A comprehensive search for microRNAs with expression profiles modulated by oncogenic KRAS: Potential involvement of miR-31 in lung carcinogenesis

KOJI OKUDELA[1], TAKESHISA SUZUKI[1], SHIGEAKI UMEDA[1], YOKO TATEISHI[1], HIDEAKI MITSUI[1], YOHEI MIYAGI[2] and KENICHI OHASHI[1]

[1]Department of Pathology, Yokohama City University, Graduate School of Medicine, Kanazawa-Ku, Yokohama 236-0004; [2]Clinical Research Institute, Kanagawa Prefectural Cancer Center Hospital, Asahi-ku, Yokohama 241-8515, Japan

Received February 17, 2014; Accepted May 13, 2014

DOI: 10.3892/or.2014.3339

Abstract. Small non-protein coding RNAs that regulate messenger RNA levels, namely microRNAs (miRNAs), have been implicated in the pathogenesis of various diseases. The purpose of the present study was to identify essential miRNAs involved in lung carcinogenesis. Previous studies demonstrated that an investigation into the downstream targets of oncogenic KRAS could be used as a strategy to elucidate the molecular mechanisms involved in lung cancer; therefore, we examined the expression profiles of mRNAs modulated by oncogenic KRAS in the present study. We focused on miR-31 from the miRNAs that were differentially expressed, and evaluated its potential role in the development of lung cancer. miR-31 was upregulated not only by oncogenic KRAS, but also by oncogenic EGFR. The expression of miR-31 was markedly attenuated in some lung cancer cell lines by deleting its host gene locus. The restoration of miR-31 in lung cancer cell lines that lost its expression attenuated their growth activities. The knockdown of miR-31 expression in lung cancer cell lines retaining its expression enhanced anchorage-independent growth activity. These results suggest that miR-31 may be a suppressor that regulates an essential oncogenic pathway, the loss of which may promote lung carcinogenesis.

Introduction

Lung cancer is one of the most common causes of cancer-related mortality in the developed world (1,2). Recurrence has been reported in a large proportion of lung cancer patients in spite of successful resection of the primary tumor (1,2). Although some lung tumors are known to be sensitive to conventional chemotherapeutic agents or certain molecular targeting agents, many are not (3,4). Thus, further understanding of the molecular mechanisms involved in lung carcinogenesis is essential for developing novel therapeutic strategies.

Our previous studies, which involved a comprehensive search for the downstream targets of oncogenic *KRAS*, identified important molecules involved in lung carcinogenesis (3,5). Mutations in driver oncogenes, such as *KRAS*, *EGFR*, *BRAF*, and *ALK*, were found to mutually and exclusively occur, and each of these mutations has been shown to transmit the common essential oncogenic signal that promotes carcinogenesis (6-8). Therefore, the downstream targets of oncogenic *KRAS* participate not only in *KRAS*-mediated carcinogenesis, but also in other oncogene-mediated carcinogeneses (3,5). Thus, an investigation into the downstream targets of oncogenic KRAS is considered to be an available strategy that can identify common important molecular mechanisms involved in lung cancer.

Small non-protein coding RNAs that regulate messenger RNA levels, namely microRNAs (miRNAs), have been identified and subsequently implicated in the pathogenesis of various diseases. The present study investigated the expression profiles of miRNAs modulated by oncogenic KRAS in airway epithelial cells. We focused on miR-31 from the miRNAs that were differentially expressed, and evaluated its potential role in the development of lung cancer.

Materials and methods

Cell lines and culture. An immortalized human airway epithelial cell line [16HBE14o, simian virus 40 (SV40)-transformed human bronchial epithelial cells] described by Cozens *et al* (9) was kindly provided by D.C. Grunert (California Pacific Medical Center Research Institute). A sub-clone of 16HBE14o cells, described as NHBE-T in this study, was used. Human lung cancer cell lines (A549, H358, H2087, H23, EKVX, H226, H827, H1819, H441, H4006, HOP62, H1299 and H460) and a human embryonic kidney cell line (HEK293T) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human lung cancer cell line LC2/ad was purchased from the Riken Cell Bank (Tsukuba, Japan). The human lung cancer cell lines, PC1, PC9 and HARA were

Correspondence to: Dr Koji Okudela, Department of Pathology, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
E-mail: kojixok@med.yokohama-cu.ac.jp

Key words: miRNA-31, KRAS, lung cancer, carcinogenesis

from Immuno-Biological Laboratories Co. (Gunma, Japan). The human lung cancer cell lines, TKB1, TKB2, TKB4, TKB5, TKB6, TKB7, TKB8, TKB14 and TKB20 were obtained from Dr Hiroshi Kamma via Dr Takuya Yazawa (Kyorin University School of Medicine) (10). Primary small airway epithelial cells (SAEC) were purchased from Sanko Kagaku (Tokyo, Japan).

