synthesis triggered RNA silencing in human cells *in vivo*. As seen in other model organisms, the long dsRNA with sense and antisense sequences of RMRP was processed into short dsRNA of 22-nt in length in a Dicer-dependent manner. Moreover, the one strand of the short dsRNA was loaded onto human AGO2, indicating the short dsRNA is a siRNA. The expression level of both endogenous and forcedly expressed RMRP was negatively regulated by the expression of either hTERT or Dicer, suggesting the downregulation mechanism of RMRP via post-transcriptional gene silencing (Figure 2B). These findings indicate that human retains endogenous RNAi mechanism, in which the RdRP activity of hTERT plays a key role in generating functional dsRNAs.

Recently, Kaprenov and co-workers provided additional support for the existence of a mammalian RdRP (Kapranov et al., 2010). They found a new class of gene termini-associated human RNAs, which showed extremely interesting structural characters; the RNAs were ~50-nt in length, and they had non-genetically encoded 5′ poly(U) tails as well as antisense sequences of the 3′ untranslated regions. The 5′ poly(U) tails were just followed by the antisense sequences of the very 3′ ends of known mRNAs. Based on the unique structures of the RNAs, the authors speculated the possibility of a novel RNA copying mechanism, in which RdRP produces antisense RNAs from the poly(A) tail of human mRNAs.

In addition, other proteins, such as RNA polymerase II (Lehmann et al., 2007), could also exhibit similar activities. Indeed, the RdRP in *Drosophila* was indentified from the RNA polymerase II core elongator complex subunit with no conserved amino acid domains compared with canonical cellular RdRPs (Lipardi and Paterson, 2009). The authors speculated that the human homolog of *Drosophila* RdRP, human IKAP protein, also has RdRP activity.

Conclusions and perspectives

The discovery of RNAi mechanisms has changed our understanding of the role of RNA in regulating gene expression. Many types of small non-coding RNAs, including endogenous siRNAs, contribute to the gene regulation transcriptionally and post-transcriptionally. Cellular RdRPs are indispensable to generate endogenous siRNAs in RNAi of model organisms. Although mammals have no homologs of cellular RdRPs and they have been long believed to have lost endogenous RNAi systems, our recent findings revealed that h-TERT has RdRP activity and plays a role in RNA silencing. Other groups also reported indirect evidence suggesting functional human RdRPs. These novel findings indicate that endogenous RNAi mechanisms are essential and highly preserved throughout the species, and RdRP-mediated gene regulatory mechanisms might control even more extensive events than we have imagined.

TERT and *IKAP* homologs are conserved in many eukaryotes including the model organisms with canonical cellular RdRPs. At present, it is unknown whether these novel RdRPs also function in the model organisms or not. If this is the case, the endogenous RNAi biology in these model organisms regulated by another pathway could exist. If the novel RdRPs are specific for high organisms, this could bring another simple question: why they lost canonical RdRPs? Future investigations about functional diversity of the non-canonical RdRPs in RNAi could provide us with some suggestions about this question.

RNAi has enormous potential for the treatment of many genetic and acquired diseases. Even the proteins lacking ligand-binding domains or sharing high degrees of structural homology to untargeted proteins as well as disease-causing RNAs can be targeted by the sequence-specific binding of the functional small RNAs in RNAi. Currently, several RNAi-based therapeutic approaches using exogenous si-RNAs are under evaluation in clinical trials. Because hTERT is specifically and highly expressed in malignant cells, hTERT-mediated endogenous RNAi induction could become a promising novel approach in RNAi-based cancer therapeutics with reduced adverse effects in normal cells. Further functional and biochemical analysis of hTERT and other presumable human RdRPs could expand the therapeutic option for many diseases.

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References

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell *121*, 207–221.

Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). *In vitro* analyses of the production and activity of secondary small interfering RNAs in *C. elegans*. EMBO J. *26*, 5007–5019.

Astier-Manifacier, S. and Cornuet, P. (1971). RNA-dependent RNA polymerase in Chinese cabbage. Biochim. Biophys. Acta *232*, 484–493.

Baltimore, D., Eggers, H.J., Franklin, R.M., and Tamm, I. (1963). Poliovirus-induced RNA polymerase and the effects of virus-specific inhibitors on its production. Proc. Natl. Acad. Sci. USA *49*, 843–849.

Boege, F. and Heinz, L.S. (1980). RNA-dependent RNA polymerase from healthy tomato leaf tissue. FEBS Lett. *121*, 91–96.

Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J., van Wolfswinkel, J.C., Chaves, D.A., Shirayama, M., Mitani, S., Ketting, R.F., et al. (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. Cell *139*, 123–134.

Cogoni, C. and Macino, G. (1999). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature *399*, 166–169.

Covey, S.N., Al-Kaff, N.S., Lángara, A., and Turner, D.S. (1997). Plants combat infection by gene silencing. Nature *385*, 781–782.

Cuperus, J.T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R.T., Takeda, A., Sullivan, C.M., Gilbert, S.D., Montgomery, T.A., and Carrington, J.C. (2010). Unique functionality of 22-nt

miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. Nat. Struct. Mol. Biol. *17*, 997–1003.

Gent, J.I., Lamm, A.T., Pavelec, D.M., Maniar, J.M., Parameswaran, P., Tao, L., Kennedy, S., and Fire, A.Z. (2010). Distinct phases of siRNA synthesis in an endogenous RNAi pathway in *C. elegans* soma. Mol. Cell *37*, 679–689.

Gillis, A.J., Schuller, A.P., and Skordalakes, E. (2008). Structure of the *Tribolium castaneum* telomerase catalytic subunit TERT. Nature *455*, 633–637.

Kapranov, P., Ozsolak, F., Kim, S.W., Foissac, S., Lipson, D., Hart, C., Roels, S., Borel, C., Antonarakis, S.E., Monaghan, A.P., et al. (2010). New class of gene-termini-associated human RNAs suggests a novel RNA copying mechanism. Nature *466*, 642–646.

Lee, H.C., Chang, S.S., Choudhary, S., Aalto, A.P., Maiti, M., Bamford, D.H., and Liu, Y. (2009). qiRNA is a new type of small interfering RNA induced by DNA damage. Nature *459*, 274–277.

Lehmann, E., Brueckner, F., and Cramer, P. (2007). Molecular basis of RNA-dependent RNA polymerase II activity. Nature *450*, 445–449.

Lipardi, C. and Paterson, B.M. (2009). Identification of an RNA-dependent RNA polymerase in *Drosophila* involved in RNAi and transposon suppression. Proc. Natl. Acad. Sci. USA *106*, 15645–15650.

Luo, Z. and Chen, Z. (2007). Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. Plant Cell *19*, 943–958.

Maida, Y., Yasukawa, M., Furuuchi, M., Lassmann, T., Possemato, R., Okamoto, N., Kasim, V., Hayashizaki, Y., Hahn, W.C., and Masutomi, K. (2009). An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. Nature *461*, 230–235.

Makeyev, E.V. and Bamford, D.H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Mol. Cell *10*, 1417–1427.

Nakamura, T.M., Morin, G.B., Chapman, K.B., Weinrich, S.L., Andrews, W.H., Lingner, J., Harley, C.B., and Cech, T.R. (1997). Telomerase catalytic subunit homologs from fission yeast and human. Science *277*, 955–959.

Pak, J. and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. Science *315*, 241–244.

Ridanpaa, M., van Eenennaam, H., Pelin, K., Chadwick, R., Johnson, C., Yuan, B., vanVenrooij, W., Pruijn, G., Salmela, R., Rockas, S., et al. (2001). Mutations in the RNA component of RNase MRP cause a pleiotropic human disease, cartilage-hair hypoplasia. Cell *104*, 195–203.

Salgado, P.S., Koivunen, M.R., Makeyev, E.V., Bamford, D.H., Stuart, D.I., and Grimes, J.M. (2006). The structure of an RNAi polymerase links RNA silencing and transcription. PLoS Biol. *4*, e434.

Sijen, T., Steiner, F.A., Thijssen, K.L., and Plasterk, R.H. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science *315*, 244–247.

Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N., and Maine, E.M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Curr. Biol. *10*, 169–178.

Sousa, R. (1996). Structural and mechanistic relationships between nucleic acid polymerases. Trends Biochem. Sci. *21*, 186–190.

Sugiyama, T., Cam, H., Verdel, A., Moazed, D., and Grewal, S.I. (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. Proc. Natl. Acad. Sci. USA *102*, 152–157.

van Dijk, A.A., Makeyev, E.V., and Bamford, D.H. (2004). Initiation of viral RNA-dependent RNA polymerization. J. Gen. Virol. *85*, 1077–1093.

Wang, X.B., Wu, Q., Ito, T., Cillo, F., Li, W.X., Chen, X., Yu, J.L., and Ding, S.W. (2010). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *107*, 484–489.

Wassenegger, M. and Krczal, G. (2006). Nomenclature and functions of RNA-directed RNA polymerases. Trends Plant Sci. *11*, 142–151.

Wingard, S.A. (1928). Hosts and symptoms of ring spot, a virus disease of plants. J. Agric. Res. *37*, 127–153.

Zong, J., Yao, X., Yin, J., Zhang, D., and Ma, H. (2009). Evolution of the RNA-dependent RNA polymerase (RdRP) genes: duplications and possible losses before and after the divergence of major eukaryotic groups. Gene *447*, 29–39.

