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ORIGINAL PAPER

MicroRNA-21 correlates with tumorigenesis in malignant peripheral nerve sheath tumor (MPNST) via programmed cell death protein 4 (PDCD4)

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Abstract
Purpose We investigated the miRNA profile in peripheral nerve tumors and clarified the involvement of miRNA in the development and progression of MPNST in comparison with neurofibroma (NF). In addition, we attempted to seek associations between the miRNA and their potential targets in MPNST.
Methods Global miRNA expression profiling was investigated for clinical samples of 6 MPNSTs and 6 NFs. As detected by profiling analysis, the expressions of miR-21 in clinical samples of 12 MPNSTs, 11 NFs, and 5 normal nerves, and 3 MPNST cell lines were compared using quantitative real-time reverse transcription PCR. MPNST cell line (YST-1) was transfected with miR-21 inhibitor to study its effects on cell proliferation, caspase activity, and the expression of miR-21 targets.
Results Analysis of miRNA expression profiles in MPNST and NF revealed significantly altered expression levels of nine miRNAs, one of those, miR-21, and its putative target, programmed cell death protein 4 (PDCD4), were selected for further studies. miR-21 expression level in MPNST was significantly higher than that in NF ($P < 0.05$). In MPNST cells, transfection of miR-21 inhibitor significantly increased caspase activity ($P < 0.01$), significantly suppressed cell growth ($P < 0.05$), and upregulated protein level of PDCD4, indicating that miR-21 inhibitor could induce cell apoptosis of MPNST cells.
Conclusions These results suggest that miR-21 plays an important role in MPNST tumorigenesis and progression through its target, PDCD4. MiR-21 and PDCD4 may be candidate novel therapeutic targets against the development or progression of MPNSTs.

Keywords Malignant peripheral nerve sheath tumor · MicroRNA · miR-21 · PDCD4

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Introduction

Peripheral nerves are composed of various elements that arise from the neural crest and neural tube, such as Schwann cells, perineural cells, and fibroblasts. Peripheral nerve tumors can occur anywhere in the body; most are benign, such as schwannoma or neurofibroma (NF). However, malignant tumors can also arise from peripheral nerves, the most aggressive of which is malignant peripheral nerve sheath tumor (MPNST). MPNST constitutes 5–10 % of all soft tissue sarcomas. Approximately 80 % of MPNSTs are pathologically indicated as high-grade tumors, with a high incidence of local recurrence (40–65 %) and distant metastasis (40–68 %). The five-year

survival rates of patients with MPNST at all sites are about 50 % (Enzinger and Weiss 2001). MPNST can develop either spontaneously or in association with neurofibromatosis type 1 (NF1). NF1 is an autosomal dominant neurocutaneous disorder, which progresses to MPNST in about 10 % of patients (Ducatman et al. 1986). NF1 mutations (Wu et al. 1999) are one cause of tumorigenesis in MPNST, but recent reports have shown that germline and somatic NF1 alterations are not only found in MPNST, but are also found in NF (Bottillo et al. 2009; Upadhyaya et al. 2008). Some papers have identified candidate genes in MPNST development, such as TP53 mutations (Lothe et al. 2001) and homozygous deletions of cyclin-dependent kinase inhibitor 2A (CDKN2A) (Berner et al. 1999; Nielsen et al. 1999). However, the molecular events involved in MPNST tumorigenesis have not been fully investigated.

MicroRNAs (miRNAs) are endogenous 18–25 nucleotide, non-coding single-stranded RNAs that suppress protein expression by binding to complementary sequences of messenger RNA (mRNA). miRNA can bind to incomplete complementary sequences, meaning that each miRNA can regulate up to hundreds of target mRNAs. More than 1,000 miRNAs have been identified across the various species and found to regulate development, cell differentiation, cell proliferation, and apoptosis (Zhang 2008). miRNA expression profiles are unique in tumors from different origins, and when overexpressed in malignant tumors, some miRNAs act similarly to oncogenes (Calin and Croce 2006). Conversely, some miRNAs with tumor-repressor functions are downregulated in malignancies (Boominathan 2010). Both normal and malignant tumor tissues may have specific miRNA expression patterns and show differential expression among tumor types. Although there have been many reports about the role of miRNAs in tumorigenesis, there are few reports about the role of miRNAs in soft tissue tumors.

In this study, we investigated the miRNA profile in peripheral nerve tumors and clarified the involvement of miRNA in the development and progression of MPNST in comparison with NF. In addition, we attempted to seek associations between the miRNAs and their potential targets in MPNSTs.

Materials and methods

Clinical samples

Tumor tissues were collected from patients who underwent surgical resection at the Okayama University Hospital. Normal nerves were obtained from tumor-free parts of amputated limbs. Written informed consent was obtained from each patient, and the use of specimens for analysis in this study was approved by an ethics committee at Okayama University. The tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C until subsequent analysis.

Cell culture

The MPNST cell lines, HS-Sch-2, YST-1, and NMS-2, were provided by RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of MEXT, Japan. HS-Sch-2 was cultured in Dulbecco's modified Eagle medium (Wako-chem, Osaka, Japan) containing 10 % fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) (Sonobe et al. 2000). YST-1 and NMS-2 were cultured in Roswell Park Memorial Institute-1640 medium (Wako-chem) with the same supplements (Nagashima et al. 1990; Imaizumi et al. 1998). Cells were maintained at 37 °C in a 5 % CO_2 humidified incubator and subcultured using 0.25 % trypsin every 4–5 days before reaching confluence.

miRNA expression profiling

For global miRNA expression profiling, tissues comprised of six MPNSTs and six NFs were investigated. Total RNA was extracted from the cells and tissues using ISOGEN (NIPPON GENE, Tokyo, Japan). RNA concentrations were determined by NanoDrop ND-100 (NanoDrop Products, Wilmington, DE, USA). Quantitative real-time RT-PCR (qRT-PCR) analysis was performed using TaqMan® MicroRNA Assays Human PanelEarly Access Kit (Applied Biosystems, Foster city, CA, USA), which includes 157 mature human miRNAs. PCR was conducted at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s in the ABI 7500 Fast Real-time PCR system (Applied Biosystems). Total RNA samples (5 ng) were transcribed into cDNA with specific miRNA stem-loop primers, and PCR products were synthesized from cDNA samples using sequence-specific primers from the TaqMan®MicroRNA Assays Human Panel (Applied Biosystems). The assays were normalized to U6 RNA levels.

miRNA expression in peripheral nerve tumors

An additional experiment was carried out to confirm the data of the differentially expressed miRNAs, as detected by profiling analysis. Total RNA was isolated from another set of clinical samples (12 MPNSTs, 11 NFs, and 5 normal nerves) and three MPNST cell lines. qRT-PCR for mature miRNAs was performed using *mir*Vana™ qRT-PCR miRNA Detection Kit and primers for miRNAs and U6 (Ambion, Austin, TX, USA). Real-time PCR analysis was performed with Power SYBR® Green PCR Master Mix and Mx300P™ (Agilent Technologies, Santa Clara, CA, USA).

PCR was conducted at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 30 s. All PCRs were run in duplicate, and gene expression, relative to U6, was calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$).