Plasmid construction. The pro-retrovirus vector bearing KRAS [wild-type (WLD), V12, H61, V12/S35, V12/G37 and V12/C40 mutants] (11) and PIK3CA (WLD, E545K mutant, and H1047R mutant) were previously described (12). Complementary DNA (cDNA) coding EGFR (NM_001964) and BRAF (NM_004333) was PCR-amplified using cDNA from NHBE-T cells as a template and inserted into the pro-retrovirus vector pQCXIP (BD Clontech, Palo Alto, CA, USA). Mutant EGFR (deletion: E745-A750) was PCR-amplified using cDNA from the PC9 lung cancer cell line. EGFR (L858R mutant) and BRAF (V600E mutant) were generated by the method of site-directed mutagenesis. DNA fragments including the miR-31 coding region (MIMAT0000089) flanking approximately a 100 base-pair margin in both directions was PCR-amplified and inserted into the pro-retrovirus vector pLHCX (BD Clontech). Vectors bearing a sense and antisense strand of cDNA were obtained. A pNsi-based miR-31 blocking vector was purchased from Takara Bio Inc. (Kyoto, Japan).

Retroviral-mediated gene transfer. The pro-retrovirus vectors bearing the desired constructs and the pLC10A1 retrovirus-packaging vector (Imgenex, San Diego, CA, USA) were cotransfected into HEK293T cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after the transfection, conditioned medium was recovered as a viral solution. The desired genes were introduced by incubating cells with the viral solution containing 10 μg/ml of polybren (Sigma, St. Louis, MO, USA). Cells stably expressing the desired genes were selected with 250 μg/ml of hygromycin B or 1,000 μg/ml of neomycin (both from Invitrogen). The pooled clones were used for analyses as follows.

miRNA expression profiling. miRNA expression profiles in empty vector (MOCK)-, KRAS/WLD-, KRAS/V12-, and KRAS/H61-vector transduced NHBE-T cells were comprehensively evaluated with human miRNA microarray (Human miRNA Microarray kit release 16.0, 8x60K, cat. no. G4870A; Agilent Technologies, Santa Clara, CA, USA). Total RNA was extracted from the cells immediately after completing the selection (5 days post-transduction) using the RNA easy kit (Qiagen, Hilden, Germany). Labeling and hybridization was performed according to the manufacturer's recommendations (Agilent Technologies).

Colony formation assays. Cells (appropriate count of $1.0x10^4$ to $5.0x10^4$) were seeded onto a 10 cm culture dish (Iwaki, Tokyo, Japan), and grown for 10 days. Cells were fixed with methanol and Giemsa-stained, and colonies visible in scanned images were counted.

Growth curve assays. Cells ($2.5x10^5$) were seeded onto a 10 cm culture dish, and were grown in DMEM (Sigma) with 10 FBS (Sigma) to a semi-confluent state for 5-7 days. Cells were counted, and $2.5x10^5$ cells were seeded again onto a 10 cm dish. Several passages were repeated in the same manner. The sum of population doublings (PDLs) at each point was calculated by the formula $SPDL_n = \log_2 (count_n/2.5x10^5) + SPDL_{n-1}$.

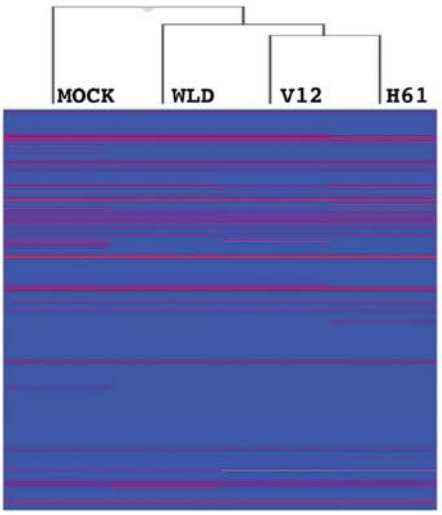

Figure 1. Empty vector (MOCK)-, wild-type KRAS/WLD, oncogenic KRAS/V12, and oncogenic KRAS/H61 were transduced into NHBE-T cells. miRNA expression in the transfectants was comprehensively evaluated with a gene chip microarray. A hierarchical clustering analysis (Ward's method) was performed and a dendrogram was described.

Soft agar colony formation assays. Cells ($1.25x10^4$) were cultured and grown in 1 ml of DMEM-based 0.3% agar (agar noble; Becton-Dickinson, Sparks, MD, USA) containing 10% FBS in 3.5 cm culture dishes (Iwaki) for 4 weeks. The agars were fixed with a buffered 4% paraformaldehyde solution, and colonies visible in scanned images were counted.

Treatment with an inhibitor for MEK and PI3K. Following infection of the retrovirus vector, cells stably expressing KRAS were selected for 3 days, and were then harvested. Cells were treated with an inhibitor for MEK (PD98059, 50 μM) or PI3K (LY294002, 50 μM) [both from Cell Signaling Technology (CST), Danvers, MA, USA] and their combination for the last 24 h before the harvest.

Comprehensive search for the downstream target of miR-31. Gene expression in the empty vector (MOCK)-, miR-31 sense strand (SS)-, and miR-31 antisense (AS) strand-transduced NHBE-T cells was comprehensively evaluated with a human gene chip microarray (SurePrint G3 Human Gene Expression 8x60K v2 Microarray kit, cat. no. G4851B; Agilent Technologies). Total RNA was extracted from the cells immediately after completing the selection (5 days post-transduction) using the RNA easy kit (Qiagen). Labeling and hybridization were performed according to the manufacturer's recommendations (Agilent Technologies). Transcripts whose signal values in the miR-31 SS-transduced cells were 5-fold higher or lower than that in both the empty vector-transduced cells and the miR-31 antisense strand-transduced cells were extracted according to the same flow chart as described in a previous study (13).