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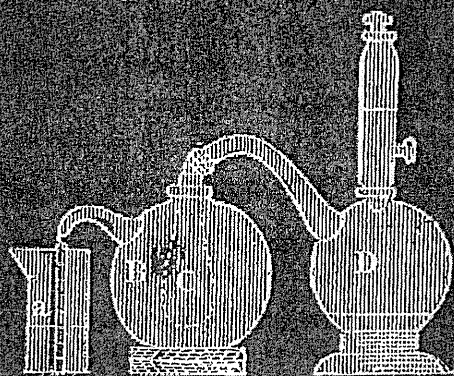

テロメレース：テロメア伸長反応と新たな機能

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正常細胞では細胞分裂のたびに染色体末端のテロメアは不完全な複製のために短縮し，このテロメア末端複製問題が細胞の老化と深く関与していることが知られている．一方で，テロメア長の維持を担う酵素であるテロメレースは90%以上のがん細胞で高発現し，末端複製問題を回避することでがん細胞の不死化に関与していると考えられている．ヒトテロメレースは，触媒サブユニットであるhTERT (Human Telomerase Reverse Transcriptase) と，鋳型RNAであるhTERC (Human Telomerase RNA component) を必要最小ユニットとし，hTERTのRNA依存性DNAポリメラーゼ（RNA-dependent DNA polymerase；逆転写酵素）活性によりテロメア末端にテロメア配列を付加することが知られている（図1）．このようにテロメア伸長をつかさどる逆転写酵素であるテロメレースの研究の歴史を振り返ると，RNA研究と切り離すことができない．RNAを内在する複合体であるテロメレースの逆転写酵素としての特徴はRNAにより規定，制御されていることがわかる．今回はRNA結合複合体としてのテロメレースがどのようにして同定されたか，また近年のテロメレース研究の新たな流れについて紹介する．

Telomere terminal transferase から reverse transcriptase へ

テロメレースは1985年GreiderとBlackburnにより，原生動物において初めて発見された酵素であり，当

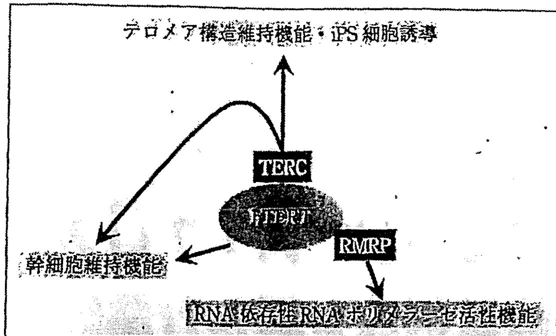

図1■hTERTの機能とhTERT結合RNA
テロメレース機能をもつhTERTはTERCと結合することによりテロメア構造維持に寄与する．近年，hTERTがテロメア構造維持作用を介してiPS細胞の誘導に関与することや，成体の組織幹細胞の機能維持，さらにRMRPと結合することにより従来の機能とは異なるRNA依存性RNAポリメラーゼ活性を示すことが明らかになっている．

初は telomere terminal transferase と記載され，染色体末端のテロメアに「terminal transferase」活性を利用してDNAを付加していく酵素と考えられていた[1]．1989年にMorinによりHeLa細胞内にヒトで初めてテロメレース複合体が発見された際にも，依然として「the human telomere terminal transferase enzyme」として報告されている[2]．すなわち，テロメレースは template independent terminal transferase 活性により染色体末端にテロメアDNAを付加すると考えられていた．しかし，この活性がRNase処理により消失するという実験結果から，テロメレース本体はRNAを内在するribonucleoproteinではないかとの仮説のもと，テロメレースを構成するRNAのクローニング競争が繰り広げられた．1989年に同じくGreiderとBlakburnにより原生動物のテロメレース複合体に内在するRNAコンポーネントが同定され[3]，この発見により，テロメレースはRNAを内在するribonucleoprotein複合体であり，テロメアの伸長は内在するRNAを鋳型として染色体末端に相補的DNAを付加するRNA依存性DNAポリメラーゼ活性によるものであるという考えの基盤が形成された．その後，原生動物から哺乳動物まで，種を超えて保存されているテロメレース複合体に内在するRNAコンポーネントが次々と同定され，TERCと命名された．これにより，テロメアを維持するテロメレースは，内在するRNAを鋳型としてDNAを付加するテロメレース逆転写酵素として種を超えて保存されているという仮説が立てられた．

Reverse transcriptase触媒サブユニットの同定

続いて，クローニング競争の標的となったのは，テロメレース逆転写酵素の触媒サブユニットである．テロメレース逆転写酵素の触媒サブユニットとして，酵母ではEst2が知られていたこと，またテロメレースは種を超えて保存されているとの仮説から，Est2のホモログとして，データベースを駆使したコンピュータ解析によりその遺伝子探索が行なわれた．その結果，1997年Cech[4]とWeinberg[5]のグループが，それぞれ独立に，ヒトテロメレース逆転写酵素の触媒サブユニットのクローニングに成功した．当時，それぞれのグループが報告した触媒サブユニット（hTRT：Cechのグループ，hEst2：Weinbergのグループ）は，予想通り逆転写酵素としてのモチーフを有するレトロウイルス逆転写酵素などと近縁の酵素であることが明らかとなった（現在，これらはhuman telomerase reverse transcriptase (hTERT) として名称が統一されている）．このようにして，テロメレースは内在するRNAを鋳型としてDNAを付加するRNA dependent DNA polymerase（逆転写酵素）であることが明らかとなった（図1）．

テロメレースとiPS細胞の誘導，幹細胞の維持

このような変遷でテロメア伸長酵素としてのヒトテロメレース研究が進められてきたが，近年，テロメレース研究の新たな流れが報告されるようになってきた．これまでにもテロメレースにテロメア構造維持機構以外の機能が存在する可能性を示唆する報告はあったが[6]，中でも，前述したテロメレース複合体の触媒サブユニットであるTERTがiPS細胞（induced pluripotent stem cell；人工多能性幹細胞）の誘導や幹細胞の機能維持に関わるのではないかという報告が相次いだ[7~9]（図1）．

2009年Marionらは，マウスの体細胞から作製したiPS細胞において，TERTの誘導とともにテロメア伸長が起こり，それが多能性の獲得と相関することを見いだした[7]．マウス胎仔繊芽細胞（MEF）にOct-3/4，Sox2，Klf4，c-Mycの4因子を導入することにより誘導したiPS細胞，またはc-Mycを除いた3因子を導入することにより誘導したiPS細胞ではともにテロメレースの再活性化が見られ，iPS作製の由来となったMEFよりもテロメア長が伸長していることが確認された．しかし，このiPS細胞のテロメア長は，由来となったMEFのテロメア長よりは長いものの，遺伝的に同じ性質を有すると考えられている胚性幹(ES)細胞と比べると短いこと

が確認された．ところが，非常に興味深いことに，このiPS細胞を培養し続けるとES細胞とほぼ同等のテロメア長をもつようになることが確認されている．このことは，テロメア伸長反応の大部分はリプログラミング後に起こるということを強く示唆するものであり，iPS細胞の未分化状態維持にテロメレースが関与していることが示唆される．

また彼らは，iPS細胞の誘導にテロメレース活性が必須であることを，テロメレース活性に不可欠なRNAコンポーネントであるTERCをノックアウトしたマウスを用いて解析した．まず，テロメア長が十分である第1世代の $Terc^{-/-}$ マウス由来のMEFからiPS細胞を作製したところ，野生型MEFを用いた場合と同等の効率でiPS細胞が誘導できることを確認した．しかし，この第1世代 $Terc^{-/-}$ マウス由来のiPS細胞ではテロメア伸長が見られなかったことから，iPS細胞誘導後のテロメア伸長反応はテロメレースによるものだと推測することができる．さらに，前述のように第1世代の $Terc^{-/-}$ マウス由来のMEFからiPS細胞を誘導することができた一方で，第2・3世代の $Terc^{-/-}$ マウス由来のMEFからiPS細胞誘導を試みたが，その誘導効率は劇的に減少することが確認された．これらの結果は，テロメレース活性のないMEFにおけるテロメア短小化がiPS細胞の誘導を抑制する原因であることを示唆している．しかし，iPS細胞誘導効率が低い第2世代の $Terc^{-/-}$ マウスと $Terc^{-/+}$ マウスを掛け合わせた第3世代 $Terc^{-/+}$ マウス由来のMEFではiPS細胞誘導効率は回復することが確認され，iPS細胞の誘導効率はテロメレース活性に依存することが強く示唆されている．

また，2005年Sarinらは，マウステロメレースの触媒サブユニットであるmTERTを毛嚢のバルジ領域幹細胞に特異的に発現させることで，著しい体毛の成長を示すことを見いだした[9]．毛嚢は成長期(anagen)，退行期(catagen)，休止期(telogen)を経て，バルジ領域幹細胞へのシグナル刺激によって休止期から成長期へと移行し体毛が合成される．TERTはこの休止期のバルジ領域幹細胞で機能し，毛嚢を速やかに成長期へと移行させ，体毛の顕著な成長をひき起こす．この現象にはテロメレース活性に不可欠なTERCの存在が必要でないことから，TERTのテロメレース活性以外の新規機能，とりわけ幹細胞機能維持におけるTERTの重要性が示された．さらに同じグループは，この体毛の顕著な成長が，TERTとクロマチンリモデリング複合体の構成因子であるBRG1によるWnt/β-catenin/Mycシグナル伝達経路の活性化によるものであることを明らかにした[10,11]．これら一連の報告は，TERTがテロメア構造維持機能とは別に幹細胞機能制御に直接的に関わっていることを示した重要な報告といえる．同様の発見は同時期に他のグループからも報告されているが，一方はこの機能にテロメレースのRNAコンポーネントであるTERCが必要であると主張し[8]，別のグループは幹細胞機能維持にはTERCは必要なく，従来から知られるテロメレース複合体の機能とは独立した機能であると主張している[9]．この点は非常に重要な問題であるが，現時点では明確な答えは出ていない（図1）．また，mTERTノックアウトマウスとmTERCノックアウトマウスで同じ表現型が認められることから[12]，はたしてTERTがTERCと独立した機能を本当に有するかどうか今なお多くの議論があり，今後も引き続き解析が必要である．

このような背景のもと，近年TERTの新規機能を制御する新たなパートナーの存在が示唆されている[13]．最近では，FuらがTERT複合体の精製を行ない，従来同定されていなかったタンパク質分子群を複合体内に同定し注目されている[14]．これらのタンパク質群の中には，幹細胞機能維持に関わることが示唆される分子と近縁のタンパク質なども存在し，これらの生物学的な意義の解析は非常に興味深い．さらにTERCにかわりTERTと結合し得るRNAの同定の試みは，RNAを制御するTERTの新たな機能の発見につながった[15]．