Knockdown of miR-21 in MPNST cells

The MPNST cell line, YST-1, was used to suppress miR-21 expression. Cells were transfected with an anti-miR™ miRNA inhibitor, miR-21 (Ambion), an miR inhibitor negative control #1 (scrambled-oligo-transfected controls), or FAM-labeled miR negative control inhibitor (mock), using siPORT™ NeoFX™ Transfection Agent (Ambion). YST-1 cells were transfected with 30 and 60 nM FAM-labeled miRNA inhibitor, and the ratio of FAM-labeled cells was calculated to assess transfection efficiencies. Twenty-four hours (h) after transfection, total RNA was extracted from the cells and miR-21 levels were assessed using qRT-PCR. For cell activity analysis and the caspase assay, cells were seeded into 96-well plates at 10,000 cells per well and treated with miRNA inhibitors, negative controls, and mock. The degree of apoptosis was assessed 48 h after transfection using the Caspase-Glo™ 3/7 Assay (Promega, Madison, WI, USA). Cell proliferation was measured 72 h after transfection using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega). The rate of absorbance was determined using a GloMax™ 20/20 Luminometer (Promega), and the results are presented as fold change compared to their respective untreated controls (mock).

Bioinformatics analysis

To understand the mechanism of action of miR-21 in MPNSTs, database research using TargetScan 5.1 (http://www.targetscan.org), MicroCosm Targets V5 (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and PicTar (http://pictar.mdc-berlin.de/) was used to predict the targets of miR-21. These three programs each use a different algorithm to identify highly complementary sites and are widely used for miRNA target prediction. Therefore, genes predicted by all three algorithms were considered to be targets.

Western blot analysis

Cell lysates for Western blot analyses were prepared from cells 72 h after transfection and from clinical samples of MPNSTs, NFs, and normal nerves. Total protein from cell lysates (15 μg/lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 % gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with Blocking Reagent (TOYOBO, Osaka, Japan) and incubated with anti-PDCD4 (ROCKLAND, Gilbertsville, PA, USA) and anti-beta actin (Sigma, St. Louis, MO, USA) at a dilution of 1:1,000 in CanGet Signal Immunoreaction Enhancer Solution (TOYOBO) for 1 h at room temperature. The membranes were washed with washing buffer and incubated with horseradish peroxidase-conjugated anti-mouse (diluted 1:10,000, R&D Systems) or anti-rabbit (diluted 1:50,000, Bethyl Laboratories, Montgomery, TX, USA) secondary antibodies for 1 h at room temperature. Immunoreactive proteins were detected using the ECL Detection System (GE Healthcare, Buckinghamshire, UK). Densitometry was carried out using Image J software (NIH, Bethesda, MD, USA), and protein content was normalized according to β-actin content.

Statistical methods

The expression levels were analyzed by Student's *t*-test. *P* values <0.01 were considered statistically significant in miRNA expression profiling and *P* values <0.05 in other analyses. Statistical analysis was performed using StatView software, version 5.0 (SAS Institute Inc., Cary, NC, USA). For miRNA expression profiling, a cluster analysis was performed using TM4 MeV 4.4 software via the open-source MeV software tool available from http://www.tm4.org/mev.html (Saeed et al. 2003).

Results

miRNA expression profiling

We compared the miRNA expression profiles of six MPNSTs with those of six NFs and identified nine miRNAs that showed significantly different expression levels between the tumors (Table 1); the expression levels of miR-21, miR-135b, miR-152, miR-130b, miR-92, and miR-15b were increased in MPNST, whereas the expression levels of miR-125b, miR-127, and miR-302d were increased in NFs. Unsupervised hierarchical clustering, based on the expression levels of these nine miRNAs, almost segregated MPNSTs from NFs and was not dissociable between patients with NF1 and non-NF1 (Fig. 1).

Validation of miRNA expression profiling

As a confirmation of profiling analysis, we quantified expressions of miR-21, miR-135b, miR-125b, miR-127, and miR-302d in clinical samples from NFs, MPNSTs, and normal peripheral nerves, and three MPNST cell lines, since there were statistical differences with more than twofold change between these miRNAs expression in

Table 1 miRNA expression profiling in peripheral nerve tumors

MPNST>NF	Fold change	*P* value	NF>MPNST	Fold change	*P* value
miR-21	**2.5**	**0.0002**	**miR-125b**	**3.6×10^2**	**0.0008**
miR-135b	**7.3**	**0.0020**	**miR-127**	**1.5×10^3**	**0.0037**
miR-152	1.5	0.0023	**miR-302d**	**9.8**	**0.0097**
miR-130b	1.6	0.0030			
miR-92	1.6	0.0040			
miR-15b	1.5	0.0083			

The expression pattern of 9 miRNAs was significantly different between NF and MPNST

Bold; 5 miRNAs that showed statistical difference with more than twofold change between the expression in MPNSTs and in NFs were further analyzed to validate miRNA expression profiling

MPNST malignant peripheral nerve sheath tumors, *NF* neurofibroma. Values represent each miRNA expression level in MPNSTs and NFs samples. *P* value is significant at <0.01

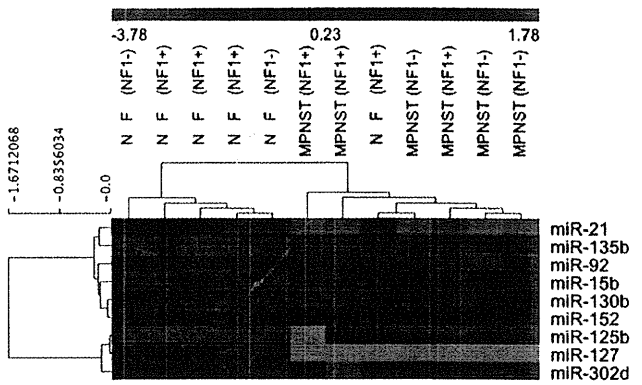

Fig. 1 Hierarchical clustering. miRNA expression was examined in 6 MPNSTs and 6 NFs. Twelve samples were clustered according to the expression profile of 9 miRNAs that were differentially expressed between MPNSTs and NFs. *P* values <0.01 were considered statistically significant. The *colored bar* at the top denotes the relative expression values seen in the cluster table

MPNSTs and in NFs (Table 1). Among 5 miRNAs, the expression level of miR-21 in clinical samples of MPNSTs was significantly higher than that in NFs and normal nerves (Fig. 2a). In contrast, miR-21 expression in NFs was similar to that in normal nerves. The expression of miR-21 in MPNST cell lines was also significantly higher than that in normal nerves (Fig. 2f). Based on these results, we proceeded with further analysis on miR-21 function in MPNSTs and NFs.

miR-21 inhibitor in MPNST cells

We characterized the function of miR-21 in MPNST cells. YST-1 cells were transfected with a specific miR-21 inhibitor to silence miR-21 expression. Transfection efficiency, as a ratio of FAM-labeled miRNA-positive cells, was 31 and 22 %, respectively, at concentrations of 30 nM and 60 nM (Fig. 3). We conducted further analysis using miR-21 inhibitor at concentration of 30nM, which resulted in better transfection efficiency. The expression level of miR-21 in YST-1 cells transfected with miR-21 inhibitor was significantly reduced to 66 % of that in the mock-transfected cells ($P < 0.05$, Fig. 4a). A caspase assay showed that transfection with miR-21 inhibitor significantly increased apoptosis in YST-1 cells by 150 % when compared to mock-transfected cells ($P < 0.01$, Fig. 4b). Cells transfected with miR-21 inhibitor showed significantly lower cell proliferation (80 %) than mock-transfected cells ($P < 0.05$, Fig. 4c).