Table I. MicroRNAs differentially expressed by the oncogenic KRAS.

Name	Accession	MOCK	KRAS/WLD	KRAS/V12	KRAS/H61
Upregulated					
hsa-miR-1238	MIMAT0005593	4.53	8.45	11.49	9.58
hsa-miR-31	MIMAT0000089	6.40	5.96	16.53	12.68
hsa-miR-191*	MIMAT0001618	5.27	9.33	11.24	9.82
hsa-let-7i	MIMAT0000415	109.44	98.74	224.70	164.02
hsa-miR-31*	MIMAT0004504	15.01	13.44	22.80	19.14
hsa-miR-29b*	MIMAT0004514	2.70	2.66	7.31	3.25
hsa-miR-29b	MIMAT0000100	95.87	66.36	141.79	100.44
hsa-miR-625	MIMAT0003294	4.70	4.03	5.85	4.74
hsa-miR-940	MIMAT0004983	12.07	11.92	15.11	12.11
Downregulated					
hsa-miR-30c	MIMAT0000244	16.95	12.52	12.13	9.82
hsa-miR-15b	MIMAT0000417	208.10	154.26	148.56	102.32
hsa-miR-28-5p	MIMAT0000085	5.66	4.09	3.99	3.60
hsa-miR-30a*	MIMAT0000088	8.71	6.70	5.73	4.91
hsa-miR-23b	MIMAT0000418	8.93	6.36	5.87	4.78
hsa-miR-130a	MIMAT0000425	55.99	39.19	36.31	26.76
hsa-miR-210	MIMAT0000267	41.26	28.93	26.16	22.94
hsa-miR-16	MIMAT0000069	157.08	110.57	96.19	73.35
hsa-miR-30e	MIMAT0000692	6.70	4.47	4.03	3.68
hsa-miR-196a	MIMAT0000226	27.48	19.75	15.85	11.31
hsa-miR-4286	MIMAT0016916	264.56	205.35	150.73	138.68
hsa-miR-15a	MIMAT0000068	57.49	40.25	31.70	29.54
hsa-let-7b	MIMAT0000063	104.48	76.06	54.29	39.47
hsa-miR-205	MIMAT0000266	116.34	74.88	59.41	50.10
hsa-miR-34a	MIMAT0000255	36.32	24.83	16.71	19.56
hsa-miR-1246	MIMAT0005898	52.63	36.00	23.66	21.83
hsa-miR-27b	MIMAT0000419	12.85	8.09	5.49	4.76
hsa-miR-429	MIMAT0001536	11.46	7.44	4.71	3.73

Normalized expression levels in transfectants are shown. Accession, gene bank accession no.; WLD, wild-type.

Results

miRNA expression profiling modulated by oncogenic KRAS. A comprehensive evaluation of the expression of miRNA revealed that oncogenic KRAS-transduced cells had different expression profiles from empty vector- and WLD KRAS-transduced cells, as KRAS/V12- and KRAS/H61-transduced cells were classified into a distant branch from the others on a dendrogram described by hierarchical clustering analysis (Fig. 1). The miRNAs that had higher or lower levels in oncogenic KRAS (KRAS/V12 and KRAS/H61)-transduced cells than in both mock (MOCK)- and WLD KRAS (KRAS/WLD)-transduced cells are listed in Table I. Quantitative RT-PCR analysis confirmed that the transduction of oncogenic KRAS induced the largest change in let-7i and miR-31 (Fig. 2). Therefore, we focused on these two miRNAs.

Let-7i and miR-31 expression in lung cancer cell lines. Let-7i and miR-31 expression levels were analyzed by quantitative RT-PCR analysis. Let-7i was expressed at various levels in all the cells examined. Let-7i levels were higher in cancer cell lines than in non-cancerous immortalized cell lines and primary airway epithelial cells (Fig. 3). In contrast, miR-31 expression levels were lower in cancer cell lines, and some cell lines [27.5%, 11/40, the loss of miR-31 expression was frequently detected in LCC cell lines (80.0%, 4/5)] completely lost its expression (Fig. 2). These results prompted us to further investigate the potential significance of the loss of miR-31 expression in lung carcinogenesis.

Effect of the restoration and knockdown of miR-31 on cell growth. The restoration of the miR-31 SS, but not the antisense strand, markedly suppressed the growth of cancer cell lines that lost miR-31 expression, as it decreased the formation of

