXA3-specific siRNA by using a transfection reagent, Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Twenty-four hours after the treatment, the medium was replaced and then the cells were transfected with pUC19/C_JPNAT using FuGENE HD (Promega, Madison, WI). Finally, the medium was replaced at 24 h after transfection and the samples were collected 2 days later.

2.3. Establishment of Tet-inducible FOXA-expressing cells

Tet-inducible FOXA-expressing HuH-7 cells were established using a Retro-X™ Tet-On Advanced Inducible Expression System (Takara-Bio Inc., Shiga, Japan). Briefly, we infected HuH-7 cells with a retrovirus vector, pRetroX-Tet-On Advanced, and used G418 to select the cells with stable RetroX-Tet-On Advanced HuH-7 clones. We next infected the clone with a retrovirus vector, either pRetroX-Tight-Pur-FOXA1, FOXA2 or FOXA3 and selected the cells with puromycin to generate Tet-inducible FOXA-expressing HuH-7 cells (HuH-7/Tet/FOXA). HuH-7/Tet/FOXA cells were plated on a collagen-coated plate at a density of 6×10^4 cells/cm^2 and precultured in 10% tetracycline-free FBS (Takara) containing DMEM for 24 h, and then the medium was replaced with ±1 μg/ml doxycycline (dox)-containing medium to induce FOXA expression. At the same time point, cells were transfected with pUC19/C_JPNAT using FuGENE HD. The medium was replaced at 24 h after transfection and samples were collected 3 days later.

2.4. Western blot analysis

Total cellular protein was extracted with RIPA buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was determined using a BCA protein assay kit (Thermo). Five micrograms of total protein extract was subjected to SDS–PAGE. After the electrophoresis, proteins that had migrated on the gel were transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with a skim milk solution. The membrane was first incubated with the primary Ab and then with the horseradish peroxidase-conjugated secondary Ab. The protein bands were visualized by using a Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA). The intensity of each band was quantified with image analyzer (Image J, NIH, Bethesda, MD, USA).

2.5. Detection of HBV RNA

Total RNA was extracted from cells by using Isogen reagent (Nippon Gene, Tokyo, Japan). Total RNA was treated with RNase-free DNase I (Promega) to remove contaminated plasmid DNA. Northern blot was performed to detect HBV transcripts. Five micrograms of DNase-treated total RNA was subjected to agarose/formaldehyde gel electrophoresis, then transferred onto Hybound P$^+$ membrane (GE). HBV RNA was hybridized with DIG-labeled 0.4 kb HBV DNA probe designed at X ORF, then detected by DIG detection kit (Roche). Real-time RT-PCR was performed to analyze precore and pregenomic RNA (pgRNA) levels by the fluorescent dye SYBR Green I method using the SYBR Premix Ex Taq, Perfect Real Time (Takara) with a LightCycler Nano System (Roche Diagnostics, Basel, Switzerland). The primer pairs for precore RNA or precore/core RNA were designed according to previous report [15]. The level of pgRNA was calculated by subtracting the value of precore RNA from that of precore/core RNA.

2.6. Detection of capsid associated HBV DNA

Intracellular capsid HBV DNA was detected by Southern blot as described previously with minor modifications [16,17]. Briefly, cells were lyzed with 1% NP-40, 1 mM EDTA, 50 mM Tris–HCl (pH7.5) and protease inhibitor cocktails (Roche), then centrifuged to remove nuclei. The supernatant was treated with DNase I, and then proteins were digested with SDS and proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Nucleic acid was purified with 2 times phenol/chloroform extractions and ethanol precipitation. Southern blot was performed by using DIG High Prime DNA Labeling and Detection Kit (Roche). DIG-labeled 3.2 kb whole HBV genome (C_JPNAT) was used to detect HBV replicative intermediates.

2.7. Detection of HBV DNA in the culture supernatant

The supernatant of HuH-7 cells after transfection of HBV plasmid was centrifuged at 15 000 rpm for 5 min to remove cell debris. The supernatant was treated with DNase I in the presence of 100 mM $MgCl_2$ and 10 mM $CaCl_2$ at 37 °C, then the reaction was stopped by the addition of EDTA. Viral DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). Real-time PCR was performed to detect HBV DNA with the specific primers described previously [18].

2.8. Analysis of host gene expression

Real-time RT-PCR was performed to detect host gene expression as described elsewhere. The primer pairs used in this experiment were showed in supplementary Table S1.

2.9. Statistical analysis

Data represent the mean ± standard error of at least triplicate experiments. *P*-value were determined by Student's *t*-Test. $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. FOXA gene silencing increased HBV replication

To clarify the role of FOXA2 in HBV replication, we performed silencing of the FOXA2 gene using a FOXA2-specific siRNA in human-hepatoma derived HuH-7 cells. We confirmed all FOXA members were expressed at the protein level in HuH-7 cells. With FOXA2-specific siRNA treatment, FOXA2 protein expression in HuH-7 cells was obviously suppressed (Fig. S1a). Cell growth was not changed in FOXA2-specific siRNA-treated cells (Fig. S1d). Under these conditions, we observed the expression of 3.5 kb, 2.1 kb and 0.7 kb HBV RNA were increased in FOXA2-specific siRNA-treated cells by Northern blot analysis (Fig. 1a). We could not compare the expression of 2.4 kb RNA because the expression level was low in our experimental system. We further investigated the expression of precore/pg RNA by real time-RT-PCR using their specific primers. Although precore RNA was not changed by FOXA2 gene silencing, the expression of pgRNA was elevated in FOXA2-specific siRNA-treated cells (Fig. 1b). The expression ratio of precore/pg RNA was decreased by FOXA2 gene silencing (Fig. 1b). The HBV replicative intermediates were increased in cells treated with FOXA2-specific siRNA (Fig. 1c). The synthesis of small S proteins (gp27 and p24) was elevated in FOXA2-specific siRNA-treated cells (Fig. 1d). Secreted HBV DNA in the culture medium from FOXA2-specific siRNA-treated cells was significantly elevated in comparison with that from control siRNA-treated cells (Fig. 1e). These results indicated that HBV replication was elevated in FOXA2 siRNA-treated cells.