Functional analysis of miR-21 in MPNST cells

In order to further investigate the action of miR-21 in MPNST, we next sought to determine the miR-21-target RNA. The potential miR-21-targets were predicted using three different algorithms. Overall, 1,000 hits were found in microcosm, 210 in TargetScan, and 175 in PicTar, with 22 overlapping targets (Table 2). Among these mRNAs, we noticed PDCD4, a previously reported target of miR-21 that is thought to act as a tumor suppressor gene (Young et al. 2010; Lankat-Buttgereit and Göke 2009). We examined whether miR-21 inhibits the protein synthesis of PDCD4. Western blot analyses demonstrated that an miR-21 inhibitor increased PDCD4 protein synthesis by approximately 40 % in transfected cells (Fig. 5), although PDCD4 mRNA levels were unchanged (data not shown). Next, we examined the expression of PDCD4 protein in clinical samples of MPNSTs, NFs, and normal nerves. Protein expression levels of PDCD4 were twofold higher in NFs than in MPNSTs ($P < 0.05$, Fig. 6) and threefold higher in normal nerves than in MPNSTs ($P < 0.01$); however, once again, there were no significant differences in PDCD4 mRNA levels among them (data not shown).

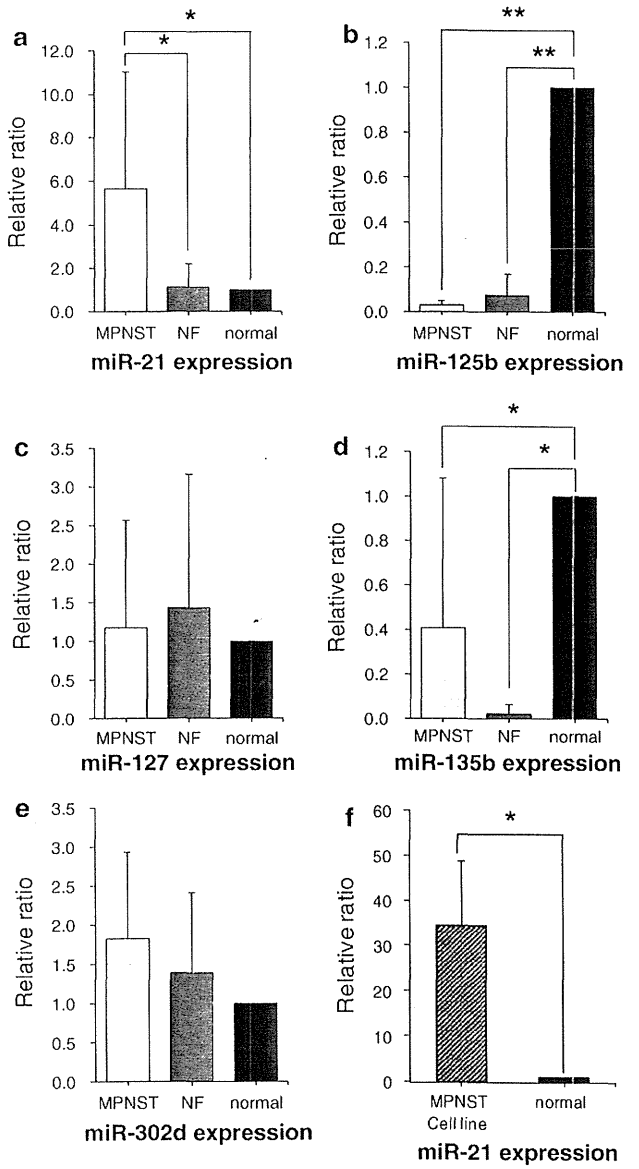

Fig. 2 Verification of 5 miRNAs expression using quantitative RT-PCR in clinical samples from MPNSTs, NFs, and normal nerves; **a** miR-21, **b** miR-125b, **c** miR-127, **d** miR-135b, **e** miR-302d. MPNST cell lines. The expression level of miR-21 in clinical samples from MPNSTs was significantly higher that than in NFs. miR-21 expression in MPNST cell lines was also significantly higher than that in normal nerves (**f**). $*P < 0.05$, $**P < 0.01$

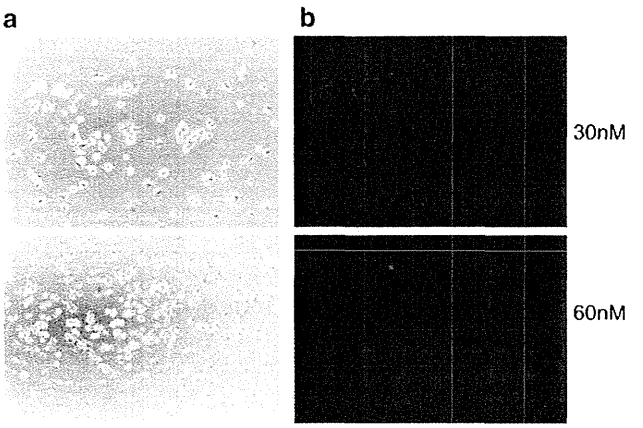

Fig. 3 Monitoring transfection efficiency in YST-1 cells. Transfection efficiencies were calculated by the ratio of FAM-labeled cells via **a** phase-contrast and **b** fluorescence imaging

Discussion

In the present study, miRNA expression profiling demonstrated that nine miRNAs were differentially expressed between NFs and MPNSTs. These nine miRNAs were considered candidate genes that could influence the tumorigenesis of MPNST or malignant conversion in NF. Previous papers have shown that these miRNAs are associated with tumor progression in other malignant tumors: miR-125b in breast cancer (Zhang et al. 2011; Saetrom et al. 2009), bladder cancer (Huang et al. 2011), and liver cancer (Alpini et al. 2011); miR-152 in gastrointestinal cancer (Chen et al. 2010); miR-130 in chronic myelogenous lymphoma (Suresh et al. 2011); miR-127 in diffuse large B-cell lymphoma (Robertus et al. 2009); miR-92 in neuroblastoma, breast cancer, and acute leukemia (Haug et al. 2011; Al-Nakhle et al. 2010; Tanaka et al. 2009); and miR-15b in malignant melanoma (Satzger et al.2010). Recent studies have also focused on the potential roles of miRNA in cancer prognosis, chemosensitivity, and multidrug resistance in bone and soft tissue sarcomas as well as other forms of malignancy (Greither et al. 2011; Song et al. 2010; Missiaglia et al. 2010). MPNST is a rare malignant tumor, and an optimal treatment strategy, including chemotherapy, has not yet been confirmed. Our small set of MPNST patients underwent various treatments, but we could make no correlations between clinical outcome of our patients and miRNA expression.