テロメレースであるTERTの新機能の発見：RNA依存性RNAポリメラーゼとして機能するhTERT

近年，RNAは遺伝子発現を多様なメカニズムで制御することが明らかとなってきた．その中核となる分子が二本鎖RNAである．RNAサイレンシングは20〜30塩基長の小さなRNAによる遺伝子発現抑制機構であるが，RNAサイレンシングを起こす代表的な分子にsiRNA (small interfering RNA)があり，内因性のsiRNAは長鎖二本鎖RNAの切断によって合成される[16,17]．一本鎖RNAを鋳型に二本鎖RNAを合成するRNA依存性RNAポリメラーゼ(RNA-dependent RNA polymerase：RdRP)は内因性siRNAの合成に関わる重要な分子として植物や線虫，分裂酵母などのモデル生物での発現が知られていたが，哺乳類ではRdRPの存在は立証されていなかった．TERTの新規機能について検討を行なっていた筆者らは，TERTが系統遺伝学的[4]および構造学的[18]にRNAウイルスのRdRPと近縁にあることに注目し，hTERTがRdRPとして機能するのではないかと推測して解析を行なった．

まず，hTERCに代わる新規hTERT結合RNAを検

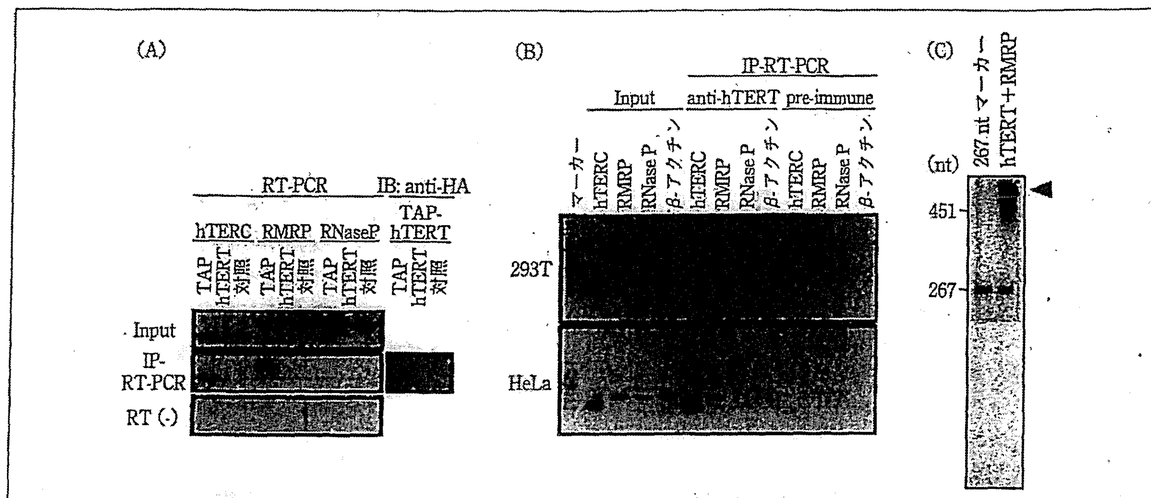

図2■hTERT-RMRP複合体はRdRP活性を有する（文献15より転載・改変）
過剰発現させたhTERT (A) および内在性hTERT (B) の免疫沈降によりhTERTとRMRPとの結合が確認された．(C) hTERTを用いた*in vitro* RdRP assayによって267塩基のRMRP（センス鎖）から合成された長鎖ヘアピン型RNA（矢印）．合成ヘアピン型RNAはRMRPのセンス配列とアンチセンス配列をともに含み，RMRPの約2倍の長さを有する．

索し，RMRP (RNA component of mitochondrial RNA processing endoribonuclease) を同定した (図2-A, B)．RMRPは267塩基のsnoRNA (small nucleolar RNA) に分類されるnon-coding RNAであり，低身長を主症候とする遺伝子性疾患である軟骨毛髪低形成 (Cartilage and Hair Hypoplasia) の原因遺伝子であることが報告されている[19]．hTERTとRMRPとの複合体のRNA合成能について*in vitro*での解析を行なったところ，この複合体は長鎖RNAの合成が可能であることがわかった（図2-C)．そこでhTERTとRMRPにより合成される長鎖RNAについて詳細に検討した結果，hTERTはRMRP（センス鎖）を鋳型としてRMRPの相補鎖（アンチセンス鎖）を合成し，合成されたRNAは長いヘアピン型の二本鎖RNAであることがわかった．つまり，hTERTは一本鎖RNAを鋳型に二本鎖RNAを合成するRdRP活性を有することが証明されたのである．

次に，細胞内におけるhTERTのRdRPとして機能について検討を行なった．hTERTとRMRPをともに発現している細胞から抽出したRNAを用いて行なったノーザンブロッティングにより，細胞内にもRMRPのセンス鎖＋アンチセンス鎖からなる長鎖ヘアピン型RNAの存在が確認された (図3-A)．さらに，RMRPのアンチセンス鎖合成は，hTERTの発現のある細胞でのみ認められることがわかった．これらより，hTERTは細胞内でもRdRP活性によりRMRPのアンチセンス鎖を合成していることが示唆された．

さらに，hTERTがもつRdRP活性の生物学的意義を考える上で，興味深い現象が見つかった．ウイルスベクターを用いてRMRPの過剰発現を試みたところ，hTERTの発現のある細胞でのみRMRPの細胞内総発現量が減少したのである（図3-B)．さらに，hTERTの発現のない細胞にhTERTを導入するとRMRPの発現は減り，逆にhTERTを発現している細胞でその発現を抑制するとRMRP発現量が増加した．これらの結果は，hTERTがRMRPに対し転写後抑制をかけている可能性を示唆していた．前述のように，RNAサイレンシングの主体は長い二本鎖RNAから切り出された20〜30塩基長の短い二本鎖RNAである．そこで，hTERTのRdRP活性によって合成されたRMRP由来の長鎖ヘアピン型RNAも機能性の短い二本鎖RNAへと切断されているのではないかと考え検討したところ，細胞内にRMRPに由来する22塩基の短い二本鎖RNAが存在することを突き止めた（図3-C)．この短い二本鎖RNAは長鎖二本鎖RNAを切断するDicerに依存して発現しており，RNAサイレンシングを起こす際に結合するAGO2タンパク質とともに存在していることが確かめられた．以上の結果，テロメレース逆転写酵素として知られたhTERTはテロメレースとは異なるRdRP活性を有し，長鎖ヘアピン型RNAの合成とそれに引き続く内因性siRNAの合成を介してRNAサイレンシングに関与することが立証されたのである（図3-D)．

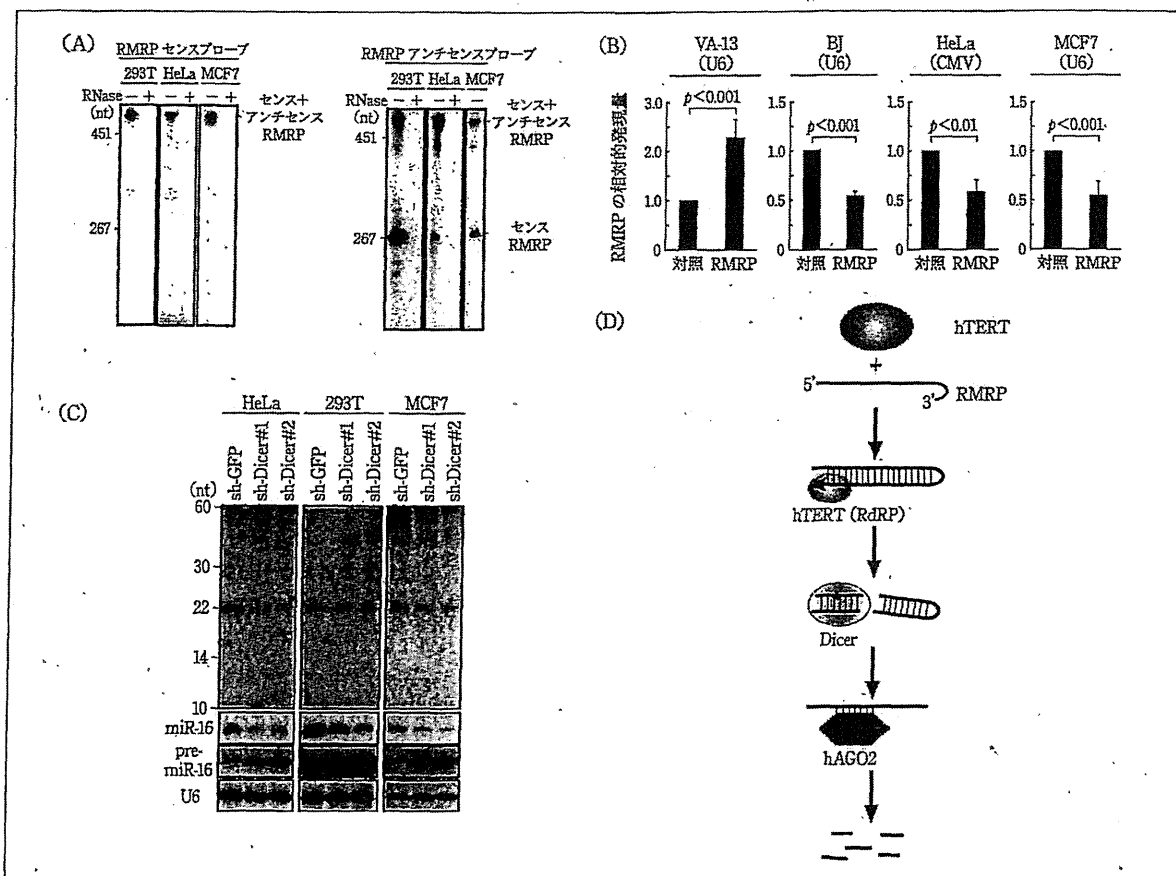

図3■hTERTのRdRP活性とRNAサイレンシング（文献15より転載・改変）
(A) hTERT発現細胞におけるセンス＋アンチセンス型RMRPの発現. (B) hTERT発現細胞ではRMRP発現ベクターの導入によりRMRPの総発現量が減少する. (C) Dicer依存性に発現するRMRP特異的な内在性 siRNA. (D) hTERTが仲介するRNAサイレンシングのモデル. hTERTはRdRP活性によりRMRPの相補鎖を合成. 合成されたヘアピン型二本鎖RNAをDicerが切断、切り出された内在性siRNAはhAGO2に取り込まれ, 相補的な配列をもつRNAの発現を抑制

おわりに

テロメレースとしてのhTERTはこれまでにも分子標的医療や再生医療分野などで臨床応用に向けた様々な研究がなされてきたが, テロメレースが新たな機能を有するとすれば, 多くの新知見が得られるはずである. 今回, hTERTの新規機能としてRdRP活性を見いだしたことにより, 癌細胞内での選択的なsiRNA合成技術やRNAの増幅技術など新たなアイデアが創出される可能性がある.