We next investigated the role of other FOXA members, FOXA1 and FOXA3, in HBV replication (Fig. 2). FOXA1 and FOXA3 protein expression was suppressed with FOXA1- and FOXA3-specific siRNA, respectively (Fig. S1b). Cell growth was decreased in FOXA1-specific siRNA-treated cells and slightly decreased in FOXA3-specific siRNA-treated cells (Fig. S1e). HBV replication was increased in both FOXA1- and FOXA3-specific siRNA-treated cells, as indicated by HBV RNA expression (Fig. 2a), pgRNA expression (Fig. 2b), HBV replicative intermediates (Fig. 2c), small S protein level (Fig. 2d) and the supernatant HBV DNA level (Fig. 2e). Since the redundant function was observed in the individual FOXA-specific siRNA treatment, we investigated the effect of combination treatment of each FOXA-specific siRNA on the HBV replication (Fig. 2f). The supernatant HBV DNA level was 4-fold increased in all FOXA-specific siRNA mixture treated cells. The results of a series of FOXA gene-silencing experiments showed that HBV replication was elevated in HuH-7 cells treated with FOXA siRNA, but the phenotype was slightly different among FOXA members.

3.2. Induction of FOXA reduced HBV replication

To further study the role of FOXA in HBV replication, we established dox-inducible FOXA expressing HuH-7. We investigated whether the expression of each type of FOXA was induced by dox treatment (Fig. S1c). Cell growth was not changed by the induction of each FOXA gene (Fig. S1f). HBV transcription and replication were strongly suppressed by the induction of either FOXA1 or FOXA2 gene, and slightly suppressed by the induction of FOXA3 (Fig. 3a and c). FOXA members inhibited pgRNA expression rather than precore RNA expression (Fig. 3b). Interestingly, the ratio of

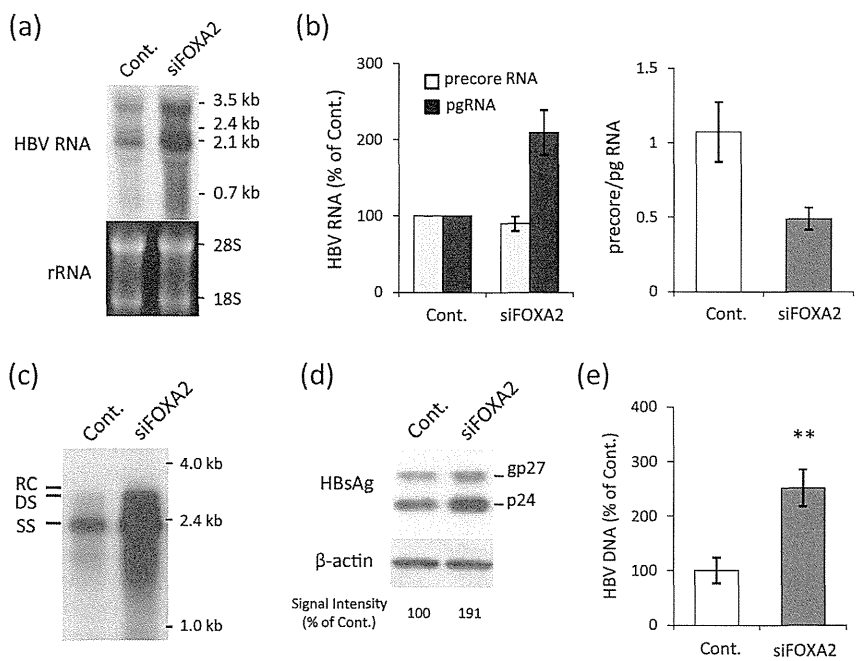

Fig. 1. FOXA2 gene silencing promoted HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of that in cells treated with control siRNA (Cont.). The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot analysis. The bands gp27/p24 were indicated as small S protein respectively. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of control siRNA (Cont.). (e) HBV DNA in the culture medium at 3 days after HBV plasmid transfection (= 4 d after siRNA transfection) was quantified by real-time PCR after DNase I treatment. Data were expressed as the percent of that in cells treated with control siRNA (Cont.).

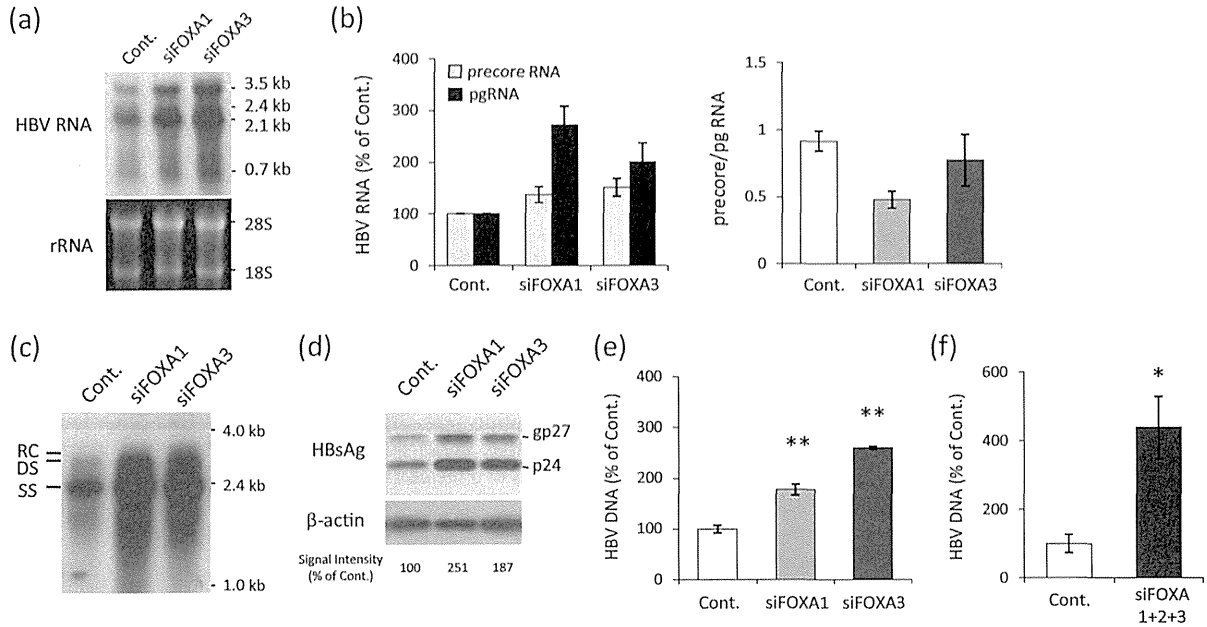

Fig. 2. FOXA1 and FOXA3 gene silencing promoted HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of that in cells treated with control siRNA (Cont.). The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot. The bands gp27/p24 were indicated as small S protein. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of control siRNA (Cont.). (e) HBV DNA in the culture medium at 3 days after HBV plasmid transfection (= 4 d after siRNA transfection) was quantified by real-time PCR. Data were expressed as the percent of that in cells treated with control siRNA (Cont.). (f) HuH-7 cells were transfected with 50 nM of control (Cont.) or combination of FOXA1 (10 nM), FOXA2 (30 nM) and FOXA3 (10 nM)-specific siRNA (siFOXA1 + 2 + 3) by using a transfection reagent. HBV DNA in the culture medium at 3 days after HBV plasmid transfection was quantified by real-time PCR. Data were expressed as the percent of that in cells treated with control siRNA (Cont.).