Two studies have examined miRNA in MPNSTs. Subramanian et al. (2010) assessed miRNA expression profile in 23 peripheral nerve sheath tumors (including six MPNSTs) and identified 10 miRNAs that were up or downregulated in comparison with NF and schwannoma. In particular, the authors demonstrated that miR-34a transcript levels may depend on p53 activation, and they showed that apoptosis could be induced in MPNST cells transfected with miR-34a. Since we only compared miRNA expression profiles in MPNSTs with those of NFs, the current study could not identify differences in miR-34a expression between the tumors. In the second study, Chai et al. (2010) reported that the expression level of miR-10b was higher in NF1-associated MPNST than in non-NF1-associated MPNSTs and concluded that miR-10b may play an important role in NF1 tumorigenesis through targeting neurofibromin and RAS signaling. Our MPNST samples

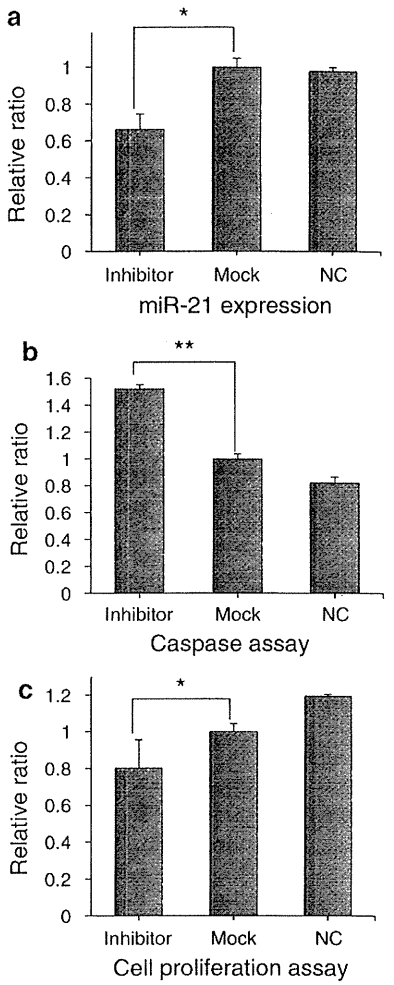

Fig. 4 Influence of miR-21 inhibitor in YST-1 cells. **a** miR-21 expression level in transfected cells. The expression level of miR-21 was significantly downregulated 24 h after transfection with miR-21 inhibitor in YST-1 cells. **b** Apoptotic activity using caspase 3/7 assay 48 h after transfection. Transfection with anti-miR-21 significantly increased apoptosis. **c** Cell proliferation assay using CellTilter-Glo 72 h after transfection. Cells transfected with miR-21 inhibitor showed significantly lower proliferation. *Inhibitor* miR-21 inhibitor, *NC* negative control. $*P < 0.05$, $**P < 0.01$

included non-NF1-associated samples, and we analyzed both NF1 and non-NF1. This may explain why our miRNA profiling did not suggest the importance of miR-10b in MPNST tumorigenesis.

Higher expression of miR-21 was detected in several types of cancers as well as soft tissue tumors (Folini et al. 2010; Zhang et al. 2008), and miR-21 could be a key regulator of oncogenic processes (Si et al. 2007; Selcuklu and Donoghue 2009). Cioffi et al. (2010) revealed that miR-21 overexpression contributes to vestibular schwannoma cell proliferation and prognosis. In leiomyoma, miR-21 was overexpressed and regulated TGF-β-RII, E2F1, PTEN, and PDCD4 (Pan et al. 2010). In our study, the

Table 2 Potential targets of miR-21

Target gene	Gene name
ASPN	Asporin
BTG2	BTG family, member 2
C17orf39	Chromosome 17 open reading frame 39
C4orf16	Chromosome 4 open reading frame 16
CCL1	Chemokine (C-C motif) ligand 1
CHD7	Chromodomain helicase DNA binding protein 7
CNTFR	Ciliary neurotrophic factor receptor
JAG1	Agged 1 (Alagille syndrome)
MATN2	Matrilin 2
NFIB	Nuclear factor I/B
NTF3	Neurotrophin 3
PCBP1	Poly(rC) binding protein 1
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
PELI1	Pellino homolog 1 (*Drosophila*)
PITX2	Paired-like homeodomain 2
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
SOX2	SRY (sex determining region Y)-box 2
SPRY1	Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)
SPRY2	Sprouty homolog 2 (*Drosophila*)
STAG2	Stromal antigen 2
TGFBI	Transforming growth factor, beta-induced, 68kDa
WWP1	WW domain containing E3-ubiquitin protein ligase 1

1,000 hits were found in microcosm, 210 in TargetScan, and 175 in PicTar. Twenty-two potential targets were found using all three algorithms

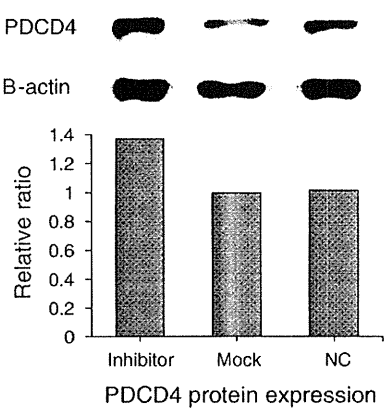

Fig. 5 PDCD4 protein expression in YST-1 cells. Western blotting detected that transfection with miR-21 inhibitor upregulated PDCD4 expression. The membranes were blotted with PDCD4 and GAPDH antibody. Intensities of each protein band were detected using ECL Detection System and analyzed using Image J software (NIH). The results are presented as fold change compared to their respective untreated controls (mock). *Inhibitor* miR-21 inhibitor, *NC* negative control

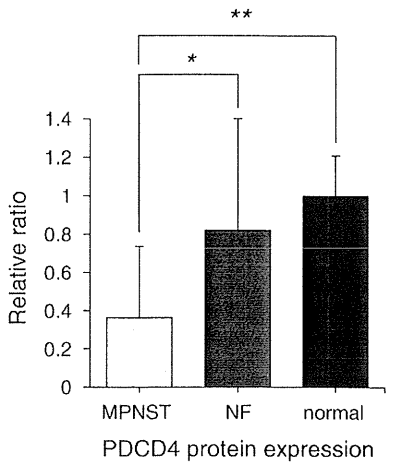

Fig. 6 PDCD4 protein expression in clinical samples of MPNSTs, NFs and normal nerves. The expression of PDCD4 protein in NFs and normal nerves was significantly higher than that in MPNSTs. $*P < 0.05$, $**P < 0.01$

expression of miR-21 was significantly higher in MPNSTs than in NFs and normal nerves, suggesting that the miR-21 might be an important progression factor in the malignant development of peripheral nerve tumors.

From the database search, we focused on PDCD4 as the target of miR-21, since PDCD4 expression has been reported in several malignant tumors (Wei et al. 2009; Chen et al. 2003; Afonja et al. 2004) and is also targeted by miR-21 (Qi et al. 2009; Frankel et al. 2008; Asangani et al. 2008). In the current study, miR-21 inhibitor induced caspase 3/7 activity in the MPNST cell line and decreased cell proliferation, despite moderate transfection efficiency. Furthermore, PDCD4 was upregulated by the miR-21 inhibitor in MPNST cells. PDCD4 was upregulated by the miR-21 inhibitor in many malignant tumors (Gaur et al. 2011; Si et al. 2007; Chan et al. 2005). The expression of PDCD4 is increased during apoptosis (Lankat-Buttgereit and Göke 2009; Allgayer 2010). The overexpression of PDCD4 might induce apoptosis via caspase cascade (Lankat-Buttgereit and Göke 2009; Zhang et al. 2006). These results suggested that inhibition of miR-21 could increase PDCD4 expression and lead to apoptosis of tumor cells through caspase activation in MPNSTs. PDCD4 has been also known as the tumor suppressor that inhibits neoplastic transformation, tumor progression, and translation and is decreased in human carcinogenesis (Young et al. 2010; Lankat-Buttgereit and Göke 2009; Wei et al. 2009; Frankel et al. 2008). The present study demonstrated that PDCD4 expression was suppressed in clinical samples from MPNST patients when compared to samples from NF patients. The overexpression of miR-21 could suppress PDCD4 function in MPNST and result in decreasing apoptosis and increasing cell proliferation as an oncogene.