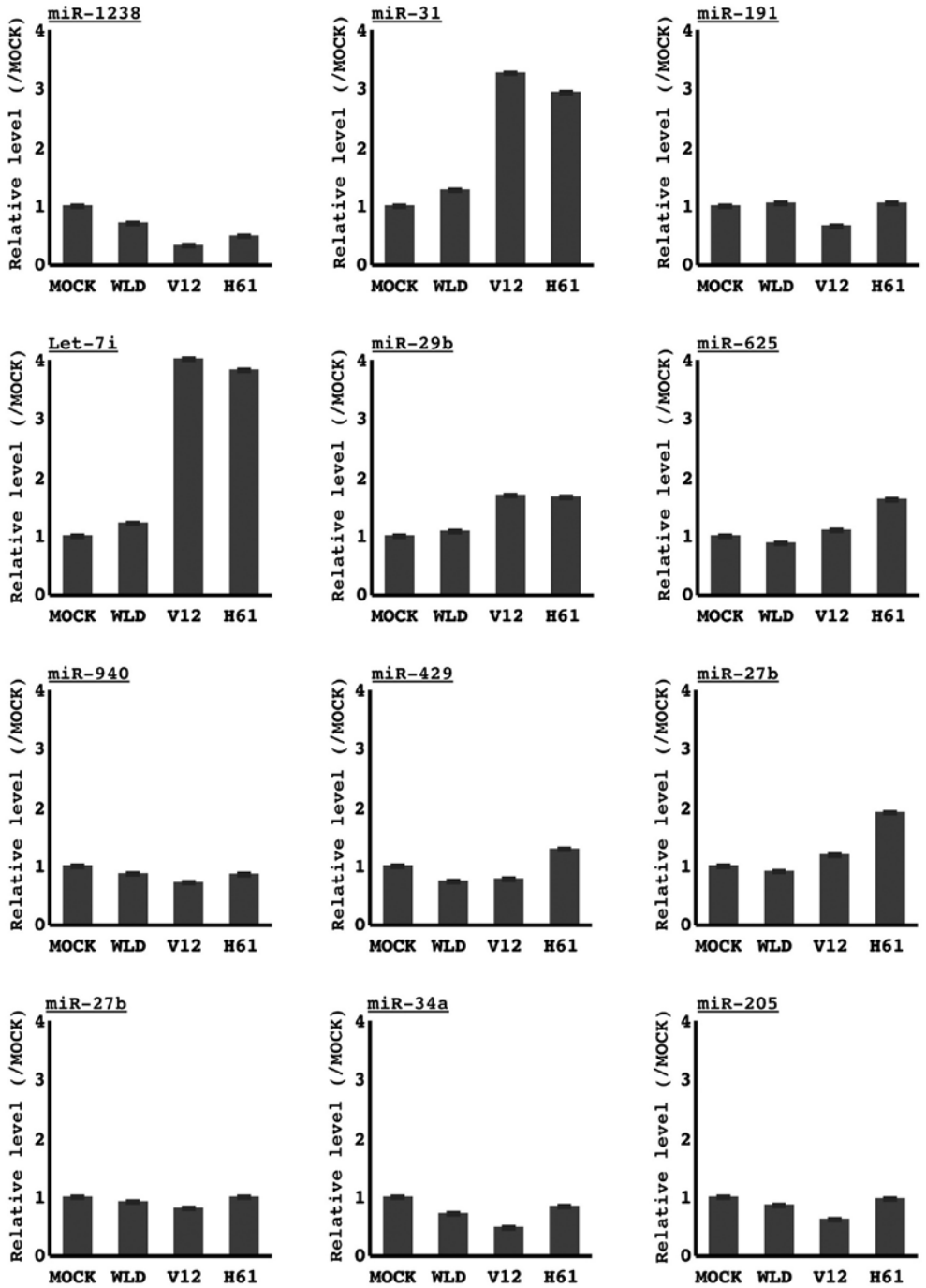

Figure 2. Level of miRNA whose expression is modulated by oncogenic KRAS. The copy number of the targeted miRNA and U6 snRNA was measured by quantitative RT-PCR. The level of miRNA normalized to that of U6 snRNA is presented. MOCK, empty vector-transduced NHBE-T; WLD, wild-type KRAS-transduced NHBE-T; V12, oncogenic mutant of KRAS/V12-transduced NHBE-T; H61, oncogenic mutant of KRAS/H61-transduced NHBE-T.

colonies and prolonged the doubling time (Fig. 4, TKB1 and H441 cells were examined. A representative result in TKB1 cells is shown). On the other hand, the knockdown of miR-31 expression in cell lines that retained its expression resulted in an enhancement in their growth activities in both ordinary media and soft agar (Fig. 5, HARA and A549 cells were examined. A representative result in HARA cells is shown).

Comprehensive search for potential genes modulated by miR-31. A comprehensive evaluation of gene expression profiles revealed that miR-31 SS-transduced cells had a different expression profile from empty vector (mock)- and miR-31 antisense strand (AS)-transduced cells, as miR-31SS-transduced cells were classified into the most distant branch on a dendrogram described from a hierarchical clustering analysis (Fig. 6). Transcripts with expression levels that were >5-fold higher in miR-31SS-transduced cells than in mock- and miR-31AS-transduced cells were extracted (Tables II and III).

Involvement of different oncogenic pathways in the induction of miR-31. The expression of miR-31 was also induced by the transduction of oncogenic EGFR [deletion mutation (deletion:

Table II. Upregulated transcripts.