（おわり）

文献

1) C. W. Greider & E. H. Blackburn : *Cell*, **43**, 405 (1985).
2) G. B. Morin : *Cell*, **59**, 521 (1989).
3) C. W. Greider & E. H. Blackburn : *Nature*, **337**, 331 (1989).
4) T. M. Nakamura *et al.* : *Science*, **277**, 955 (1997).
5) M. Meyerson *et al.* : *Cell*, **90**, 785 (1997).
6) S. A. Stewart *et al.* : *Proc. Natl. Acad. Sci. USA*, **99**, 12606 (2002).
7) R. M. Marion *et al.* : *Cell Stem Cell*, **4**, 141 (2009).
8) I. Flores *et al.* : *Science*, **309**, 1253 (2005).
9) K. Y. Sarin *et al.* : *Nature*, **436**, 1048 (2005).
10) J. Choi *et al.* : *PLoS Genet*, **4**, e10 (2008).
11) J. I. Park *et al.* : *Nature*, **460**, 66 (2009).
12) X. Yuan *et al.* : *Genes Cells*, **4**, 563 (1999).
13) E. H. Blackburn : *Nature*, **436**, 922 (2005).
14) D. Fu & K. Collins : *Mol. Cell*, **28**, 773 (2007).
15) Y. Maida *et al.* : *Nature*, **461**, 230 (2009).
16) K. Okamura *et al.* : *Nature Rev. Mol. Cell. Biol.*, **9**, 673 (2008).
17) H. Siomi *et al.* : *Nature*, **457**, 396 (2009).
18) A. Gillis *et al.* : *Nature*, **455**, 633 (2008).
19) M. Ridanpaa *et al.* : *Cell*, **104**, 195 (2001).

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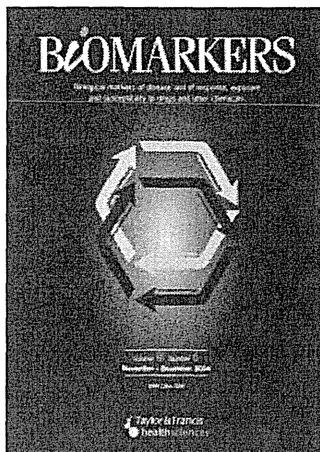

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MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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ORIGINAL ARTICLE

MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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Abstract

We identified that microRNA expression changed dynamically during liver development and found that miR-500 is an oncofetal miRNA in liver cancer. miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissue. Most importantly, an increased amount of miR-500 was found in the sera of the HCC patients. In fact, miR-500 levels in sera of the HCC patients returned to normal after the surgical treatment in three HCC patients. Our findings reveal that diverse changes of miRNAs occur during liver development and, one of these, miR-500 is an oncofetal miRNA relevant to the diagnosis of human HCC.

Keywords: *miRNA; miR-500; hepatocellular carcinoma, liver development, diagnosis*

Introduction

MicroRNAs (miRNAs) are small RNA molecules of 21–25nt that have the potential to a play central role in physiological and pathological processes, including cell differentiation, apoptosis and oncogenesis (Ambros 2004, Esquela-Kerscher et al. 2006). The biogenesis of miRNAs involves nucleolytic processing of precursor transcripts, which are transcribed from different genomic locations as long primary transcripts (pri-miRNA) by RNA polymerase II in the nucleus (Lee et al. 2004). Pri-miRNAs are processed by the RNase-III family of an enzyme, Drosha, to a ~70nt precursor called the pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 and then cleaved in the cytoplasm by Dicer to ~22nt double-strand mature miRNA (Han et al. 2006, Lund et al. 2004, Ketting et al. 2001). A single strand of the mature miRNA is assembled into effecter complexes called miRNPs (miRNA-containing ribonucleoprotein particles), which share a considerable amount of similarity with an RNA-induced silencing complex (RISC) (Nelson et al. 2004). They induce gene suppression post-transcriptionally by inducing mRNA degradation or by regulating the translational efficiency of mRNA (Bartel 2004).

Several reports have shown the importance of miRNA functions in tissue development. More recent reports, in particular those regarding comprehensive microRNA profiling analysis, have shown that miRNAs are expressed in a tissue-specific manner and their expression altered

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in the process of development, such as cardiogenesis and haematopoiesis (Chen et al. 2006, 2004). For example, miR-1, which is expressed specifically in cardiac and skeletal muscle, is essential for cardiac morphogenesis and conduction (Zhao et al. 2007). Another study showed that miR-181a regulates intrinsic antigen sensitivity during T-cell development (Li et al. 2007). Another important aspect of miRNA study is the association of its gene targets and disease, which have been investigated by many researchers. Mir-17-92 polycistron has been designated as oncomiR-1 (He et al. 2005), and let-7 family miRNAs and miR-34 function as tumour suppressors (Johnson et al. 2005, Yu F et al. 2007, He et al. 2007); moreover, a number of studies have given evidence that several miRNAs are associated with carcinogenesis and regulate the expression of cancer-related genes.

Although emerging evidence suggests that several miRNAs are involved in the process of liver development (Esau et al. 2006, Fu et al. 2005, Gramantieri et al. 2007), the roles of miRNAs in hepatogenesis and their possible relation to hepatocarcinogenesis have not been thoroughly examined. In this study, to investigate liver development from the biological aspects of microRNA, we performed a mouse miRNA microarray carrying 340 miRNA probes. We report that some of these miRNAs are strongly expressed, and that dynamic changes in their expression profile are observed in the process of liver development. We also show that miR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in hepatocellular carcinoma (HCC) tissue. Thus, dynamic miRNA regulation is an important feature as an oncofetal non-coding small RNA relevant to the diagnosis of human liver cancer.

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Materials and methods

RNA extraction

C57BL/6J mice were used in this study. Total RNA from mouse liver tissues (embryo (E) 14, E16, E18, neonate and adult), *in vitro* fetal hepatocyte cultured samples (days 0, 1, 3, 5 and 7), and liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7, and Hep3B) were extracted using the mirVana™ miRNA Isolation Kit (Ambion, Tokyo, Japan). Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Locked nucleic acid (LNA)-based miRNA microarray

The miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0, was applied to detect the expression of mouse miRNA (Exiqon, Vedbaek, Denmark). Total RNA samples were collected from fetal (E14, 16 and 18), neonate and adult (8-week-old) mice ($n = 7–10$). Total RNA samples (2000 ng) from liver tissue and reference (Universal control, which is made from mouse tissue mixtures) were labelled with the Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit according to the procedure described by the manufacturer (Exiqon). Hybridisation and normalisation were performed according to the miRCURY™ LNA array manual, and image analysis of the miRCURY™ LNA array microarray slides was acquired using an Agilent Technologies Microarray Scanner and Agilent Feature Extraction 9.1 (Agilent Technologies, Tokyo, Japan). A hierarchical cluster was produced from microarray data using a Euclidean distance calculation based on Ward's methods by GenMaths software (Applied Maths). All the miRNA microarray data are shown in Supplementary Table 1 (see the online version of this article).

Cell culture

Liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7 and Hep3B) were cultured in liquid culture with Dulbecco's modified eagle medium (DMEM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and a 1% antibiotic antimycotic solution (Invitrogen, Tokyo, Japan). The cells were maintained *in vitro* at 37°C in a humidified atmosphere with 5% CO_2.

Patients and RNA specimens

Liver tissues were obtained surgically with informed consent from patients at the National Cancer Center Hospital (Tokyo, Japan). The study was approved by the Institutional Review Board of the National Cancer Center Research Institute. Liver tissue total RNAs were extracted from 40 HCC patients and their associated non-cancerous tissue. The clinical data and pathological diagnosis are summarized in Supplementary Table 2 (see the online version of this article).

Real-time polymerase chain reaction

Total RNAs of approximately 100 ng were reverse-transcribed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Real-time quantitative polymerase chain reaction (PCR) amplification of the cDNA template was done using Taqman Universal PCR Master Mix (Applied Biosystems) in

an ABI PRISM 7300 (Applied Biosystems). The PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Taqman probes for human and mouse miRNA were used to assess the expression levels of miRNA (mmu-miR-101b, ID 4373159; mmu-122a, ID 4373151; mmu-miR-142-5p, ID 4373135; mmu-miR-223, ID 4373075; mmu-miR-451, ID 4373360; has-miR-346, ID 4373038; has-miR-500, ID 4373225; Applied Biosystems). The expression levels were normalised against U6 (RNU6B, ID 4373381; Applied Biosystems) or total RNA volume.