The expression of PDCD4 mRNA did not differ in the MPNST cell line or in clinical samples, as the expression of PDCD4 protein was decreased. Asangani et al. (2008) also reported that PDCD4 mRNA was unaltered by inhibition of miR-21 as opposed to a significant change in PDCD4 protein in colorectal cancer. miRNAs can bind to complementary sites in the mRNA target to negatively regulate target gene expression at the post-transcriptional level (Zhang 2008; Bartel et al. 2004). We proposed that the main mechanism of miR-21-induced PDCD4 suppression might be post-transcriptional, as suggested previously (Asangani et al. 2008).

In conclusion, our miRNA expression profiling and validation analyses suggest that miR-21 plays an important role in MPNST tumorigenesis and progression through its target, PDCD4. Together, these results indicate that miR-21 and PDCD4 may be candidate novel therapeutic targets against the development or progression of MPNSTs.

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Conflict of interest None declared.

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6．マイクロRNAによるがん転移予防への展開 -miR-143による骨肉腫肺転移抑制効果とその標的遺伝子の同定-

尾﨑充彦・杉本結衣

悪性腫瘍の発生および進展にマイクロRNAの発現異常との関連が報告されている。さらに，腫瘍組織におけるマイクロRNA量を調節することにより病態を改善できることが報告されつつあり，治療に向けた取り組みが進められている。

本稿では，ヒト骨肉腫細胞の浸潤能を制御するマイクロRNAを核酸医薬としてモデルマウスへ全身投与することにより，転移抑制効果を示すことに成功したデータを紹介するとともに，マイクロRNAという観点からの転移メカニズム解明に向けたわれわれの解析データについて概説する。

はじめに

日本における死因第一位の「がん」。早期診断，新規治療法や新薬の開発により，原発巣に限局したがんは治癒可能となりつつある一方で，がん治療を困難にしている「がん転移」については，いまだ確立された治療法はない。「転移を制するものはがんを制する」と言われるように，がん治療の最大の目標は転移を制御することと言っても過言ではない。がんの転移は，原発巣において増殖したがん細胞が，既存組織の破壊を伴いながら遊走・浸潤した後に脈管内へ進入し，原発巣とは異なる臓器や組織において新たな腫瘍組織（転移巣）を形成するといった様々な現象が多段階で連続的に生じる過程を経て成立している。そして転移巣を形成したがん細胞は，原発巣のそれと比較し悪性度を増しており，種々の治療法に抵抗性をもつこと，さらに転移巣が形成される場所やその多発性により外科的切除が困難となるケースが多い。したがって，転移巣に対する有効な治療法および転移そのものに対する予防法の開発が臨床上重要な課題となっている。

Ⅰ．骨肉腫について

骨肉腫は骨原発の悪性腫瘍であり，日本での発症数は年間200例ほどであり必ずしも多くはないが，小児に発生する原発性悪性骨腫瘍の中で最も多く，骨肉腫の6〜7割が20歳未満で発症する特徴をもっている。好発部位は膝関節や肩関節近傍の長管骨骨幹端であり，大腿骨，頸骨，上腕骨が三大好発部位である[1]。1980年以前は骨肉腫が発生した足や腕を外科的に切断する治療法が行われていたが，切断後に多くの症例において肺転移を生じ，5年生存率は10〜15％程度と極めて予後不良であった。その後，メトトレキサート，アドリアマイシン，シスプラチンおよびイホスファミド

key words
骨肉腫，浸潤，転移，転移巣，肺転移，MMP13，PAI-1，miR-143，アテロコラーゲン

などの抗がん剤による化学療法の発展により，患肢を温存できるようになるとともに5年生存率も約70%と飛躍的に向上した[2]。しかしながら，治療経過中に新たに肺転移が生じた症例や，すでに初診時に肺転移を有している患者の予後はいまだに極めて不良であり，Uribe-Boteroらの骨肉腫症例の病理解剖例における転移頻度の調査によれば，肺転移は実に98.1%に達している[3]。したがって，骨肉腫における肺転移の有無は重要な予後因子の1つであり，原発巣に対する治療に加えて肺転移をあらかじめ予防する方法の確立は骨肉腫患者，とりわけ若年で発症した患者の治療成績向上のために重要なポイントとなる。

Ⅱ．マイクロRNA-143（miR-143）による骨肉腫肺転移抑制効果

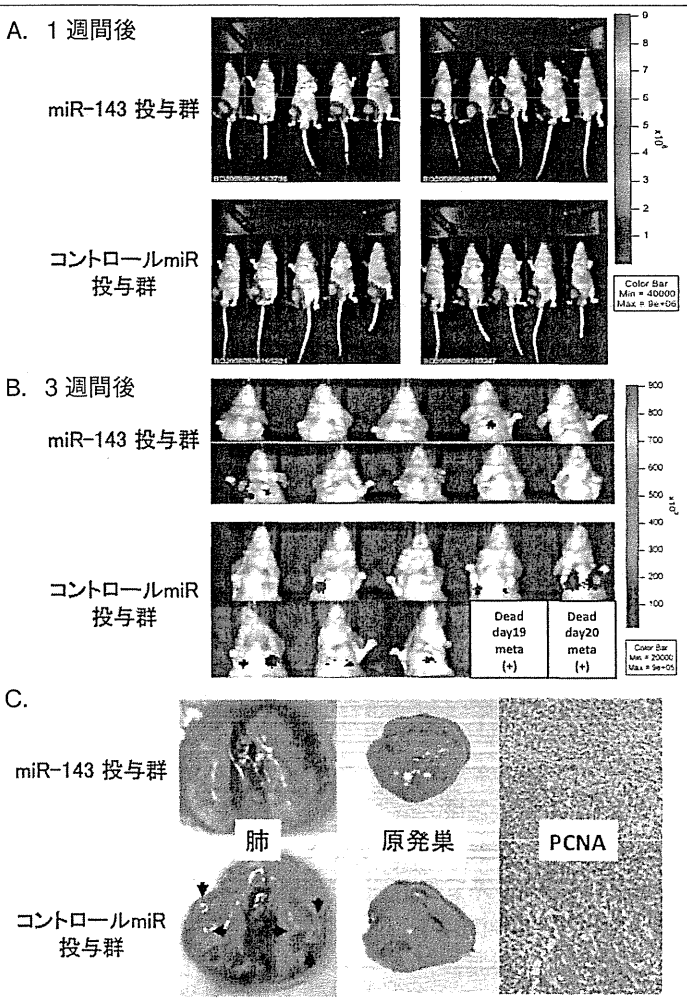
ヒト骨肉腫細胞の肺転移に関与するマイクロRNAの検索には，2種のヒト細胞株（HOSと143B）を用いた。143B細胞は，HOS細胞をウイルスによってトランスフォームした細胞であり，マウス膝関節への移植により高頻度に肺転移を生じる。一方，その親株であるHOS細胞は肺転移を示さない。すなわち，この2つの細胞株は遺伝的背景が極めて類似しているにもかかわらず，転移という現象について明らかに異なる性質を示すことから，両細胞株におけるマイクロRNA発現の差異をマイクロアレイにて検索した。HOS細胞と比較し転移能を有する143B細胞において発現量が2倍以上増加しているマイクロRNAを19種，一方，1/2以下に減少しているマイクロRNAを9種検出した[4]。前者については，その機能を阻害するアンチマイクロRNAを，後者には合成したマイクロRNAを用い143B細胞へ導入し，細胞増殖能と浸潤能をそれぞれ*in vitro*の系で解析した。その結果，マイクロRNA-143（miR-143）を143B細胞へ導入した際，細胞の浸潤能を最も強く阻害するとともに，細胞増殖に影響を及ぼさないことが明らかとなった。かかる所見は，miR-143が骨肉腫細胞の増殖には関与せず，浸潤能を負に制御している可能性を強く示唆した。