precore/pg RNA was increased only in FOXA2-induced cell (Fig. 3b). Small S proteins were decreased in FOXA1- and FOXA2-induced cells, but not in FOXA3-induced cells (Fig. 3d). Secreted HBV DNA in the culture supernatant was significantly decreased in cells overexpressing any of the FOXA members (Fig. 3e). These results indicated that FOXA induction suppressed HBV replication, but the mechanism was different among FOXA members.

3.3. Regulation of hepatic differentiation by FOXA members

Liver-enriched transcriptional factors control hepatic differentiated states in the liver and is thought to engage in crosstalk [19,20]. HNF4α is a central factor which involves in hepatic maturation and regulates many liver-specific genes, including albumin [21]. HNF4α has also been reported to be a positive regulator of HBV replication [22,23]. Therefore, we investigated the possibility that FOXA members regulated HBV replication via HNF4α and other nuclear hormone receptors by using a Tet-inducible FOXA-expressing system. HNF4α mRNA expression was significantly suppressed by approximately 50% in FOXA1- and FOXA2-induced cells (Fig. 4a). However, the induction of FOXA3 did not significantly affect the HNF4α level. We also obtained similar expression pattern regulated by FOXA members in RXRα and PPARα expression but not in HNF1α and HNF1β (Fig. 4b). These results suggested that HBV replication was negatively regulated by FOXA members, partly mediated via the downregulation of HNF4α and other nuclear hormone receptors expression.

4. Discussion

Previous studies demonstrated that all HBV promoters and enhancers contain at least one FOXA binding site [2]. In this study, we showed that the transcription of 3.5 kb, 2.1 kb and 0.7 kb RNA were regulated by FOXA members (Figs. 1a, 2a, and 3a). 3.5 kb RNA contains precore RNA and pgRNA. The former codes HBeAg, which is reported as a negative regulator for HBV [24]. The latter codes core and polymerase and also acts as a template for HBV DNA, so that pgRNA directly serves for HBV replication [25]. Actually, the mutations, A1762T and G1764A, which was frequently observed in chronic hepatitis B patients, suppressed precore RNA expression and shows high HBV replication [26]. Therefore, the change of the expression ratio of precore/pg RNA was important for HBV replication. Here we showed that FOXA members negatively regulate pgRNA expression rather than precore RNA expression (Figs. 1b, 2b, and 3b). However, the effect of FOXA on the precore/pg RNA ratio was somewhat different among members. Our results demonstrated that FOXA2 caused the greatest effect for precore/pg RNA ratio in both FOXA2 gene silencing and induction studies. On the contrary, FOXA3 showed less effect for precore/pg RNA ratio than other members (Figs. 2b and 3b). The studies using non-hepatic cell lines, which supported HBV replication by introducing nuclear hormone receptors, showed that FOXA1 and FOXA2 antagonize HBV replication [22]. It is also reported that FOXA1 and FOXA2 directly interfered with the elongation rate of pgRNA [12]. These results suggested that FOXA members negatively regulate HBV transcription at various transcriptional steps, but their contributions were different among members.

The HBV surface antigen is composed of large, middle, and small S proteins. The large S protein is transcribed from 2.4 kb preS1 RNA, whereas middle and small S proteins are transcribed from 2.1 kb preS2/S RNA. Different promoters, S1p and S2p, independently regulate these RNAs, respectively [1]. We had expected that HBV surface antigens would be activated by FOXA members, because FOXA activated both S1p and S2p in reporter

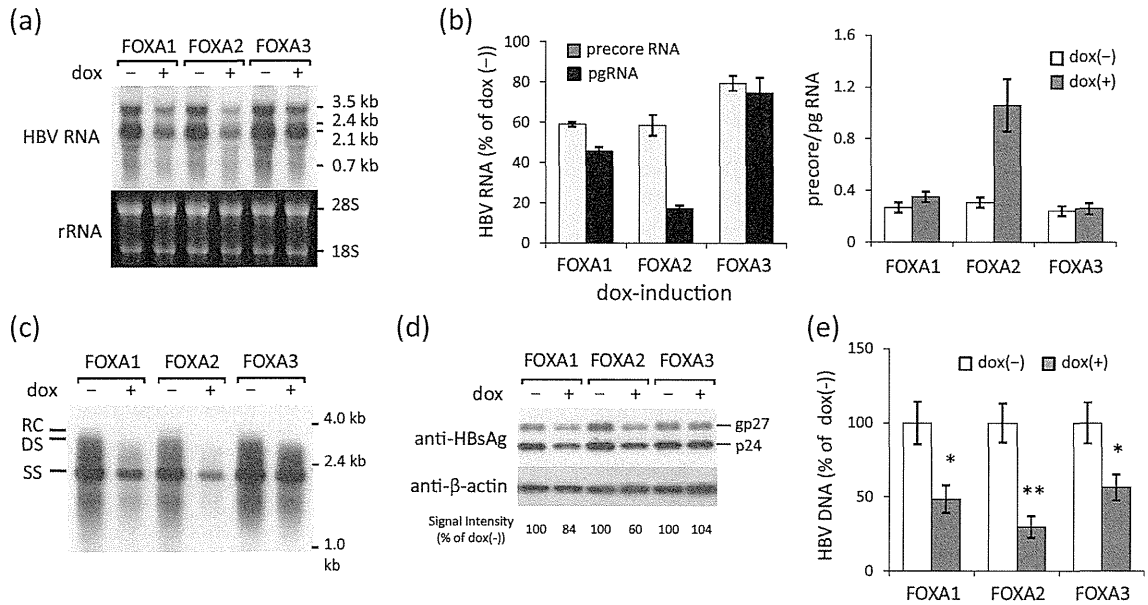

Fig. 3. Dox-induction of FOXA expression reduced the HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of a percentage of that in dox-untreated. The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot. The bands gp27/p24 were indicated as small S protein. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of dox-untreated (dox(−)). (e) HBV DNA in the culture media at 4 day after HBV plasmid transfection was quantified by real-time PCR. Data were expressed as a percentage of that in the medium from dox-untreated (dox(−)) cells.