RNA isolation from human serum samples

Whole blood samples were obtained from patients with HCC at the Kyoto University (Kyoto, Japan). All of the donors or their guardians provided written consent and ethics permission was obtained for the use of all samples. Blood samples were taken before and after completion of surgery. Serum samples were stored at -80°C until analysis. For serum RNA isolation, total RNA was isolated using Isogen (Nippon Gene, Japan), according to the manufacturer's instructions.

Measurement of serum miRNA levels by using TaqMan qRT-PCR assays

A fixed volume of 5 µl of RNA solution (14 ng) was used as input into the reverse transcription reaction. Input RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems) in a small-scale reverse transcription reaction (comprising 2 µl of H_2O, 1 µl of 10x reverse-transcription buffer, 0.2 µl of RNase inhibitor (20 units ml^{-1}), 0.1 µl of 100 mM dNTPs, 0.7 µl of Multiscribe reverse transcriptase and 5 µl of input RNA), using a Tetrad2 Peltier Thermal Cycler (BioRad, Tokyo, Japan) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Teverse transcription product (4.75 µl) was combined with 5.25 µl of PCR assay reagents (comprising 5 µl of TaqMan 2x Universal PCR Master Mix, No AmpErase UNG and 0.25 µl of TaqMan miRNA assay) to generate a PCR of 10.0 µl of total volume. Real-time PCR was performed as described above. Serum levels of miR-16 were measured as internal normalisation control as they were not significantly different between controls and patients in prostate cancer and colorectal cancer (Mitchell et al. 2008).

Statistical analysis

The results are given as mean ± SD. The Student's *t*-test was performed for statistical evaluation; $p<0.05$ or $p<0.001$ was considered significant.

Table 1. MicroRNAs (miRNA) abundantly expressed in liver development.

Liver stage	MiRNA name[a]
E14	miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-346, miR-374-5p, miR-451, miR-486, miR-500
E16	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-295, miR-346, miR-367, miR-374-5p, miR-451, miR-464, miR-471, miR-486, miR-500, miR-547
E18	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-324-3p, miR-374-5p, miR-451, miR-486
Neonate	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-463
Adult	miR-21, miR-22, miR-29a, miR-29b, miR-29c, miR-101a, miR-101b, miR-122a, miR-126-5p, miR-192, miR-374-5p

[a]miRNAs are listed in ascending order. E, embryo.

Results

Analysis of the global expression levels of miRNA in the process of liver development by LNA-based miRNA microarray

To examine how the expression profile of miRNA changed in the process of mouse liver development, we performed an LNA-based miRNA microarray at different developmental stages. Total RNAs from E14, 16, 18, neonate and adult liver were isolated and labelled with Hy3, and total RNAs of universal control consisted of several tissue mixtures labelled with Hy5 as a common reference. After normalisation of the miRNA expression, the number of high- and low-expressed miRNAs at different time stages was counted. High-expressed miRNA represents twofold or more upregulated miRNA, and low-expressed miRNA represents twofold or more downregulated miRNA, when compared with an average expression level of all miRNAs (see Supplementary Figure 1 in the online version of this article). Throughout all developmental stages of the liver, most of the miRNA expression levels were classified as low-expressed miRNA; in contrast, the number of high-expressed miRNAs was quite limited and are listed in Table 1. These data indicated that expression levels of the general miRNAs were very low and that a limited number of miRNAs were highly expressed in mouse liver development.

Differential expression patterns of miRNAs in the process of mouse liver development

To determine differentially expressed miRNA and to quantify the expression changes in the process of liver

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development, hierarchical unsupervised clustering analysis was performed using microarray data of E14, 16, 18, neonate and adult mouse liver. The case cluster analysis of the microarray data indicated a similarity of clusters from the viewpoint of the expression pattern between E14 and E16 fetal liver and between neonate and adult liver (Figure 1), indicating that the miRNA expressions changed depending on the developmental stage. These results indicated that expression of most of the miRNAs was regulated precisely in the process of liver development.

The expression pattern of miRNA selected from highly expressed miRNAs (Table 1) was verified by real-time PCR to show the accuracy of miRNA expression acquired from the microarray analysis. The left panels of Figure 2 present the results of microarray analysis for five miRNAs (miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451). Expressions of miR-101b and miR-122a were low at the early stage of liver development and were upregulated during maturation. In contrast, expressions of miR-142-5p, miR-223 and miR-451 were high at the early stage of liver development and were already known as miRNAs expressed in haematopoietic cells (Chen et al. 2004, Zhan et al 2007, Johnnidis et al 2008). The right panels of Figure 2 are the results of real-time PCR for the same set of miRNAs. In comparison to the microarray results and the real-time PCR results, these data obtained from two different methods showed approximately similar expression patterns of miRNAs, confirming the validity of our microarray analysis.

Interestingly, miRNAs (miR-142-5p, miR-451 and miR-223) expressed in haematopoietic cells were highly expressed at the early stages (E14 and E16) and then gradually downregulated in the process of liver development (Figure 2). Because whole fetal liver is a haematopoietic organ and a large number of haematopoietic cells are contained there, this also indicated the accuracy of expression profiling of miRNA in the process of liver development by LNA-based microarray.

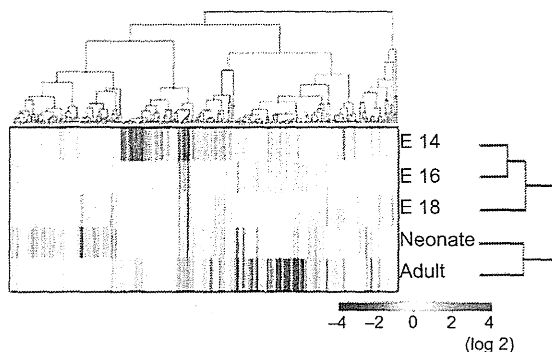

Figure 1. A global expression pattern of miRNA in the process of mouse liver development. The data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on Ward's methods. The liver samples are aligned vertically: embryo (E) 14, E16, E18, neonate and adult. Samples were linked by the dendrogram shown on the right to highlight the similarity in their miRNA expression patterns. The expression profile of each miRNA is depicted in the respective row. The expressions of miRNA are linked by the dendrogram shown on the top to highlight the similarity in their expression patterns.

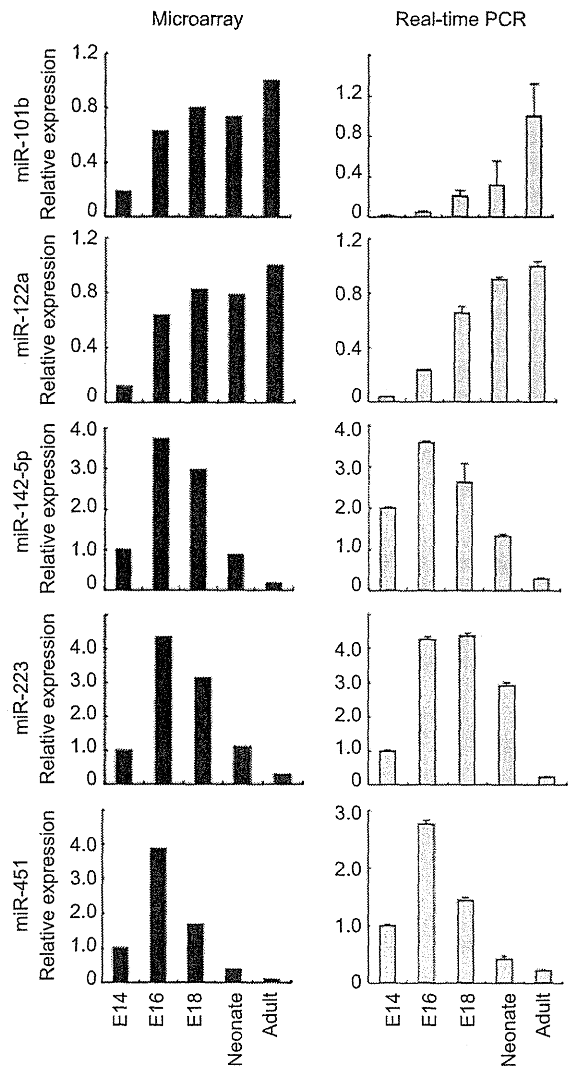

Figure 2. Differential expression of selected miRNA in mouse liver development by microarray and real-time polymerase chain reaction (PCR). miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were selected from highly expressed miRNAs to confirm the expression levels of microarray analysis by real-time PCR. The left panels represent the miRNA expression levels by microarray analysis. The right panels represent the miRNA expression levels by real-time PCR. The expression profile is compared for mouse fetal (embryo (E) 14, E16 and E18), neonate and adult liver. In the graphs of miR-142-5p, miR-223 and miR-451, the expression level of E14 fetal liver is set to 1.0. Real-time PCR analyses were performed in triplicate and expression values are normalized with total RNA volume. Data are shown as mean ± SD.

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Differential expression patterns of cancer-related miRNAs in the process of mouse liver development

Interestingly, when analysing the expression patterns of the hierarchical clustering data in detail, we found that the expression of several let-7 miRNA family known as 'tumour suppressor miRNA' was upregulated, and, in contrast, the expression of miRNAs known as 'potential oncogenes' which are involved in cell proliferation, was downregulated in the process of liver development. Therefore, to reveal the expression pattern of cancer-related miRNAs in the process of mouse liver development, the expression profile of 21 selected miRNAs (11 miRNAs as oncogenes and 10 miRNAs as tumour suppressors) is summarized in Figure 3. Many oncogenic miRNA expressions, such as those of miR-17-5p, miR-20, and miR-92, tended to decrease in the process of mouse liver development (Figure 3A). In contrast, except for let-7d* and let-7e, the expression pattern of the let-7 miRNA family was elevated in the process of liver development (Figure 3B). This study provides evidence that the expression of oncogenic miRNA is downregulated and that the expression of tumour suppressor miRNA is upregulated in the process of liver development.