そこで，*in vitro*で得られたデータを*in vivo*で確認するため，ヒト骨肉腫細胞自然肺転移モデルを作製しmiR-143の転移抑制効果を検討した。この解析には，非観血的に腫瘍の生着，増殖および転移巣の形成を同一個体にて経時的に観察可能な*in vivo* imaging system（IVIS，Xenogen社）を用いた。ルシフェラーゼ遺伝子を導入した143B細胞をマウス膝関節へ移植し，IVISにて原発巣の形成を確認した（図❶A）。このマウスに対し，miR-143を低濃度のアテロコラーゲン（最終濃度0.05%）を担体として，3日おきに尾静脈より9回投与した。コントロール群（10匹）では，143B細胞移植3週間後に8匹（80%）で肺転移を生じ，そのうち2匹は肺転移による呼吸不全により3週間以内に死亡していた。一方，miR-143投与群で肺転移を生じたマウスは3週間後に10匹中2匹（20%）にとどまっており，miR-143の全身投与により骨肉腫細胞の原発巣からの肺転移が明らかに抑制されたことが示された（図❶B）。さらに，マウスを解剖し原発巣を摘出した結果，miR-143投与群とコントロール群で腫瘍重量に差はなく，さらに組織学的にPCNA（proliferation cell nuclear antigen）発現を指標とした腫瘍増殖活性を免疫組織化学的に検索した結果，両群間に差は見出せなかった（図❶C）。以上の結果は，miR-143の全身投与により骨肉腫細胞の増殖能とは無関係に腫瘍細胞の肺転移抑制効果を示しており，*in vitro*においてmiR-143が骨肉腫細胞の増殖には関与せず浸潤能を負に制御しているデータが，*in vivo*においても裏づけられたことを示した。したがって，miR-143の標的遺伝子群を明らかにすることにより，骨肉腫細胞の浸潤・転移に特異的に関わる分子を同定し，その転移メカニズムを明らかにできると考えられた。

Ⅲ．miR-143標的分子群の同定と転移抑制メカニズムの探索

マイクロRNAは，22塩基程度のゲノムにコードされたタンパクをコードしない小さなRNA分子であり，標的とするmRNAの主として3'側非翻訳領域に存在するシード配列を認識して結合し，mRNAの分解あるいはペプチド鎖伸長抑制などにより，mRNAからタンパクへの翻訳を阻害する。

図❶ miR-143による骨肉腫肺転移抑制効果（*in vivo* イメージングによる評価）と原発巣における腫瘍細胞増殖活性（文献4より改変）



A. ヒト骨肉腫細胞（143B細胞）をマウス膝関節内へ接種することにより，接種部位に原発巣を形成する。
B. 腫瘍細胞接種3週間後，コントロールmiRを経静脈的に全身投与した群では10匹中8匹に肺転移巣が確認されたが，miR-143投与群では，肺転移が抑制されている。
C. 肺転移巣は，コントロールmiR投与群でのみ肉眼的に観察された（矢印）。miR-143原発巣の重量および腫瘍細胞増殖活性は，miR-143投与の有無にかかわらず同程度であった。 （グラビア頁参照）

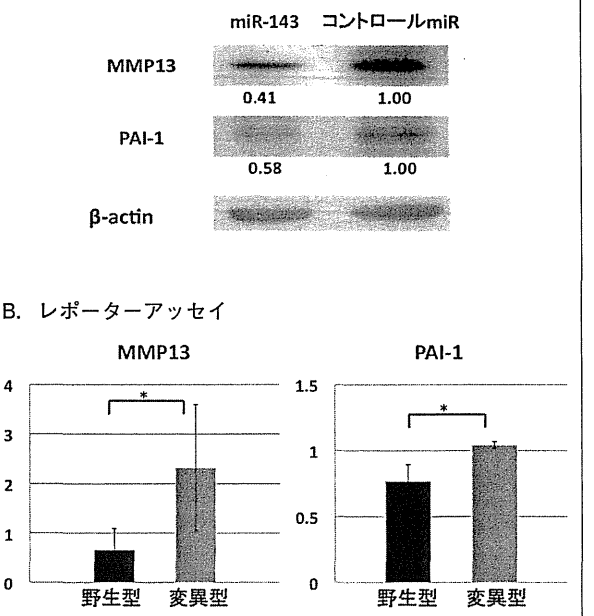
ヒトにおいて現在1500を超えるマイクロRNAが同定されており（miRBase 18）[5]，タンパク質をコードする遺伝子の約1/3の発現が，これらマイクロRNAによって制御されていると推定されている[6]。1種のマイクロRNAが標的とするmRNAは複数存在し，多数のタンパクにおいて緩やかな発現量の低下を示すことが報告されており，まさに遺伝子発現の「ファインチューナー」として細胞内環境を精巧に制御していると考えられる[7]。

本稿で紹介したmiR-143は，転移性の骨肉腫細胞において発現低下を示しており，この細胞へmiR-143を導入することでその標的遺伝子群の発

現を減弱させ，結果として浸潤・転移を抑制していると考えられる。そこで，ヒト骨肉腫細胞におけるmiR-143標的遺伝子の探索を行った。文献的にはヒト大腸がん細胞株およびBリンパ腫細胞株を用いた検討により，miR-143の標的遺伝子としてK-RASおよびERK5がすでに報告されていた[8][9]。しかしながら143B細胞において，miR-143がK-RASおよびERK5を標的としている結果を得ることはできなかった。そこで143B細胞におけるmiR-143標的遺伝子群を包括的に回収するため，①抗Ago2抗体を用いた免疫沈降（Ago2 IP）法および②Labeled miRNA pull-down（LAMP）法をそれぞれ行った[4]。前者は，マイクロRNAが標的とするmRNAと結合してRNA-induced silencing complex（RISC）へ取り込まれることを利用し，RISCの構成タンパク質の1つであるAgo2タンパクに対する抗体で免疫沈降し標的遺伝子群を回収する系である。免疫沈降とRNA精製にはそれぞれmiRNA isolation kit, human Ago2（和光純薬）とmirVana（Ambion社）を用いた。他方，後者は143B細胞を超音波破砕にて溶解した後，ジゴキシゲニン標識したmiR-143と混和し標的mRNAと結合させ，抗ジゴキシゲニン抗体にて回収する系である。LAMP法は，Hsuらによってゼブラフィッシュの胚を用いて行われた報告[10]を元に，われわれが哺乳類細胞用へ独自に改良した方法である。