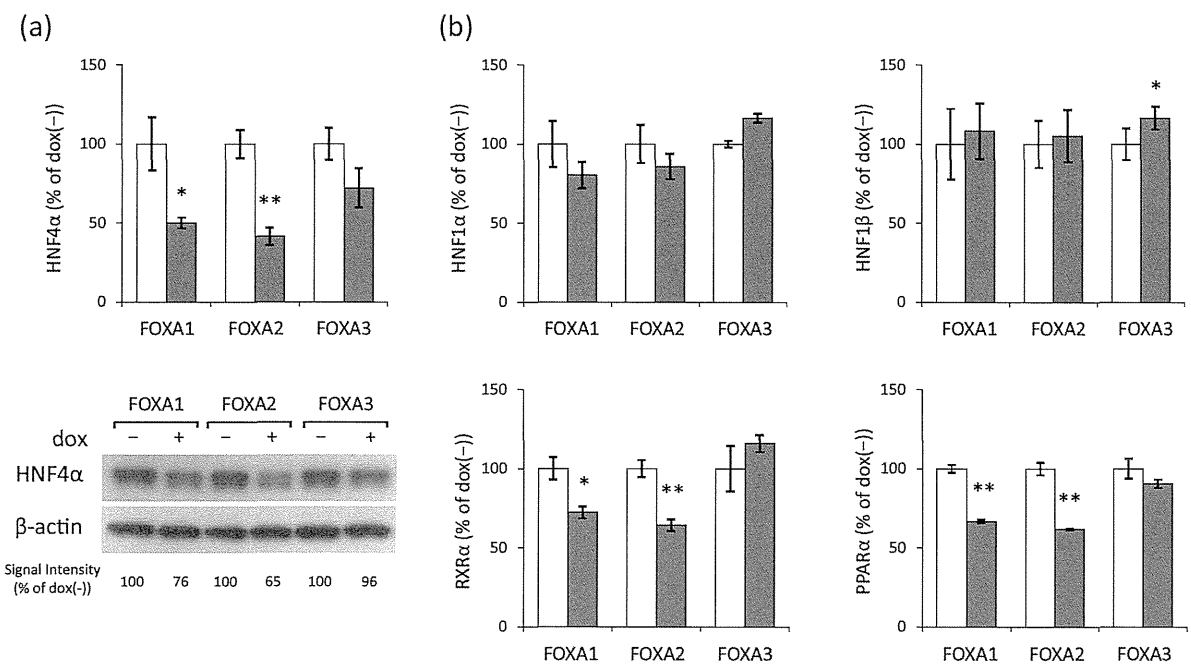

Fig. 4. Effect of FOXA induction by dox on the expression of liver enriched transcriptional factors in HuH-7 cells. (a) The expression of HNF4α was analyzed by real-time RT-PCR (graph) and Western blot (lower panel). The expression of HNF4α mRNA was expressed as a percentage of dox-untreated (dox(−)) cells. The protein band intensity was quantified by densitometric analysis and indicated below each lane. The value of HNF4α was normalized to that of β-actin, and expressed as percent of dox-untreated (dox(−)) cells. (b) The expression of HNF1α, HNF1β, RXRα, and PPARα mRNA were measured by real-time RT-PCR. Data were expressed as a percentage of dox-untreated (dox(−)) cells.

assays [10,11]. However, our results using 1.24-fold genome-length HBV indicated that only the small S protein was downregulated by FOXA members, especially FOXA1 and FOXA2. This was due to the methodological differences between the reporter assay and HBV replication system using a 1.24-fold HBV genome. As a report regarding HBV enhancer [27,28], HBV transcription thought

to be regulated by its multiple enhancers. In this respect, the studies using over genome-length HBV were thought to be more suitable for understanding the mechanism of HBV replication.

Liver function is controlled by the set of liver-enriched transcriptional factors [29]. FOXA members are also key regulators for liver development and liver-specific functions [5,30]. Based on the studies using knockout mice for various FOXA members, the function of FOXA in those events is thought to differ among members [31]. In this study, we found that the suppression of HNF4α, PPARα and RXRα expression was observed only in FOXA1- or FOXA2-expressing cells (Fig. 4). These nuclear hormone receptors are important for HBV replication [22]. As for HNF4α, the reduction of HNF4α expression by TGF-β1 resulted in the suppression of HBV replication [32]. The replication of HBV was inhibited by HNF4α-specific siRNA in HepG2 cell transfected plasmid containing 1.3-fold HBV genome [33]. These results suggested that FOXA1 and FOXA2 had indirect pathways leading to the suppression of HBV replication via nuclear hormone receptors. Moreover, we observed that the regulation of small S expression was different between FOXA1/2 and FOXA3 (Fig. 3). Because there were no HNF4-binding sequences in Sp2 [1], the regulation of small S by FOXA was thought to be independent of HNF4α. FOXA members bind similar DNA sequences via highly conserved Forkhead box motifs, but their gene regulation differs among various cell types [31,34]. These results suggested that the different regulatory roles between FOXA1/2 and FOXA3 in small S expression consisted of not only direct binding to the HBV genome but also indirectly regulation through FOXA target genes. Further studies will be needed to address these questions.

It has been reported that the infection of HBV in vitro was restricted only in differentiated-hepatocytes, such as human primary hepatocytes [35]. The development of HBV-susceptible cells has been attempted using HepaRG cells [36], HuS-E/2 cells [37], and umbilical cord matrix stem cells [38]. These results indicated that the differentiated state of these cells was important for viral infection. However, a method of persistent HBV infection using the established cell lines has not been developed yet. One of the reasons is that the HCC cell lines alter hepatic differentiated states, including by changing the expression of hepatic transcriptional factors, to maintain tumor phenotypes [4,39]. Here, we showed that the changes of FOXA expression levels altered the replication of HBV in HuH-7. These results suggested that the control of liver-enriched transcriptional factors in HCC cell lines is important for the development of effective HBV replication in cell culture systems.

In conclusion, we demonstrated that all FOXA members negatively regulated in HBV replication via downregulation of the level of HBV transcripts. Small S proteins were decreased in FOXA1- and FOXA2-, but not in FOXA3-induced cells. We also reported that the downregulation mechanism was different among FOXA members. It is hoped that these results will contribute to the establishment of a persistent HBV replication system, which could lead to the development of effective antiviral therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.03.022.