Expression of miR-500 is high in human fetal liver

As reported above, the expression levels of oncogenic miRNAs were downregulated in liver development. We tried to identify new miRNA candidates that act as an oncogenic miRNA in the liver from the microarray data. As a first step toward the elucidation of the role of miRNAs in liver carcinogenesis, we focused on downregulated miRNAs during liver maturation, which are possibly related to cell proliferation; high expressions of miR-140, miR-346, miR-411, miR-470 and miR-500 were detected at an early stage (E14) of liver development and downregulated at the late developmental stages (E16 and E18) (Figure 4A). Among these, miR-500 and miR-346 expressions were remarkably downregulated during development; thus, we concentrated on miR-500 and miR-346, which could be expected to be a potential target relevant to fetal liver development to control the time and spatial expression of sets of mRNA.

In the next step, the occurrence of miR-500 and miR-346 was assessed in human fetal and adult liver. Real-time PCR analysis revealed that the expression of miR-500 in human fetal liver, but not that of miR-346, was significantly higher than that in normal adult liver (Figure 4B and Supplementary Figure 2A (see online version of this article)). Taken together, as miR-500 expression was downregulated in human adult liver, our data suggest that miR-500 is developmentally associated with human fetal hepatocyte specification and functions. The

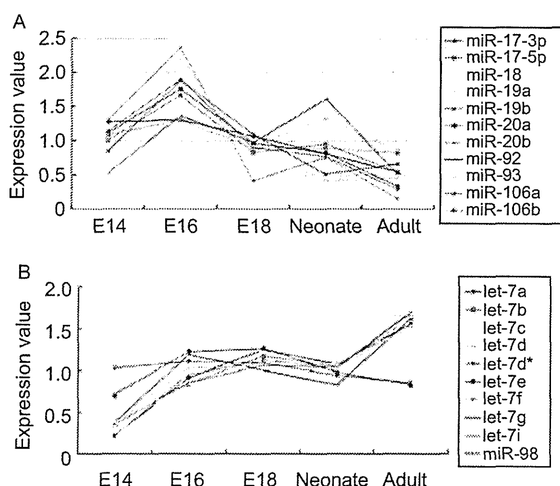

Figure 3. Expression patterns of cancer-related miRNAs in the process of mouse liver development. (A) Expression pattern by a microarray analysis (each sample: n=7-10) of miRNA that may act as an oncogene. (B) The expression pattern by the microarray analysis (each sample: n=7-10) of the let-7 family miRNAs functioned as a tumour suppressor. Expression levels are normalised by average expression value of each miRNA and shown in the graph.

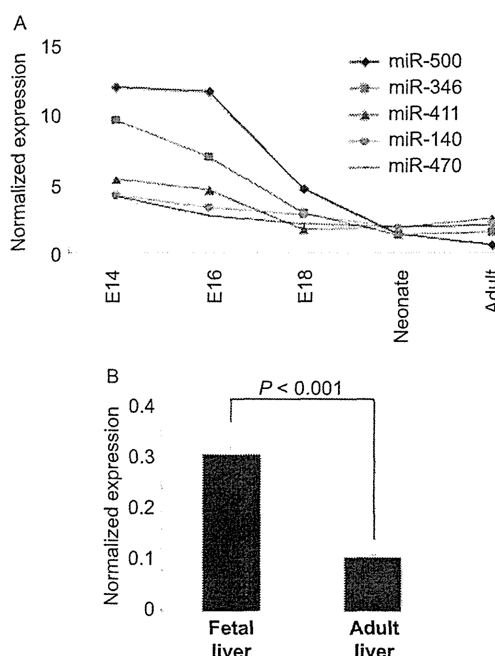

Figure 4. The expression of miR-500 is higher in the fetal stage than in the adult stage (A). The expression profile of miRNA decreased in the process of mouse liver development. Expression values are based on microarray data. (B) Expression of miR-500 in human fetal and adult liver. Real-time polymerase chain reaction analyses were performed in triplicate. Expression values are normalised with U6 snRNA value. The data represent the mean ± SD, $p < 0.001$.

results of our ongoing knock-down analysis of miRNA in liver cancer cells will be presented in a future work.

Expression of miR-500 is high in human liver cancer

We next examined the expression level of miR-500 in six human liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander) to assess whether miR-500 acts as an oncofetal miRNA and found that it increases 2.4- to 47.6-fold more in Alexander, JHH-7, HepG2, Huh-7 and Hep3B than in normal liver (Figure 5A); in contrast, no detectable amount of miR-500 was found in Li-7. On the other hand, the expression levels of miR-346 in the six liver cancer cell lines were not high (see Supplementary Figure 2B in the online version of this article). To evaluate the potential of miR-500 as an oncofetal miRNA, the expression levels of human miR-500 were analysed by real-time PCR in 40 pairs of malignant neoplasias of hepatocyte lineage (T) and adjacent non-tumorous tissue (NT). Differences in the miR-500 expression level were statistically significant ($p < 0.001$) between T and NT (Figure 5B), but miR-346 expression was not significantly changed (see Supplementary Figure 2C in the online version of this article). Some of the samples exhibited remarkably high expression levels of miR-500, and 45% (18/40 patients) of the samples showed 1.2- to 8.6-fold higher upregulation in the cancerous samples than in each non-tumorous sample and

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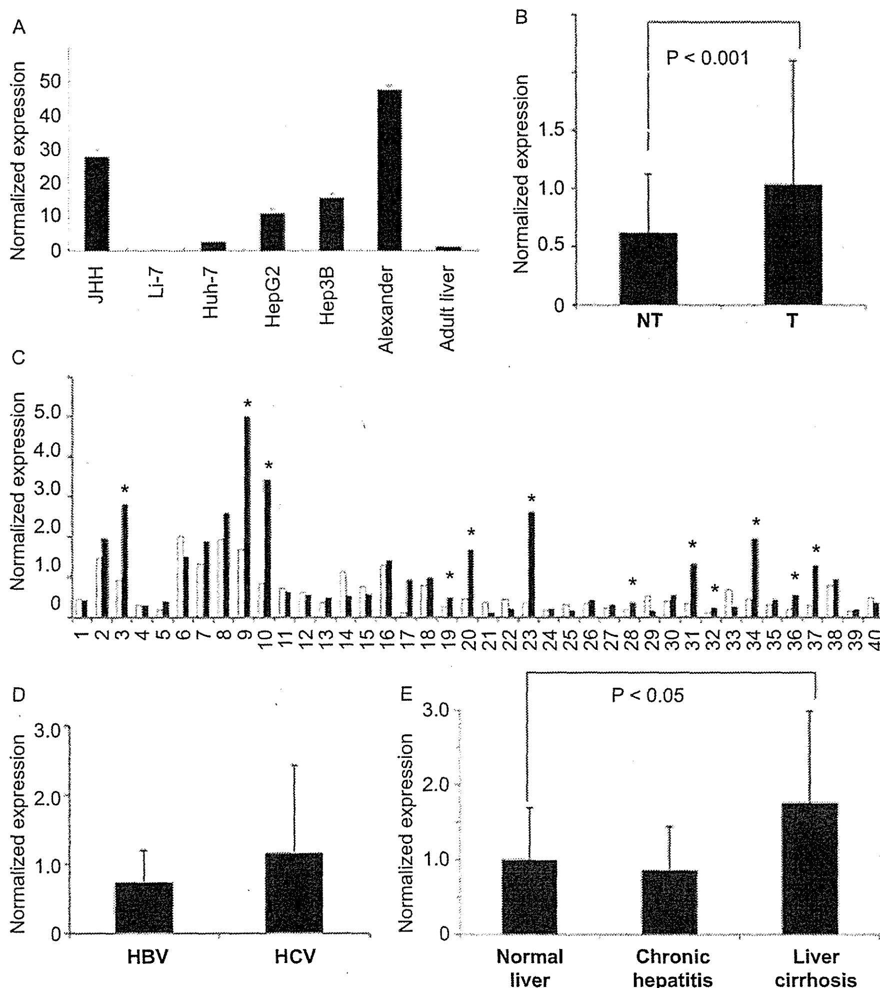

Figure 5. The expression of miR-500 is clearly upregulated in human liver cancer (A) miR-500 expression abundantly detected in liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander). The expression level of normal liver is set to 1.0. The data represent the mean ± SD. (B) Forty pairs of hepatocellular carcinoma (HCC) patients (tumour (T) and non-tumour (NT)) were analysed by real-time polymerase chain reaction of human miR-500. The data represent the mean ± SD, $p < 0.001$. (C) Expression levels of miR-500 in each patient (T and NT). Samples of 12 patients (*) showed twofold or more upregulation in HCC. (D) Expression levels of miR-500 in hepatitis B virus (HBV, $n = 10$) and hepatitis C virus (HCV, $n = 26$). (E) miR-500 expression was upregulated in liver cirrhosis ($n = 17$) more than normal liver ($n = 11$) and chronic hepatitis samples ($n = 19$). The data represent the mean ± SD, $p < 0.05$. Expression values are normalised with U6 snRNA value.

12 patients showed more than 2.0-fold higher expression (30%) (Figure 5C). Based on the clinical data and pathological diagnosis (see Supplementary Table 2 in the online version of this article), there is no significant difference in miR-500 expression between hepatitis virus B and C infection (Figure 5D). Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, but not chronic hepatitis (Figure 5E), suggesting that miR-500 expression was upregulated during cirrhosis development. Thus, although only limited samples expressed miR-500 higher, miR-500 might be useful as a biomarker in the early stage of liver cancer.

Expression profiling of miR-500 in HCC patient serum

Recently, it has been reported that miRNAs are circulating in serum (Chim et al. 2008, Gilad et al. 2008) and tumour-derived miRNAs such as miR-155, miR-21, miR-15b, miR-16 and miR-24 are detected in the plasma and serum of tumour patients (Mitchell et al. 2008, Lawrie et al. 2008). In fact, an increased amount of miR-500 was found in the sera of three out of ten HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. To determine whether or not serum levels of miR-500 truly reflect the presence of cancer in the HCC patients, the presence of miR-500 in the sera of three human HCC patients, post- and presurgical treatment, was also assessed. As can be seen in Figure 6, elevated serum levels of miR-500 in the three HCC patients were significantly reduced after surgery and returned to normal levels. These results expect that the miR-500s abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions.