		MOCK		miR-31SS		miR-31AS			
Symbol	Accession	Signal	Flag	Signal	Flag	Signal	Flag	miR-31SS/MOCK	miR-31SS/AS
NUDT10	NM_153183	0.0119	A	1.1978	P	0.0124	A	100.8360	96.2198
RIPK3	NM_006871	0.0114	A	1.0102	P	0.0121	A	88.4318	83.3022
C2CD4B	NM_001007595	0.0245	A	0.5805	P	0.0115	A	23.6809	50.5356
SERPINI1	NM_005025	0.0112	A	0.4145	P	0.0116	A	37.0894	35.6275
KRTAP10-11	NM_198692	0.0123	A	0.4065	P	0.0116	A	33.1742	35.1714
SLC24A4	NM_153647	0.0357	A	1.0444	P	0.0314	A	29.2833	33.2618
CCDC140	NM_153038	0.0110	A	0.3725	P	0.0115	A	33.9644	32.4076
PLA2R1	NM_007366	0.0103	A	0.2964	P	0.0106	A	28.7619	27.8916
HTR3D	NM_182537	0.0106	A	0.3012	P	0.0111	A	28.3353	27.0245
CXCR2P1	NR_002712	0.0128	A	0.2771	P	0.0103	A	21.7125	27.0160
STEAP2	NM_001244945	0.0099	A	0.2577	P	0.0102	A	25.9085	25.1678
GSG1L	NM_001109763	0.0148	A	0.2657	P	0.0108	A	17.9319	24.5156
PLAC9	NM_001012973	0.0111	A	0.2532	P	0.0109	A	22.7315	23.2710
MPPED2	NM_001584	0.0119	A	0.2784	P	0.0124	A	23.4476	22.5310
NKX2-8	NM_014360	0.0322	A	0.3877	P	0.0179	A	12.0409	21.6246
ZMAT1	NM_001011657	0.0112	A	0.2303	P	0.0116	A	20.6436	19.8318
CYYR1	NM_052954.2	0.0255	A	0.2456	P	0.0128	A	9.6355	19.2358
MYL1	NM_079420	0.0095	A	0.1850	P	0.0098	A	19.3932	18.7914
ENTPD1	NM_001776	0.0106	A	0.2072	P	0.0111	A	19.5553	18.6842
OTOL1	NM_001080440	0.0111	A	0.2118	P	0.0115	A	19.1511	18.4960
BPIFB4	NM_182519	0.0106	A	0.2031	P	0.0111	A	19.1675	18.3275
STARD6	NM_139171	0.0104	A	0.1980	P	0.0109	A	18.9708	18.2463
FAM70A	NM_017938	0.0100	A	0.1856	P	0.0103	A	18.6179	18.0836
SLC34A2	NM_006424	0.0117	A	0.2084	P	0.0121	A	17.8522	17.1757
TCL6	NR_028288	0.0119	A	0.2186	P	0.0128	A	18.3689	17.0699
TMEM64	NM_001008495	0.0401	A	0.4789	P	0.0290	A	11.9343	16.5116
SLC25A21	NM_030631	0.0109	A	0.1860	P	0.0113	A	17.0261	16.4283
FABP7	NM_001446	0.0139	A	0.1728	P	0.0107	A	12.4500	16.2039
ADAMTSL2	NM_014694	0.0249	A	0.1737	P	0.0110	A	6.9690	15.7766
OR2W5	NM_001004698	0.0112	A	0.1846	P	0.0117	A	16.4571	15.7667
MLANA	NM_005511	0.0094	A	0.1372	P	0.0098	A	14.5261	13.9842
LDLRAD2	NM_001013693	0.0119	A	0.1733	P	0.0124	A	14.6248	13.9382
OR5K1	NM_001004736	0.0095	A	0.1334	P	0.0098	A	14.0985	13.5742
TMPRSS4	NM_019894	0.0283	A	0.1621	P	0.0122	A	5.7287	13.2791
GLT25D2	NM_015101	0.0105	A	0.1410	P	0.0109	A	13.4839	12.8923
FMN2	NM_020066	0.0088	A	0.1484	P	0.0120	A	16.7833	12.4044
SLC3A1	NM_000341	0.0154	A	0.2346	P	0.0207	A	15.2038	11.3567
AMICA1	NM_153206	0.0185	A	0.2714	P	0.0247	A	14.6324	10.9989
FAM186B	NM_032130	0.0919	P	1.1598	P	0.1056	P	12.6267	10.9792
SNORD115-2	NR_003294	0.0116	A	0.1296	P	0.0123	A	11.1625	10.5393
PAX5	NM_016734	0.0110	A	0.1198	P	0.0114	A	10.9324	10.5101
MAGEC2	NM_016249	0.0137	A	0.1294	P	0.0125	A	9.4181	10.3264
IPW	NR_023915	0.0221	A	0.3631	P	0.0353	A	16.3945	10.2860
TNFSF11	NM_033012	0.0107	A	0.1121	P	0.0111	A	10.5095	10.0805
SIGLECP3	NR_002804	0.0627	P	0.3595	P	0.0359	A	5.7295	10.0019
WDR49	AK097556	0.0096	A	0.0992	P	0.0099	A	10.3910	9.9812
SATL1	NM_001012980	0.0120	A	0.1243	P	0.0127	A	10.3606	9.7853

Table II. Continued.