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PLOS ONE

Chronic Hepatitis B Prevalence among Children and Mothers: Results from a Nationwide, Population-Based Survey in Lao People's Democratic Republic

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Abstract

Background: Hepatitis B is regarded as a serious public health issue in Lao People's Democratic Republic (Lao PDR), a Southeast Asian country. However, disease epidemiology among the general population is not well known, and thus a nationwide cross-sectional survey for hepatitis B surface antigen (HBsAg) prevalence in children and their mothers was conducted.

Methods and findings: We applied three-stage cluster sampling using probability proportionate to size. After randomly selecting child (5 to 9 years old) and mother (15 to 45 years old) pairs from the selected villages, questionnaires and HBsAg rapid tests were conducted. Data from 965 child and mother pairs were analyzed. Multivariate logistic regression analyses were used to investigate the independent association of individual background characteristics for the odds of being HBsAg positive. In total, 17 children and 27 mothers were HBsAg positive. HBsAg prevalence was estimated to be 1.7% (95% confidence interval: 0.8%-2.6%) in children, and 2.9% (95% confidence interval: 1.7%–4.2%) in their mothers after taking sampling design and weight of each sample into account. Mother's infection status was positively associated with HBsAg positivity in children ($p<0.001$), whereas other potential risk factors, such as ethnicity, proximity to health centers, and history of surgery, were not. There were no significant associations between mother's HBsAg status and history of surgery, and other sociodemographic factors.

Conclusions: Despite the slow implementation of the hepatitis B vaccination program, HBsAg prevalence among children and their mothers was not high in Lao PDR compared to reports from neighboring countries. The reasons for the differences in prevalence among these countries are unclear. We recommend that prevalence surveys be conducted in populations born before and after the implementation of a hepatitis B vaccination program to better understand the epidemiology of hepatitis B.

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Introduction

More than two billion people have been infected with hepatitis B worldwide, and among these individuals, more than 350 million suffer from chronic hepatitis B virus (HBV) infection [1,2,3]. Infection with HBV results in 600,000 to 1.2 million deaths per year due to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2,4]. HBV is responsible for 60% to 80% of the world's hepatocellular carcinoma cases, one of the major three causes of death in Africa, Asia, and the Pacific Rim, and accordingly, has been categorized as a Group 1 carcinogenic agent to humans by the International Agency for Research on Cancer [5].

The prevalence of hepatitis B differs throughout the world. Southeast Asian countries have been estimated to have a chronic HBV infection rate of more than 8% before the introduction of hepatitis B vaccination [6]. The Western Pacific region of the World Health Organization (WHO), to which most of the Southeast Asian countries belong, is assumed to have a high prevalence of hepatitis B [7]. Specifically, the prevalence is estimated to be 9% to 12% among women of childbearing age [8] and 8% to 10% among children in pre-vaccine era [9]. The WHO

estimates that the region has 28% of the global population, while it accounts for almost half of all chronic hepatitis B infections worldwide [10].

Hepatitis B vaccination, especially within 24 hours after childbirth, is considered the most effective and efficient preventive measure against hepatitis B infection [3,11]. Based on these assumptions, the WHO set goals to lower the prevalence of chronic hepatitis B among children over 5 years of age to 2% by 2012 and 1% by 2017. To achieve these goals, the WHO plans to increase immunization coverage to 65% for the birth dose and 80% for the third dose of the hepatitis B vaccine [7].

Lao People's Democratic Republic (Lao PDR) is a Southeast Asian country, located in the center of the Indochina peninsula. The country is landlocked and surrounded by China, Vietnam, Cambodia, Thailand, and Myanmar. The neighboring countries report relatively high hepatitis B prevalence compared to other parts of the world. For example, a survey from two provinces in Cambodia reported a hepatitis B surface antigen (HBsAg) prevalence of 7.7% (95% CI: 6.2%–9.3%) among healthy volunteer adults [12]. Another population-based survey in a province in rural Vietnam found that 18.8% (95% CI: 15.7%–21.9%) of adults and 12.5% (95% CI: 9.7%–15.3%) of infants were HBsAg positive at the time of the survey [13]. Thus, Lao PDR has been regarded as one of the hyperendemic countries for hepatitis B for quite some time and is ranked as a priority country by the WHO [7,9] despite a lack of data on the prevalence in a representative population. Pre-vaccine era prevalence was estimated as 11.8% [4], 8–10% [9], or 8% or more [6] for Lao PDR and Indochina countries. In response to this situation, Lao PDR has implemented the hepatitis B vaccine into the routine immunization program since 2002 (at 6, 10, and 14 weeks after birth), as well as birth dosing since 2004. The birth dosing was initiated at referral hospitals in the capital city, and then gradually expanded into rural hospitals (2006), and eventually home deliveries (2010). However, since then, no direct investigation has been conducted, and thus a nationwide survey is warranted [7,9]. The routine immunization coverage is reported as 56% for BCG, 50% for the third DPT, 50% for the third hepatitis B, 40% for measles, and 46% for oral polio vaccine in 2007, when a proportion of target children were born [14].

The primary objective of the present study was to estimate the chronic HBV infection rates by measuring the seroprevalence of HBsAg among children aged 5 to 9 years, and their mothers aged 15 to 45 years.

Methods

Ethical considerations

The survey protocol was reviewed and approved by the Ethical Committee of the Ministry of Health, Lao PDR, and the institutional review board of the National Center for Global Health and Medicine, Japan (NCGM-G-001130-00). Access to selected households was granted by the Ministry of Health, and the provincial and district government authorities.

After obtaining approval to conduct the survey from local authorities, surveyors explained the purpose of the survey to village leaders, selected participants, and their caregivers, assured them that all information would be strictly confidential and that no names would be gathered, and that there would be no benefit or penalties for agreeing or refusing to participate. Written informed consent was obtained from each mother on behalf of her child for each pair. Written informed consent was obtained from legal representatives (next of kin, caregivers, or guardians) when mothers were illiterate. The respondents' names were not recorded on the questionnaire sheets.

Study population

The target population was children aged 5 to 9 years (date of birth: January 2, 2002 to January 1, 2007) and their mothers aged 15 to 45 years (date of birth: January 2, 1966 to January 1, 1997) living in the selected cluster at the time of the survey. The reasons for this selection criteria are: 1) the national and regional hepatitis control policy target is to reduce chronic hepatitis B prevalence among children aged 5 years or older [7]; 2) Lao PDR does not have reliable HBsAg prevalence data among healthy adults, and mothers of childbearing age are considered the major source of hepatitis B infection for children; and 3) our pilot survey revealed that between 20 and 25 mother and child pairs can be practically sampled from each village.