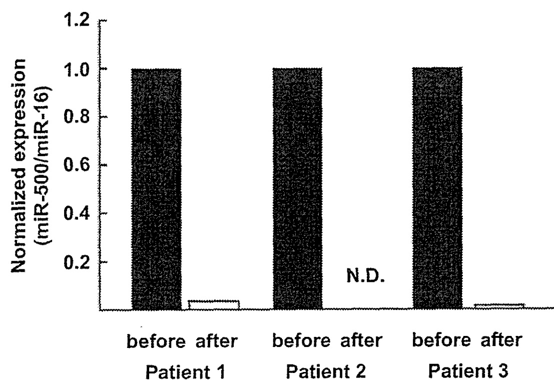

Figure 6. Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients (n=3) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not detected.

Discussion

Using a global miRNA expression profile in mouse liver development analysed by an LNA-based miRNA microarray, our data indicate that dynamic changes in miRNA expression occur in mouse liver development. However, the number of high-expressed miRNAs was quite limited at all developmental stages of the liver. This finding is also consistent with several reports that dominant miRNA expression is rigidly controlled in a developmental stage-specific and tissue- or cell-type-specific manner (Chen et al 2006, Shan et al. 2007). For example, it has been reported that the expressions of miR-1 and miR-133 are high and specific in adult cardiac and skeletal muscles and modulate skeletal muscle proliferation and differentiation by negatively regulating the histone deacetylase-4 or serum response factor (Chen et al. 2006). On the other hand, expression levels of the general miRNA are low at all stages of liver development. However, our data indicate that the expression pattern of some of the low-expressed miRNAs, including let-7 family, also dramatically change in the process of mouse liver development (Figure 4B). Using this platform, the overall regulation of individual miRNAs of sequential stages of liver development was determined, providing us with a useful baseline for understanding the developmental dynamics of liver miRNA expression.

In this study, we identified a novel cancer biomarker candidate, miR-500, which was designated as an oncofetal miRNA in the early stage of liver cancer, because miR-500 expression is highly expressed in a fetal liver and downregulated in the developmental process and then upregulated in the process of liver cirrhosis. When the expression profile of miR-500 in human tissues was examined, its expression was not specific in the liver and was broadly detected in all tissues (see Supplementary Figure 3 in the online version of this article). However, the expression level of miR-500 is high at the early stages of liver development in mice and humans. Furthermore, miR-500 was abundantly expressed in human liver cancer cell lines (JHH-7, Huh-7, HepG2, Hep3B and Alexander) and liver cancer tissues. Interestingly, six miRNAs (mir-532, 188, 362, 501, 660, 502) in addition to miR-500 make a cluster within a 10-kb distance from miR-500, and their expressions could be modulated by the same transcriptional regulatory unit. However, the levels were not remarkably changed during mouse liver development. Therefore, by analysing these miRNAs together, miR-500 might be a better biomarker in HCC.

We tried to test the effect of miR-500 using liver cancer cell lines. In a knock-down analysis of miR-500 with miR-500 LNA, significant changes in cell proliferation and colony formation were not observed in both Alexander and JHH-7 cells (see Supplementary Figure 5A and B in the online version of this article). Likewise, mature

miR-500 were transfected into Li-7 cells, which did not express miR-500 and we found there are no significant differences in cell proliferation (see Supplementary Figure 5C in the online version of this article). Although our data indicated that miR-500 did not affect cell proliferation in liver cancer cell lines, there might be a close association between tissue development and carcinogenesis in the fields of miRNA. For detailed analysis of function of miR-500, we await for generation of miR-500 knockout mice.

As several groups have reported that levels of certain circulating miRNA are associated with clinical characteristics in diseases (Gilad et al. 2008, Lawrie et al. 2008), our data suggest that miR-500 was circulating in the sera of the HCC patients and miR-500 levels in sera of the HCC patients returned to normal after the surgery. Although our results are promising for miRNA-based HCC screening, there are several limitations in this study and we suggest: (1) as the sample size is quite small, further validation that miR-500 could be a reliable marker for HCC in a large cohort is necessary; (2) use of better controls to determine whether or not serum miR-500 levels are changed due to the trauma of surgery; (3) it is desirable to examine whether serum miR-500 levels change in patients with chronic hepatitis and liver cirrhosis; (4) it is necessary to compare if serum miR-500 could be better than earlier diagnostic methods such as serum α-fetoprotein.

The differential expression patterns of miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were determined by miRNA microarray and real-time PCR analysis. The specific expression of miR-122 in the liver has previously been described by several research groups. Esau et al. (2006) reported that miR-122 was a key regulator of lipid metabolism in the liver, regulating increased hepatic fatty acid oxidation, a decrease in hepatic fatty acid and cholesterol synthesis rates by reductions of several lipogenic genes. Interestingly, two groups demonstrated evidence that the hepatitis C virus genome has predicted binding sites of miR-122 and that miR-122 positively regulated the replication hepatitis C virus in human liver (Jopling et al. 2005, Randall et al. 2007). In addition to miR-122a, we found that miR-101b expression was upregulated in mouse liver development. Furthermore, upregulation of miR-101b and miR-122a expression was observed in the *in vitro* cultured of fetal hepatocytes treated with OsM and Dex (see Supplementary Figure 4A–C in the online version of this article). It has been reported that miR-101 is related to the immune system and megakaryocytopoiesis (Yu D et al. 2007, Garzon et al. 2006); however, the role of miR-101 in the liver has not yet been examined.

During early development in mice, haematopoietic stem cells emerge in the aorta/gonado/mesonephros region and then the stem cells migrate and expand in the fetal liver before haematopoiesis takes place in the bone marrow by the time of birth. Although most of the miRNAs that we observe in the liver developmental process are constitutively expressed, specific miRNAs are enriched at distinct stages of haematopoietic development. We found that the expression of miR-142-5p, miR-223 and miR-451 was downregulated in the process of liver maturation. As it has been reported that miR-142-5p and miR-142-3p are highly expressed in all haematopoietic tissues (Chen et al. 2004), miR-142 may thus play a critical role at the early stage of haematopoiesis. The expression of miR-223 was mainly detected in bone marrow and negatively regulated myeloid progenitor proliferation and granulocytic differentiation and activation (Johnnidis et al. 2008). In addition, miR-451 expression was upregulated during erythroid differentiation, and gain- and loss-of-function studies disclosed that miR-451 was related to erythroid maturation (Zhan et al. 2007).

Recent studies have indicated that a decrease of mature miRNA expression by impaired miRNA processing accelerates tumorigenesis and that a global reduction of miRNAs is observed in human cancers, suggesting that the role of overall miRNAs is to guard against oncogenic transformation (Kumar et al. 2007, Lu et al. 2005). In particular, the let-7 family is broadly known as a tumour suppressor. It has been reported that a decrease of let-7 expression was observed in human lung cancer and that let-7 negatively regulates the expression of H-ras and *HMGA2* oncogenes in breast cancer cells (Johnson et al. 2005, Yu F et al. 2007, Takamizawa et al. 2004). In addition, miR-16 was also reported as a tumour suppressor by inducing apoptosis mediated by Bcl-2 and modulating the cell cycle (Cimmino et al. 2005, Linsley et al. 2007). In a study of liver carcinogenesis, a decrease of miR-122 expression was observed in rat liver tumour (Kutay et al. 2006). Consistent with this report, miR-122a and miR-101b expression levels in 40 pairs of malignant neoplasias of hepatocyte lineage and adjacent non-tumorous tissue were reduced significantly ($p<0.05$, $n=40$) in tumour samples (see Supplementary Figure 4D in the online version of this article). However, in previous studies, it has been revealed that specific miRNAs acted as oncogenes, as their overexpression facilitates cancer progression. For example, miR-17-92 polycistron was overexpressed in lymphomas, lung cancers and colorectal cancers and enhanced cell proliferation (He et al. 2005, Hayashita et al. 2005). Furthermore, the copy number and expression level of miR-155 and its non-coding RNA transcript BIC were greatly increased in B-cell lymphomas (Eis et al. 2005). Our data show that the expression profile of oncogenic miRNAs was downregulated and, vice versa, the expression of tumour-suppressor miRNAs was upregulated in the process of liver development (Figure 4). This suggests that elevated oncogenic miRNAs are important

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at the early developmental stage of the liver because, in this period, cell proliferation is frequent; in contrast, upregulation of tumour suppressor miRNAs is essential for preventing abnormal cell proliferation at the late stage of liver development. Therefore, our data suggest that the tight regulation of expression of cancer-related miRNAs (both oncogenic miRNAs and tumour suppressor miRNAs) occurred during normal liver development.

Finally, we have documented dynamic changes in miRNA expression that were found in the process of mouse liver development and some of them behaved as an oncofetal miRNA in HCC. Although little is known about the expression regulations, targets or roles of miRNAs in the liver, the expression profiles of miRNA in development could be informative with respect to the elucidation of the process of the development and diagnosis of cancer because the expression of some of the cancer-related miRNAs dramatically changed. Further studies on the differential expression of miRNA in liver development could contribute to a better understanding of the process of liver development and embryonic haematopoiesis and could facilitate the discovery of candidate miRNAs for cancer diagnosis and therapeutic targets in liver cancer.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

Ambros V. (2004). The functions of animal microRNAs. Nature 431:350-5.

Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-97.

Chen CZ, Li L, Lodish HF, et al. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83-6.

Chen JF, Mandel EM, Thomson JM, et al. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228-33.

Chim SS, Shing TK, Hung EC, et al. (2008). Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 54:482-90.

Cimmino A, Calin GA, Fabbri M, et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102:13944-9.

Eis PS, Tam W, Sun L, et al. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 102:3627-32.

Esau C, Davis S, Murray SF, et al. (2006). miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. Cell Metab 3:87-98.

Esquela-Kerscher A, Slack FJ. (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6:259-69.