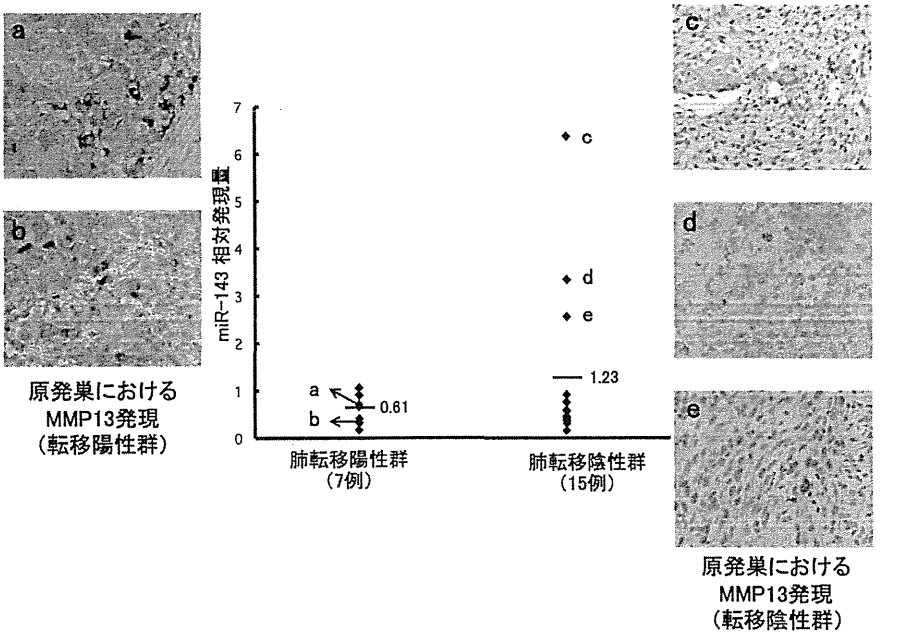
この2種の包括的回収法によって得られたRNAプールをそれぞれマイクロアレイによって解析し，共通する遺伝子として78遺伝子を検出した。さらに，浸潤・転移に関わる遺伝子群を絞り込み，*in vitro*の系において確認したところ，PAI-1（plasminogen activator inhibitor-1）およびMMP13（matrix metalloproteinase 13）が標的遺伝子であることを突き止めた。143B細胞へのmiR-143導入によりPAI-1およびMMP13タンパク発現量が減少することをウエスタンブロッティング法により明らかにするとともに，レポーターアッセイによりmiR-143が両遺伝子のシード配列を直接認識してレポーター活性を抑制することを示した（図❷）。PAI-1のシード配列はmRNAの3'UTRに存在しているが，興味深いことにMMP13のシード配列は3'UTRにはなく，ORF（open reading frame）内に存在することが明らかとなった（杉本ら，未発表データ）。シークエンスデータを元に作成されている各種データベースにおいて，miR-143の標的遺伝子候補にMMP13は含まれておらず，われわれの解析結果は「マイクロRNAの標的分子群の検索および同定において，*in silico*データを十分に活用したうえで，ウェット系の解析により最終的に証明することが重要であること」を強く示唆している。加えて，過去にmiR-143の標的として報告されたK-RASやERK5が少なくとも骨肉腫細胞株143Bにおいて標的遺伝子であることが確認できなかっ

図❷　miR-143標的遺伝子の確認（文献4より）



A. 143B細胞にmiR-143を導入することにより，MMP13およびPAI-1タンパク発現量が減少した。
B. ルシフェラーゼ遺伝子の下流にシード配列を含むORF領域（MMP13）あるいは3'UTR（PAI-1）をつなげたプラスミドを用い，miR-143導入によるレポーター活性を解析した。野生型のシード配列をもつプラスミドを導入した場合，miR-143によるレポーター活性の抑制効果が確認され，シード配列に変異を入れた場合はその活性抑制効果がキャンセルされた（*$P<0.05$）。

図❸ ヒト骨肉腫原発巣におけるmiR-143およびMMP13発現解析（文献4より）



ヒト骨肉腫原発巣22例よりRNAを抽出し，リアルタイムPCR法によりmiR-143発現量を定量した結果，転移陰性群（15例）と比較し転移陽性群（7例）で低値を示した。さらに原発巣におけるMMP13発現を免疫組織化学的に検索した結果，転移陰性群ではMMP13陽性腫瘍細胞が散見されるが，転移陰性群の中でmiR-143高発現を示す症例ではMMP13陽性腫瘍細胞は極めて乏しかった。

（グラビア頁参照）

たことから，特定のマイクロRNAの標的遺伝子は組織や細胞種ごとに異なっている可能性を示しており，今後マイクロRNAの機能解析を進めていくうえで常に考慮すべきポイントであると考えられた。

Ⅳ．ヒト骨肉腫臨床材料を用いた検討

次にわれわれは，ヒト骨肉腫臨床材料（22例の原発巣）におけるmiR-143発現の検証を試みた（図❸）。全例とも初診時において転移は陰性であったが，うち7例については原発巣切除後の治療経過中に肺転移を生じ（転移陽性群），他方15例は術後1～9年間において転移陰性の症例（転移陰性群）であった。両群において，その原発巣におけるmiR-143発現量をリアルタイムPCR法にて定量した結果，前者と比較して後者においてmiR-143発現量が高値を示す傾向にあり，とりわけ転移陰性例のうち3例ではmiR-143発現量が高値を示した。MMP13タンパク発現を免疫組織化学的に解析した結果，miR-143発現量が乏しい転移陽性群ではいずれの症例においてもMMP13陽性腫瘍細胞が散見されたが，転移陰性群のmiR-143高発現例3例はいずれもMMP13陽性腫瘍細胞が検出されないか極めて乏しいことが示された。腫瘍細胞の骨溶解作用にMMP9活性化の関与が知られているが，このMMP9の活性化にはMMP13が直接関わっていることが報告されている[11]。したがって，ヒト骨肉腫原発巣においてmiR-143発現の低下が，その標的遺伝子であるMMP13発現量増加に関与し，おそらくMMP9の活性化を経て浸潤・転移を生じている可能性が示唆された。換言すれば，この浸潤・転移能を有するmiR-143低発現骨肉腫細胞にmiR-143を補充することで，転移関連分子の発現や活性化を阻害し，肺転移を抑制する結果に

なったというメカニズムが推察された。

おわりに

本稿では，miR-143によるヒト骨肉腫細胞の転移抑制効果およびその標的分子群の一部を同定したデータを中心に述べた。転移抑制効果はモデル動物を用いたデータであるが，マイクロRNAを核酸医薬として用いることで，ヒト骨肉腫の肺転移予防に向けた新たなブレイクスルーがもたらされる可能性がある。また，腫瘍細胞の浸潤・転移を制御するマイクロRNAの標的遺伝子群を明らかにすることで，新たな「がん転移特異的な分子標的」を見出すことが期待できる。

本研究成果は，落谷孝広博士，竹下文隆博士，小坂展慶博士（国立がん研究センター研究所）との共同研究である。また，臨床材料をご提供いただいた川井章博士，小林英介博士（国立がん研究センター中央病院整形外科）に深謝いたします。

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New Technology

界面活性剤様ペプチドA6Kを担体としたsiRNAによるがん治療

A6K, a synthetic surfactant-like peptide for siRNA-based cancer therapy

松田　範昭

Key Words：siRNA, peptide surfactant, DDS

Abstract

界面活性剤様ペプチドA6KはsiRNAと安定なコンプレックスを形成し，siRNAにリボヌクレアーゼへの強い耐性を生じさせる。コンプレックスを生体内に局所投与したところ，siRNAは分解されにくく，1週間以上に渡って遺伝子発現の抑制効果を示した。現在，A6Kを担体としたsiRNAによるがん治療に向けて，国立がん研究センターによりトリプルネガティブ乳がん患者の疾患制御遺伝子を対象とした臨床適用を目指しているところである。