Calculation of sample size

The equation used to calculate the required sample size is as follows [15,16]:

$$n = Z^2 \times p(1-p)DEFF \times 2/(d^2 \times RR)$$

where n = sample size
Z = significance level for 95% confidence
p = expected prevalence
$DEFF$ = design effect
d = precision
RR = response rate

The sample size (n) of 961 was calculated on the basis of an expected HBsAg seroprevalence (p) of 5%, a 5% level of significance (Z), precision (d) of ±2.0%, design effect ($DEFF$) of 2.0, two strata, and response rate (RR) of 95%. For field practicability, we requested 24 survey teams to sample 21 child and mother pairs from each cluster, with the aim of gathering 1,008 pairs in total.

Survey design and sampling

The survey applied a stratified three-stage random cluster sampling design, a type of probability sampling recommended by the WHO [15,17]. The survey was carried out by 24 survey teams (two members per team). Team members were recruited from the same districts that were under investigation to implement the survey more smoothly. The survey teams consisted of epidemiology, surveillance, or laboratory staff. The survey teams were supervised by 11 national personnel (six from the National Immunization Program and five from the National Center for Laboratory and Epidemiology, Ministry of Health) as well as 13 provincial officers.

For stratified multistage cluster sampling, immunization coverage by district and population data were obtained from the National Immunization Program, the Ministry of Health, and the Department of Statistics, Lao PDR. For post-survey weight adjustment, the survey teams obtained the latest population data from village leaders or health volunteers.

All 143 districts in Lao PDR were stratified into two strata, one having high (more than 76%) and the other having low (76% or less) immunization coverage for the third diphtheria, pertussis, tetanus, and hepatitis B (DPT-HepB)vaccines as reported in 2010. For the first stage, we selected 12 districts from each stratum using probability proportionate to size (PPS) sampling based on the population census of 2005. For the second stage, we selected two villages from each selected district by PPS sampling, and 48

villages were randomly sampled in total. In the instances in which the selected village lacked a sufficient number of children or the survey team could not approach the selected village due to safety or security reasons, the nearest village on the way back to the district center was selected. For each selected village, surveyors obtained a list of households, including age and sex, primarily from the poverty reduction program data with the assistance of the village leader, women's union, and/or healthcare volunteer. From these lists, 21 mothers aged 15 to 45 years old with children aged 5 to 9 years were randomly selected using a paper-based lottery system. When a mother had multiple children aged 5 to 9 years old, the youngest child was chosen for the survey. Special attention was paid to ensure that the child's biological mother was surveyed, as adoption is common in rural Lao PDR.

The survey was carried out from January 25th to February 4th, 2012. Each survey team successfully approached their assigned villages, with the exception of one village, which could not be visited because of road difficulties. An alternative village was chosen according to the predetermined selection criteria. In total, 1,008 children and 1,008 mothers were sampled. The overall response rate for HBsAg was 100%; however, 43 pairs were excluded from the analysis due to age ineligibility. That is, one child was over 9 years of age and 33 were less than 5 years of age. Furthermore, three mothers were over 45 years of age and six were less than 15 years of age. This happened as 43 mothers confused calendar age with traditional age. In rural areas, newborns start at one year old and a year is added to their age for each passing of a Lunar New Year. The surveyors asked participants for their age in years and their date of birth, and checked that they matched. A total of 965 pairs were included for analysis.

Questionnaires

A brief face-to-face questionnaire was administered to the sampled mother. The questionnaire consisted of 25 questions in four domains of inquiry: sociodemographic background of the family (i.e., ethnicity, family head's occupation, and mother's education level), family history of liver diseases, including mother, demographic characteristics of the child (i.e., age, sex, and place of birth), and immunization records. Additionally, questions were asked regarding exposure to potential risk factors for acquiring hepatitis B infection (e.g., history of blood transfusion, surgical operation, and sharing of toothbrush). The questionnaire was developed in English, translated into Lao, back-translated into English, and then compared and revised by bilingual staff members. A small pilot test was conducted prior to the data collection.

Testing for HBsAg

We used a simple and rapid test (Alere Determine HBsAg test card; Alere Medical Co. Ltd., Chiba, Japan) rather than the traditional ELISA test, as it was better suited to use in the field [14]. The sensitivity and specificity of the test were reported as high in two Asian countries [18,19]. In Vietnam, the Determine HBsAg test validity was measured based on comparison with HBsAg EIA. Results were 100% in both sensitivity and specificity in 328 samples [18]. In China, the Determine HBsAg performance was evaluated in comparison with HBsAg EIA for 671 samples. The sensitivity was reported to be 98.9% and specificity 100% [19]. The Determine HBsAg examination kit is one of the most reliable point-of-care HBsAg tests, and is recommended by the WHO [15]. HBsAg testing was performed according to the manufacturer's instructions. Blood was collected from a finger prick using a safety lancet (BD Safety Lancet, Becton Dickinson, NJ, USA) and glass capillary tube, and the blood was applied onto the sample pad of the rapid test kit. After applying the chase buffer, surveyors assessed the results after at least 15 minutes, but no longer than 24 hours. When no control bar appeared after 15 minutes, the test results were considered invalid, and the test was repeated. Blood spots were collected onto filter paper for further testing. A 2-day training session was organized for surveyors and supervisors on the use of the rapid test and the completion of the questionnaire. To ensure the safety of the blood collection procedure, surveyors always used a new pair of latex gloves. Surveyors were instructed to place all capillary tubes and lancets into safety boxes immediately after use.

Data entry and statistical analysis

All of the completed questionnaires were brought to a centralized location and the data were entered into a Microsoft Excel 2007 spreadsheet. Data were double-entered and cross-checked. Logistic regression tests and odds ratios were used to examine the relationship between the independent variables and HBsAg results. Multivariate logistic regression was used to investigate the independent association of different household and individual characteristics with the odds of being HBsAg positive. All estimates and standard errors were calculated by taking the multistage clustered sampling design and the weight of each sample into account to give representative, unbiased results. A p value <0.05 was considered statistically significant.

In our regression analyses, we adjusted for potential confounders by using the following variables: third DPT-HepB immunization coverage at the location of current residence, mother's age, ethnic group, mother's education level, family head's occupation, and mother's HBsAg status. For multivariate logistic regression analyses, multicollinearity was tested by calculating the variance inflation factors for each independent variable, and a value of more than 10 was considered as having multicollinearity.