Fu H, Tie Y, Xu C, et al. (2005). Identification of human fetal liver miRNAs by a novel method. FEBS Lett 579:3849-54.

Garzon R, Pichiorri F, Palumbo T, et al. (2006). MicroRNA fingerprints during human megakaryocytopoiesis. Proc Natl Acad Sci U S A 103:5078-83.

Gilad S, Meiri E, Yogev Y, et al. (2008). Serum microRNAs are promising novel biomarkers. PLoS ONE 3:e3148.

Gramantieri L, Ferracin M, Fornari F, et al. (2007). Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res 67:6092-9.

Han J, Lee Y, Yeom KH, et al. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125:887-901.

Hayashita Y, Osada H, Tatematsu Y, et al. (2005). A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65: 9628-32.

He L, He X, Lim LP, et al. (2007). A microRNA component of the p53 tumour suppressor network. Nature 447:1130-4.

He L, Thomson JM, Hemann MT, et al. (2005). A microRNA polycistron as a potential human oncogene. Nature 435:828-33.

Johnnidis JB, Harris MH, Wheeler RT, et al. (2008). Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature 451:1125-9.

Johnson SM, Grosshans H, Shingara J, et al. (2005). RAS is regulated by the let-7 microRNA family. Cell 120:635-47.

Jopling CL, Yi M, Lancaster AM, et al. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309:1577-81.

Ketting RF, Fischer SE, Bernstein E, et al. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev 15:2654-9.

Kumar MS, Lu J, Mercer KL, et al. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet 39:673-7.

Kutay H, Bai S, Datta J, et al. (2006). Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 99:671-8.

Lawrie CH, Gal S, Dunlop HM, et al. (2008). Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141:672-5.

Lee Y, Kim M, Han J, et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. Embo J 23:4051-60.

Linsley PS, Schelter J, Burchard J, et al. (2007). Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 27:2240-52.

Li QJ, Chau J, Ebert PJ, et al. (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell 129:147-61.

Lu J, Getz G, Miska EA, et al. (2005). MicroRNA expression profiles classify human cancers. Nature 435:834-8.

Lund E, Guttinger S, Calado A, et al. (2004). Nuclear export of microRNA precursors. Science 303:95-8.

Mitchell PS, Parkin RK, Kroh EM, et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 105:10513-18.

Nelson PT, Hatzigeorgiou AG, Mourelatos Z. (2004). miRNP:mRNA association in polyribosomes in a human neuronal cell line. RNA 10:387-94.

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Randall G, Panis M, Cooper JD, et al. (2007). Cellular cofactors affecting hepatitis C virus infection and replication. Proc Natl Acad Sci U S A 104:12884–9.
Shan Y, Zheng J, Lambrecht RW, et al. (2007). Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Gastroenterology 133:1166–74.
Takamizawa J, Konishi H, Yanagisawa K, et al. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64:3753–6.
Yu D, Tan AH, Hu X, et al. (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature 450:299–303.
Yu F, Yao H, Zhu P, et al. (2007). Let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131:1109–23.
Zhan M, Miller CP, Papayannopoulou T, et al. (2007). MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol 35:1015–25.
Zhao Y, Ransom JF, Li A, et al. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 129:303–17.

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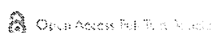

ORIGINAL RESEARCH

Screening of potential molecular targets for colorectal cancer therapy

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Abstract: Colorectal cancer is a leading cause of cancer death worldwide. To identify molecular targets for colorectal cancer therapy, we tested small interfering RNAs (siRNAs) against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colorectal cancer cells (HT-29 cells). The results indicate that the downregulation of *PSMA7* (proteasome subunit, α-type, 7) and *RAN* (ras-related nuclear protein) most efficiently induced apoptosis of HT-29 cells. *PSMA7* and *RAN* were highly expressed in colorectal cancer cell lines compared with normal colon tissues. Furthermore, *PSMA7* and *RAN* were overexpressed in not only colon tumor tissues but also the other tumor tissues. Moreover, *in vivo* delivery of *PSMA7* siRNA and *RAN* siRNA markedly induced apoptosis in HT-29 xenograft tumors in mice. Thus, silencing of *PSMA7* and *RAN* induces cancer cells to undergo apoptosis, and *PSMA7* and *RAN* might be promising new molecular targets for drug and RNA interference-based therapeutics against colorectal cancer.

Keywords: colorectal cancer, molecular target, RNAi, *PSMA7*, *RAN*

Introduction

Colorectal cancer is one of the most common cancers in women and men worldwide. Nearly 1.2 million cases of colorectal cancer were expected to occur in 2007.[1] The highest incidence rates are found in Japan, North America, parts of Europe, New Zealand, and Australia.[1] Worldwide, some 630,000 people die from colorectal cancer per year, accounting for 8% of all cancer deaths.[1] The five-year survival for persons with colorectal cancer is about 65% in Japan; however, when this cancer is detected at advanced stages, the five-year survival rate decreases to 10%,[2] necessitating effective therapeutic targets.

A tumor is characterized by uncontrolled growth and spread of abnormal cells, which invade adjacent normal tissue and spread to other organs, a process that causes death.[3] Multiple molecular alterations are involved in a transformation from a normal cell into a cancerous cell and a progression from a pre-cancerous lesion to malignant tumors.

Angiogenesis is critical in tumor growth and survival. Its inhibition is a promising target for cancer therapy.[4–6] Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis in cancer. In many human tumors including colorectal cancer, VEGF amd VEGF receptors (VEGFR) are overexpressed. Regulating the VEGF/VEGFR pathway is an effective approach to treat cancer.[6,7] A humanized anti-VEGF monoclonal antibody, Bevacizumab, is the first approved biological inhibitor against VEGF for the treatment of metastatic colorectal cancer.[4–6,8] Small-molecule tyrosine

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kinase inhibitors against VEGFRs are also being developed for cancer therapy.[4,6,9,10]

Furthermore, the survival of abnormal cells is a characteristic feature of cancer. In colorectal tumors, some signal transduction pathways drive abnormal cell growth. The most important factor promoting cell survival is epidermal growth factor (EGF).[11,12] Its signaling is a potential target for cancer therapy.[4,5] In treating colorectal cancer, a monoclonal antibody against EGF receptor (EGFR) such as a cetuximab is active,[4,5,12–14] and small-molecular tyrosine kinase inhibitors of EGFRs have been shown to be effective.[4,12]

Additionally, one of the hallmarks of human carcinogenesis is the breakdown of cell apoptotic machinery.[15] Overexpression of anti-apoptotic Bcl-2 family members frequently relates to decreased sensitivity to anticancer drugs and radiotherapy in many types of cancer.[16] The antisense oligonucleotide drug targeting Bcl-2 mRNA expression such as an oblimersen is being investgated in some cancers.[17] The BH3-domain of anti-apoptotic Bcl-2 family proteins is required for the antiapoptotic function. BH-3 mimic peptides that interfere with Bcl-2 signaling are currently under development.[16] Moreover, therapies based on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces programmed cell death, have been studied.[18] Monoclonal antibodies against TRAIL receptors with an agonistic effect on the TRAIL pathway have been generated.[18,19] Thus, inducing apoptosis is a promising approach in the development of a molecular targeted therapy for cancer.

In this paper, we focused on apoptosis induction to identify molecular targets for colorectal cancer therapy. We tested siRNAs against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colon cancer cells (HT-29 cells). The results showed that the downregulation of proteasome subunit, α-type, 7 (PSMA7) and ras-related nuclear protein (RAN) strongly caused apoptosis of HT-29 cells. PSMA7 siRNA and RAN siRNA markedly induced apoptosis in HT-29 xenograft tumor tissues in mice. This silencing of PSMA7 and RAN that induces cancer cells to undergo apoptosis suggests that PSMA7 and RAN are potential key targets for future RNA interference (RNAi)-based therapeutics against colorectal cancer.

Materials and methods

Cell culture

Five colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC), and maintained at 37 °C under 5% CO_2 in a humidified incubator. Caco-2 (human colorectal adenocarcinoma) cells were cultured in Eagle's minimum essential medium (EMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and nonessential amino acids (Invitrogen). Human colorectal carcinoma (HCT116) and human colorectal adenocarcinoma (HT-29) cells were cultured in McCoy's 5A medium (Invitrogen) containing 10% FBS. LoVo (human colorectal adenocarcinoma, derived from supraclavicular lymph node metastatic site) cells were cultured in Ham's F12 medium (Invitrogen) containing 10% FBS. T84 (human colorectal carcinoma, derived from lung metastatic site) cells were cultured in DMEM/Ham's F-12 medium (Invitrogen) containing 10% FBS. To obtain total RNA from these cell lines we plated cells at 1×10^5 cells per well (6-well plate) and culture for three days.

Design and synthesis of siRNAs

We designed siRNAs and synthesized them with four siRNA duplexes for each gene target (Dharmacon, Chicago, IL, USA). The siRNA sequences were described in Table 1.

Cell transfection array

For RNAi-based functional screening of genes, we used a reverse transfection based-cell transfection array.[20] HT-29 cells were plated into the cell transfection array in a 96-well format and transfected with siRNA. We evaluated the effects of the downregulation of genes on promotion of apoptosis, as mentioned below.

Measurement of cell proliferation

We plated HT-29 cells into a cell transfection array at a density of 5×10^3 cells per well and cultured. Three days after, we measured cell proliferation by resazurin reduction assay using CellTiter-Blue Reagent (Promega, Madison, WI, USA). Cells were incubated with CellTiter-Blue Reagent for one hour at 37 °C, and the fluorescence was then measured at 560Ex/590Em. After that, we subjected the same cell transfection array to a caspase-3/7 assay, Hoechst staining, and a cell-direct real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Measurement of caspase activity *in vitro*

Cells were incubated with the Caspase-Glo 3/7 Reagent (Promega) for one hour at room temperature, and the luminescence was then measured.

Hoechst staining

Cells on a cell transfection array were washed with phosphate-buffered saline (PBS), and a fixative and staining solution was