		MOCK		miR-31SS		miR-31AS			
Symbol	Accession	Signal	Flag	Signal	Flag	Signal	Flag	miR-31SS/MOCK	miR-31SS/AS
SLC19A3	NM_025243	0.0121	A	0.1239	P	0.0127	A	10.2372	9.7655
DPP6	BC035912	0.0249	A	0.1543	P	0.0160	A	6.2027	9.6431
OR5H2	NM_001005482	0.0156	A	0.1053	P	0.0109	A	6.7301	9.6315
CIB4	NM_001029881	0.0117	A	0.1166	P	0.0122	A	9.9576	9.5572
WDFY4	NM_020945	0.0122	A	0.1211	P	0.0128	A	9.9557	9.4762
SAMD13	NM_001010971	0.0105	A	0.1029	P	0.0109	A	9.8322	9.4019
NBPF6	NM_001143988	0.0113	A	0.1090	P	0.0117	A	9.6855	9.2810
FAM135B	NM_015912	0.0097	A	0.0922	P	0.0100	A	9.4969	9.2206
OR4A47	NM_001005512	0.0105	A	0.1659	P	0.0181	A	15.8434	9.1669
CCL22	NM_002990	0.0117	A	0.1145	P	0.0126	A	9.7614	9.0799
SLC5A4	NM_014227	0.0105	A	0.0987	P	0.0109	A	9.4252	9.0167
SH3TC1	NM_018986	0.0838	P	0.6744	P	0.0752	P	8.0521	8.9695
MYH7	NM_000257	0.0119	A	0.1112	P	0.0124	A	9.3740	8.9612
TXLNG2P	NR_045129	0.0111	A	0.1045	P	0.0117	A	9.4000	8.9166
NUBPL	NM_025152	0.0104	A	0.0950	P	0.0109	A	9.0971	8.7016
CNGA3	NM_001298	0.0094	A	0.1537	P	0.0177	A	16.2720	8.6960
EGR4	NM_001965	0.0105	A	0.0938	P	0.0109	A	8.9198	8.6280
IL24	NM_001185156	1.4258	P	9.1516	P	1.1788	P	6.4187	7.7634
RXFP1	NM_021634	0.0111	A	0.0888	P	0.0115	A	8.0062	7.7382
CCDC85C	NM_001144995	0.0116	A	0.1157	P	0.0154	A	9.9729	7.4922
TRIM36	NM_001017397	0.0108	A	0.1024	P	0.0137	A	9.4777	7.4839
TACC1	BC041391	0.0128	A	0.1323	P	0.0177	A	10.3064	7.4633
FAM24B	NM_152644	0.0140	A	0.0911	P	0.0126	A	6.5027	7.2256
ITGAL	NM_002209	0.0668	P	0.4498	P	0.0641	P	6.7361	7.0141
GMCL1P1	NR_003281	0.0104	A	0.3532	P	0.0506	A	33.9062	6.9811
IL24	NM_001185156	10.1283	P	66.0420	P	9.7012	P	6.5205	6.8076
PTPN20A	NM_001042387	0.0097	A	0.0671	P	0.0101	A	6.9039	6.6542
SRPX	NM_006307	0.0090	A	0.0602	P	0.0093	A	6.6622	6.4412
RORC	NM_005060	0.0096	A	0.0797	P	0.0124	A	8.3190	6.4372
HDAC11	NM_001136041	0.0688	P	0.5473	P	0.0856	P	7.9591	6.3925
CRHBP	NM_001882	0.0094	A	0.0623	P	0.0098	A	6.6587	6.3675
SPTSSB	NM_001040100	0.0111	A	0.0738	P	0.0117	A	6.6527	6.3147
SERPINB11	NM_080475	0.0110	A	0.1245	P	0.0201	A	11.2800	6.2069
CC2D2B	XM_003403528	0.0102	A	0.0656	P	0.0106	A	6.4287	6.2016
DNAH17	NM_173628	0.0097	A	0.1610	P	0.0263	A	16.6227	6.1282
DUOXA2	BX537581	0.0087	A	0.0552	P	0.0090	A	6.3524	6.1045
SLC35G3	NM_152462	0.0088	A	0.0557	P	0.0092	A	6.3135	6.0811
SLC17A4	NM_005495	0.0105	A	0.0667	P	0.0110	A	6.3493	6.0686
MEOX2	NM_005924	0.0118	A	0.0726	P	0.0123	A	6.1790	5.8922
CLDN8	NM_199328	0.0106	A	0.0630	P	0.0109	A	5.9634	5.7753
TMEM108	NM_023943	0.0179	A	0.2513	P	0.0440	A	13.9997	5.7122
DPP6	NM_001039350	0.0124	A	0.2982	P	0.0533	A	23.9789	5.5933
FAM164A	NM_016010	0.0103	A	0.0598	P	0.0107	A	5.8098	5.5618
GSTTP1	NR_003081	0.0118	A	0.0823	P	0.0148	A	6.9710	5.5445
OR4F6	NM_001005326	0.0118	A	0.0611	P	0.0113	A	5.1841	5.4305
HEATR7B1	XM_001721240	0.0153	A	0.1492	P	0.0277	A	9.7300	5.3768
DPYS	NM_001385	0.0100	A	0.0554	P	0.0104	A	5.5306	5.3504

Table II. Continued.

Symbol	Accession	MOCK		miR-31SS		miR-31AS		miR-31SS/MOCK	miR-31SS/AS
		Signal	Flag	Signal	Flag	Signal	Flag		
OR10Z1	NM_001004478	0.0121	A	0.0661	P	0.0127	A	5.4614	5.2210
PARP10	NM_032789	0.1746	P	1.1198	P	0.2158	P	6.4149	5.1885
PIEZO2	AK092226	0.0088	A	0.0466	P	0.0091	A	5.2934	5.0979
ACCN5	NM_017419	0.0090	A	0.0478	P	0.0094	A	5.2910	5.0893
CAPS2	AF251056	0.0105	A	0.0636	P	0.0126	A	6.0511	5.0303
RBPMS	AK057533	0.0124	A	0.0900	P	0.0180	A	7.2453	5.0094

MOCK, empty vector-transduced; miR-31SS, miR-31 sense strand-transduced; miR-31 antisense strand-tranduced NHBE-T cells; accession, gene bank accession no.; flag indicates whether gene expression was present (P) or absent (A).