All statistical analyses were carried out using STATA version 12 (Stata Corp., College Station, TX). Means and proportions were calculated using STATA's 'svy' function, with each sample weighted according to estimated population size.

Results

Socioeconomic backgrounds

The baseline characteristics of the 965 mothers and their children are summarized in Table 1. The mean age of the mothers was 29.1 years (95% CI: 26.2–33.1), and the mean age of the children was 5.8 years (95% CI: 5.4–6.3). Of the sampled children, 474 (49.4%) were male and 486 (50.6%) were female (five were unknown).

HBsAg prevalence among the general population

Of the 965 pairs included in the study, 17 children and 27 mothers were positive for HBsAg. Six child and mother pairs were HBsAg positive. The estimated prevalence was 1.7% for children (95% CI: 0.8%–2.6%) and 2.9% for mothers (95% CI: 1.7%–4.2%) after taking the sampling design and weight of each sample into account. HBsAg prevalence did not change significantly between DPT-HepB3 high and low coverage districts in both children and mothers (Table 2).

Potential risk factors

To determine whether background characteristics affect HBsAg status, we conducted multivariate logistic regression analysis in children and their mothers. In children, the mother's HBsAg status was positively associated with hepatitis B infection (Table 3),

Table 1. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old) in Lao PDR by selected background characteristics.

		n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
Mothers' age(n = 965)	15–19	4	0.41	0	0.00		0	0.00	
	20–24	85	8.80	1	1.18	0.00–3.52	3	3.53	0.00–7.53
	25–29	294	30.47	7	2.38	0.63–4.13	8	2.72	0.85–4.59
	30–34	275	28.50	6	2.18	0.44–3.92	9	3.27	1.16–5.39
	35–39	176	18.24	3	1.70	0.00–3.64	3	1.70	0.00–3.64
	40–45	131	13.58	0	0.00		4	3.05	0.07–6.04
Ethnicity (n = 963)	Low land Lao	651	67.60	9	1.38	0.48–2.28	19	2.92	1.62–4.22
	Mid land Lao	248	25.75	6	2.42	0.49–4.34	5	2.02	0.25–3.78
	High land Lao	64	6.65	2	3.13	0.00–7.51	3	4.69	0.00–10.01
[1]Transportation (n = 939)	on foot	298	31.74	1	0.34	0.00–1.00	6	2.01	0.41–3.62
	bicycle	14	1.49	0	0.00		0	0.00	
	motor bike	364	38.76	7	1.92	0.51–3.34	10	2.75	1.06–4.43
	car	183	19.49	5	2.73	0.35–5.12	6	3.28	0.67–5.88
	hand tractor	66	7.03	3	4.55	0.00–9.71	4	6.06	0.15–11.97
	other	14	1.49	0	0.00		0	0.00	
[2]Time (n = 901)	< 5 minutes	31	3.44	0	0.00		1	3.23	0.00–9.81
	5 to 15 minutes	274	30.41	3	1.09	0.15–2.33	6	2.19	0.45–3.93
	15 to 30 minutes	231	25.64	5	2.16	0.27–4.06	11	4.76	2.00–7.53
	30 to 60 minutes	209	23.20	5	2.39	0.30–4.48	4	1.91	0.04–3.79
	> 60 minutes	156	17.31	3	1.56	0.00–4.68	4	2.56	0.06–5.07
[3]Education (n = 962)	did not finish primary school	307	31.91	7	2.28	0.60–3.96	12	3.91	1.73–6.09
	primary school	374	38.88	5	1.34	0.17–2.51	10	2.67	1.03–4.32
	junior high	185	19.23	3	1.62	0.00–3.46	2	1.08	0.00–2.59
	high school	73	7.59	0	0.00		1	1.37	0.00–4.10
	college/univ	20	2.08	1	5.00	0.00–15.47	2	10.00	0.00–24.41
	other or unknown	3	0.31	1	33.33	0.00–100.00	0	0.00	
[4]Occupation (n = 963)	farmer	683	70.92	13	1.90	0.88–2.93	19	2.78	1.55–4.02
	fisherman	5	0.52	0	0.00		0	0.00	
	laborer	92	9.55	1	1.09	0.00–3.25	5	5.43	0.71–10.16
	public officer	88	9.14	1	1.14	0.00–3.40	3	6.25	1.70–10.80
	factory employee	8	0.83	0	0.00		0	0.00	
	general employee	16	1.66	1	6.25	0.00–19.57	0	0.00	
	merchant	63	6.54	1	1.59	0.00–4.76	0	0.00	
	others	8	0.83	0	0.00		0	0.00	
Mother's surgery (n = 962)	yes	95	9.88	2	2.11	0.00–5.05	3	3.16	0.00–6.74
	no	867	90.12	15	1.73	0.86–2.60	24	2.77	1.67–3.86
Child's sex (n = 960)	male	474	49.38	9	1.89	0.67–3.13			
	female	486	50.63	7	1.44	0.38–2.50			
Place of delivery (n = 961)	province hospital	207	21.54	4	1.93	0.04–3.82	6	2.90	0.59–5.20
	district hospital	105	10.93	2	1.90	0.00–4.56	5	4.76	0.62–8.90
	health center	10	1.04	0	0.00		0	0.00	
	private clinic	11	1.14	0	0.00		1	9.09	0.00–29.35
	at home	569	59.21	8	1.41	0.44–2.38	14	2.46	1.18–3.74
	in the forest	56	5.83	3	5.36	0.00–11.44	1	1.79	0.00–5.36
	other health facility	3	0.32	0	0.00		0	0.00	
Child's surgery (n = 960)	yes	22	2.29	0	0.00				

Table 1. Cont.

	n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
no	938	97.71	16	1.71	0.88–2.54			

[1]Transportation to the nearest health facility, [2] Time to the nearest health facility, [3] Mothers' completed education, [4] Family head's occupation.
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whereas the other potential risk factors were not associated according to the adjusted odds ratio. We did not obtain information regarding the type of delivery, and we did not find significant differences in HBsAg prevalence associated with delivery settings. No independent factor was positively associated with HBsAg positivity in mothers, according to the adjusted odds ratio (Table 4).

Immunization status

Written immunization records were available for 213 out of 965 children (22.1%). One hundred ninety eight children were vaccinated with three doses of hepatitis B vaccine, and 34 children were immunized on the day of birth or the following day. Five out of 213 children with immunization records were HBsAg positive (2.35%; 95% CI: 0.30–4.40%), while 12 of 752 without immunization records were HBsAg positive (1.60%; 95% CI: 0.70–2.49%). The differences between the two groups were not significant ($p = 0.46$).