界面活性剤様ペプチドA6K

マサチューセッツ工科大学（MIT）で開発された界面活性剤様ペプチドA6Kは，疎水性アミノ酸であるアラニン（A）6分子と塩基性アミノ酸であるリシン（K）1分子からなり，N末端がアセチル化，C末端がアミド化されたものである[1]。水溶液中では，C末端のリシンの側鎖が親水性で正に荷電していることにより界面活性剤様の性質を示し，疎水性部分同士が会合し，自己組織化して直径30nm程度のチューブ状の形状をとる（図1a）[2]。A6KとsiRNAを混合することにより両者は安定なコンプレックスを形成するが（図1b），負電荷のsiRNAは正電荷のA6Kチューブの外側および内側表面にイオン的に吸着しているものと考えられる。A6Kは本稿で述べるsiRNAの担体のほか，種々のタンパク質の徐放化剤としての可能性が見出されている。さらに，膜タンパク質の可溶化剤，葉緑体太陽電池の担体など，幅広い応用開発が進行中である。

siRNA/A6Kコンプレックスの生体内安定性

国立がん研究センター研究所の落谷らにより，

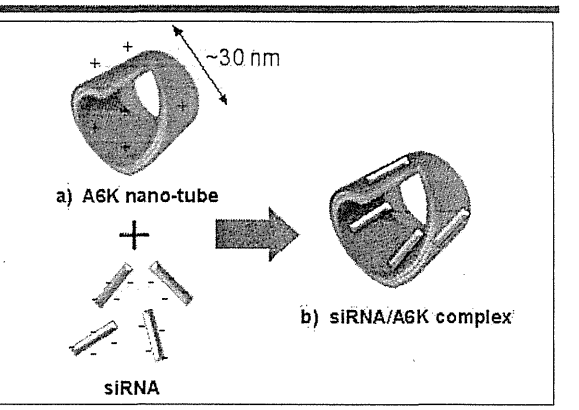

図1　界面活性剤様ペプチドA6Kの自己組織化とsiRNAとのコンプレックス形成

siRNAはA6Kとコンプレックスを形成することで，リボヌクレアーゼ（RNase）に強い耐性を持つことが*in vitro*および*in vivo*において確認されている。*in vitro*では，siRNA（Luciferase GL3 duplex）の単独およびA6Kと混合したものについてRNase Cocktail Enzyme Mixとともに37度でインキュベーションしたところ，siRNA単独では30分程度で分解されるものが，コンプレックスにおいては長時間ほとんど分解されないことが示されている。

また，*in vivo*での検証においては，ルシフェラーゼ発現前立腺腫瘍細胞（PC-3M-luc-C6）をマウス皮下に移植したモデルに，ルシフェラーゼ遺伝子を認識するGL3 siRNAとA6Kのコンプレックスを腫瘍周辺部に局所投与した。図2はGL3 siRNAの投与後のルシフェラーゼ発光強度の経時変化を示したものである。コントロールとしてA6Kのみを投与したものでは，腫瘍の増殖とともに発光強度が増大していくが，GL3 siRNA/A6Kコンプレックス投与群ではルシフェラーゼ発光が抑制された。特筆すべきは，Day 0において1回のみコンプレックスを局所投与したにもかかわらず，その抑制効

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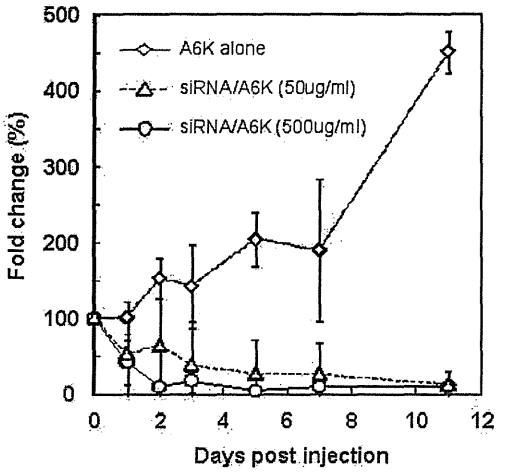

図2　PC-3M-luc-C6皮下腫瘍形成マウスへのGL3 siRNA/A6Kコンプレックスの局所投与によるルシフェラーゼ発光抑制の経時変化

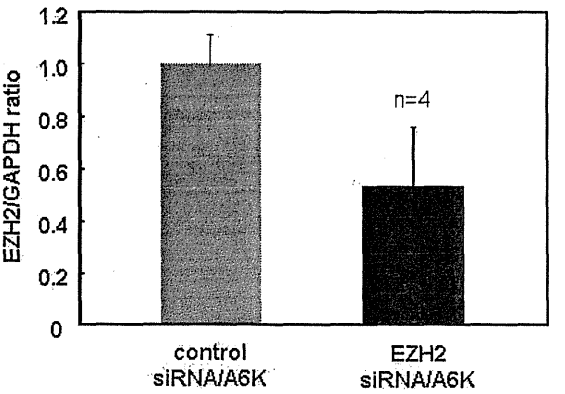

図3　PC-3M-luc-C6皮下腫瘍形成マウスへのEZH2 siRNA/A6Kコンプレックスの局所投与によるノックダウン効果

果が1週間以上に渡って継続したことである。これは，siRNAがA6Kとコンプレックスを形成することでRNaseからの分解に抵抗し，体内で長期間機能を保つことができること，また，siRNA/A6Kコンプレックスは若干の粘稠性をもつため，局所投与した位置に留まりやすく，siRNAが徐放されることで持続的に効果を発揮し続けるためであると考える。

■siRNA/A6Kコンプレックスによる腫瘍抑制

落谷らにより，マウス皮下腫瘍移植モデルへsiRNA/A6Kコンプレックスを局所投与したときにおける腫瘍抑制効果が検証された。エピジェネティックな遺伝子発現調節に関与するEZH2遺伝子をターゲットとし[3]，EZH2 siRNA（25ug）とA6Kのコンプレックスを4日おきに3回腫瘍周辺部に局所投与した。その結果，ネガティブコントロールsiRNA/A6Kコンプレックスを投与したものと比較して，腫瘍が有意に抑制された。また，図3はEZH2 siRNA/A6Kコンプレックスを3日間連続投与したときのEZH2 mRNAの定量結果を示すが，3日目に約50%のEZH2 mRNAのノックダウン効果が認められた。

■がん治療の臨床応用に向けて

siRNA/A6Kコンプレックスを用いたがん治療への臨床応用を目標に，ターゲット遺伝子として抗がん剤に対する耐性を担い[4]，さらにがん幹細胞の制御に関係するとされる[5] ribophorin II (RPN2)を選択して，国立がん研究センターと弊社にて共同研究を進めている。現在，厚生労働科学研究費補助金の支援を受けて非臨床試験を実施中であり，コンプレックスの全身投与による免疫惹起や肝機能への影響が見られないこと，イヌの自然発症乳がんへの局所投与による有効性を確認している。通常の薬物療法が効を奏しにくいトリプルネガティブ乳がんを対象疾患として，国立がん研究センター中央病院にてファーストインマン医師主導治験の実施を目指しているところである。

■謝辞

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