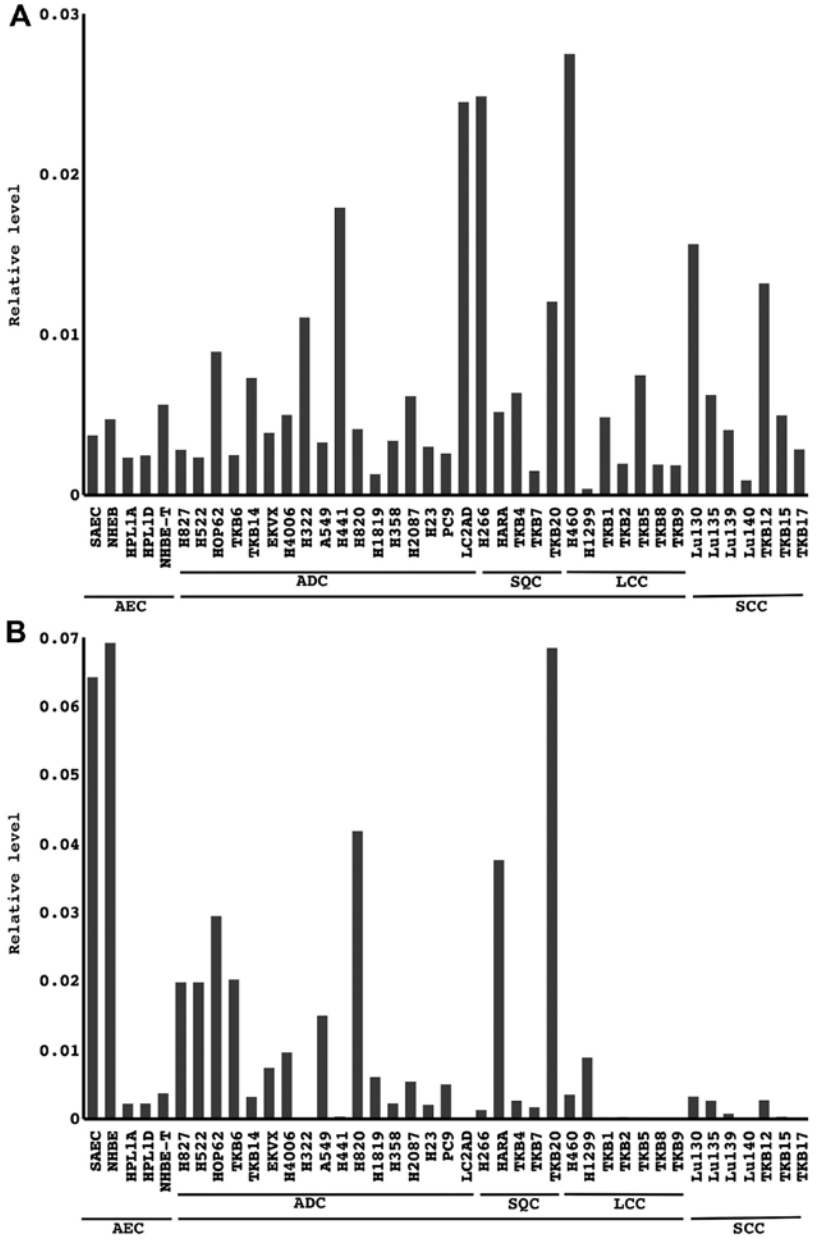

Figure 3. Level of let-7i and miR-31 in non-cancerous and cancer cell lines. The copy number of let-7i, miR-31 and U6 snRNA was measured by quantitative RT-PCR. The level of (A) let-7i or (B) miR-31 normalized to that of U6 snRNA is presented. AEC, airway epithelial cells (non-cancerous cells); ADC, adenocarcinoma cell lines; SQC, squamous cell carcinoma cell lines; LCC, large cell carcinoma cell lines; SCC, small cell carcinoma cell lines.