Discussion

HBsAg prevalence among the general population

The estimated HBsAg prevalence in the general population was much lower in both children and adults than that of previous reports from neighboring countries and Lao PDR. For example, HBsAg prevalence in adults in Cambodia, Thailand, and Vietnam was reported to be 7.7% (95% CI: 6.2%–9.3%) [12], 6 to 10% [15,20], and 18.8% (95% CI: 15.7%–21.9%) [13], respectively. Data on HBsAg prevalence amongst children was relatively scarce, and reported to be 3.5% (95% CI: 2.4%–4.8%) in Cambodia [21], and 18.4% (95% CI: 13.4%–23.4%) in Vietnam [13]. In Lao PDR, studies in blood donors, hospitalized patients, and Lao migrant workers tested in Thailand showed HBsAg prevalence of 8.73% (95% CI: 8.69%–8.77%) [22], 17.99% (95% CI: 17.81%–18.17%) [23], and 6.86% (95% CI: 6.80%–6.92%) [24] based on the given numerators and denominators in the articles, respectively.

Since the study objective was to estimate the nationwide HBsAg prevalence among the general population of Lao PDR, and thus the study design is a cross sectional survey, it is difficult to explain the reasons for the unexpectedly low prevalence. There are several potential explanations for this observation. The survey methodology used was very different from that used for blood donors, patients, and migrant workers. We used probability sampling and thus the results are representative of the whole population, whereas studies of blood donors, hospitalized patients, and migrant workers used non-probability sampling and therefore the results are restricted to these populations. The primary objective of our survey was to estimate HBsAg prevalence among the general population, so probability sampling was the most appropriate choice. Demographic conditions among the sampled population are determined by survey methodology, and therefore the results showed discrepancy. The WHO strongly recommends probability sampling for hepatitis B prevalence survey [7,15,17]. Although Lao PDR has the lowest population density of the Indochina peninsula countries [25], the precise effects on hepatitis B prevalence of the reduced frequency of human to human contact due to the country's relatively low population density and less developed infrastructure remain unclear.

The number of HBsAg positives varied from 0 to 4 per cluster. Since the sampling design of the survey aimed to estimate the prevalence in the whole country, it is difficult to determine whether these differences reflect the local endemic status.

Potential risk factors

Our survey revealed that no potential risk factors were significantly associated with the children's infection status, with the exception of the mothers' hepatitis B infection status. HBsAg prevalence surveys in other countries revealed that history of surgery [26,27], level of education [26], and ethnicity [28] were independently associated with hepatitis B infection. The reason why we could not find any potential risk factors positively associated with hepatitis B infection among children is not clear. However, it should be noted that the primary objective of the present study was to assess HBsAg prevalence, and not its risk factors. Additionally, some reports found that HIV positive individuals are positively associated with hepatitis B virus infection

Table 2. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old).

	Children's HBsAg (+)	%	95% CI	Standard error	Design effect	Mothers' HBsAg (+)	%	95% CI	Standard error	Design effect
High coverage districts (n = 486)	6	1.14	0.23–2.04	0.44	0.82	18	3.79	1.79–5.79	0.97	1.24
Low coverage districts (n = 479)	11	2.39	0.75–4.03	0.79	1.27	9	1.88	0.49–3.37	0.69	1.22
Total (n = 965)	17	1.72	0.81–2.63	0.44	1.10	27	2.93	1.65–4.20	0.61	1.28

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Table 3. Unadjusted and adjusted odds ratio for being HBsAg positive among children from five to nine years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	2.13	0.73–6.21	0.16	3.47	0.77–15.64	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	0.70	0.28–1.78	0.44	0.87	0.31–2.47	0.79
Ethnicity	Low land Lao	1(reference)					
	others	1.90	0.67–5.40	0.22	1.41	0.26–7.72	0.68
Education	none	1(reference)					
	finished primary school or upper	1.50	0.67–3.36	0.30	1.03	0.27–3.89	0.96
Occupation	white collar	1(reference)					
	blue collar	1.15	0.37–3.64	0.80	0.60	0.18–1.96	0.38
Sex	male	1(reference)					
	female	0.75	0.21–2.62	0.63	0.65	0.21–2.08	0.46
Birth place	health facility	1(reference)					
	non-health facility	0.98	0.39–2.49	0.97	0.79	0.28–2.21	0.64
Mothers' HBsAg	negative	1(reference)					
	positive	24.02	9.45–61.07	0.00	28.13	10.21–77.53	0.00

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[29,30]; however, we did not investigate HIV due to limited budget.

WHO's regional target

The interim target of the WHO is to reduce HBsAg prevalence to less than 2% in children aged at least 5 years old by 2012 [7,31]. The point prevalence is used for monitoring the control of hepatitis B. The Regional Office for the Western pacific recommended that the country conduct a national HBsAg prevalence survey to verify whether the country has reached the regional prevalence target [9]. Following these criteria, Lao PDR had already achieved its goal. However, it is unlikely that Lao PDR achieved the target through the immunization program alone because the country has the lowest immunization coverage of all countries in the region [7,9]. Considering the relatively lower HBsAg seroprevalence among the mothers compared to those reported in previous studies, it is likely that Lao PDR had a lower prevalence even before the introduction of the hepatitis B immunization program. Therefore, the final target of reducing HBsAg prevalence to less than 1% in children aged at least 5 years could be difficult to achieve if the country simply continues its current immunization policy.

A nationwide prevalence survey targeting the general population is ideally conducted before implementing the immunization

Table 4. Unadjusted and adjusted odds ratio for being HBsAg positive among mothers from 15 to 45 years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	0.50	0.20–1.28	0.14	0.47	0.19–1.16	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	1.03	0.43–2.51	0.94	0.94	0.39–2.25	0.88
Ethnicity	Low land Lao	1(reference)					
	others	0.80	0.30–2.17	0.65	0.68	0.25–1.85	0.44
Education	none	1(reference)					
	finished primary school or upper	1.68	0.70–4.01	0.23	2.04	0.89–4.68	0.09
Occupation	white collar	1(reference)					
	blue collar	1.71	0.53–5.55	0.35	1.93	0.68–5.50	0.21
History of surgery	no	1(reference)					
	yes	1.28	0.39–4.25	0.67	1.30	0.35–4.78	0.68

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