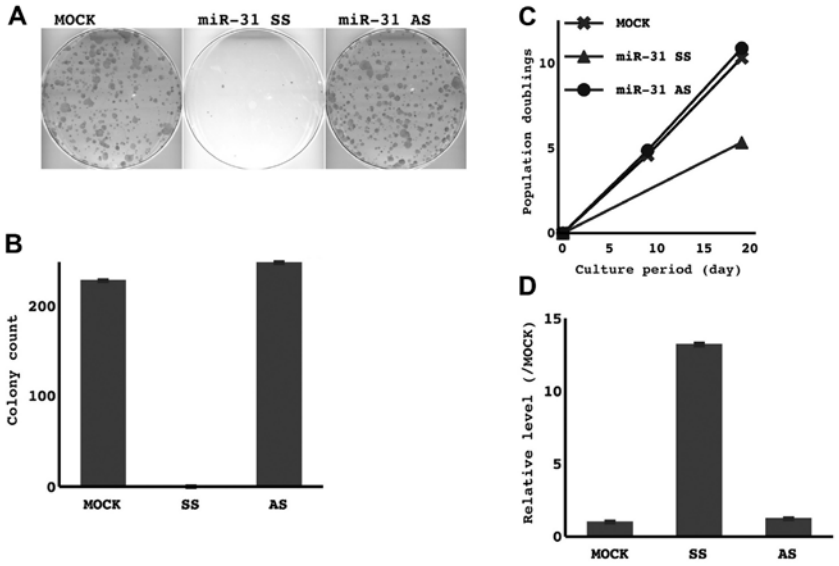

Figure 4. Biological effect of the restoration of miR-31 in lung cancers losing its expression. The representative results from the examination of TKB1 cells are presented. Empty vector (MOCK), the sense strand of miR-31 (SS), and the antisense strand of miR-31 (AS) were transduced. Following a brief selection, the surviving cells were harvested and counted, and $2.0x10^4$ were re-seeded onto a 10 cm dish. (A) After 14 days, the cells were methanol-fixed and Giemsa-stained. (B) The means and standard deviations (error bars) of colony counts from triplicate experiments are presented. (C) Cells selected were grown and passed several times. Cumulated population doublings are presented. Cells harvested immediately after the selection process were examined for the expression of miR-31 and U6 snRNA by quantitative RT-PCR. (D) The level of miR-31 normalized to that of U6 snRNA is presented.

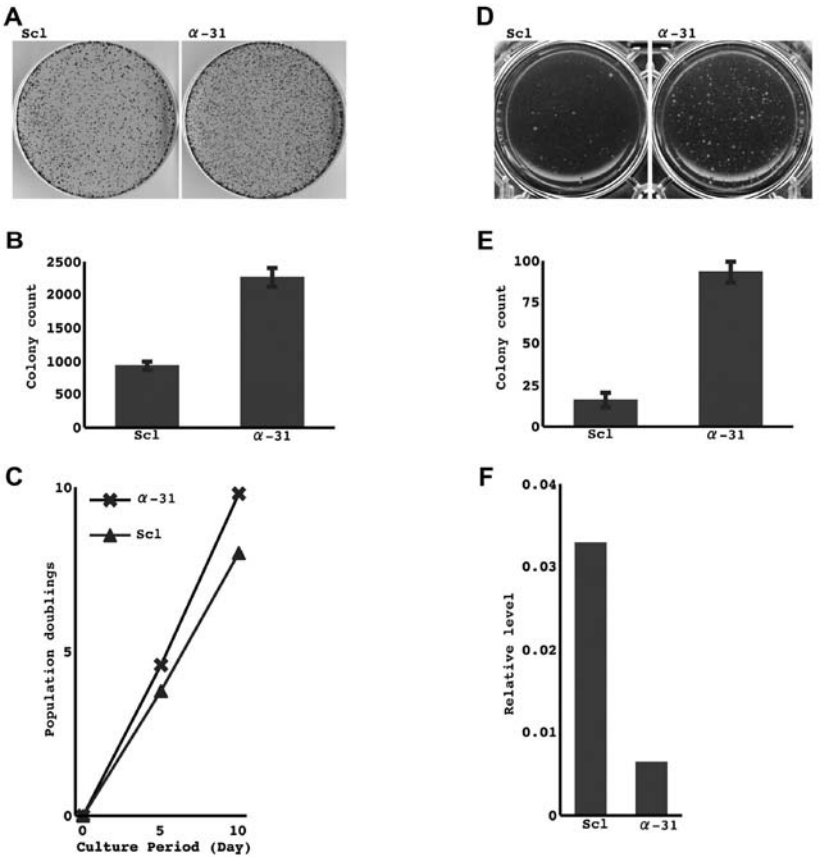

Figure 5. Biological effect of the knockdown of miR-31 in lung cancer cells retaining its expression. The representative results from the examination of HARA cells are presented. The control vector bearing the scrambled random sequence (scl) and the inhibitory vector coding short hairpin RNA targeting for miR-31 (α-miR-31) were transduced. Following a selection for 10 days, the surviving cells were harvested and counted, and $2.0x10^4$ were re-seeded onto a 10 cm dish. (A) After 14 days, the cells were methanol-fixed and Giemsa-stained. (B) The means and standard deviations (error bars) of colony counts from triplicate experiments are presented. The cells after the selection were grown and passed several times. (C) Cumulated population doublings are described. The selected cells ($1.25x10^4$) were cultured and grown in 1 ml of DMEM-based 0.3% agar containing 10% FBS in 3.5 cm culture dishes for 4 weeks. (D) The agars were fixed with a buffered 4% paraformaldehyde solution. (E) The means and standard deviations (error bars) of colony counts from triplicate experiments are presented. The cells harvested immediately after the selection process were examined for expression of miR-31 and U6 snRNA by quantitative RT-PCR. (F) The level of miR-31 normalized to that of U6 snRNA is presented.

Table III. Downregulated transcripts.

		MOCK		miR-31SS		miR-31AS			
Symbol	Accession	Signal	Flag	Signal	Flag	Signal	Flag	miR-31SS/MOCK	miR-31SS/AS
DYTN	NM_001093730	58.1445	P	7.8986	P	169.4681	P	0.1358	0.0466
MGP	NM_000900	0.2369	P	0.0116	A	0.2249	P	0.0490	0.0516
SAMD9L	NM_152703	0.1643	P	0.0149	A	0.2193	P	0.0908	0.0680
SAA2	NM_001127380	0.3662	P	0.0299	A	0.3758	P	0.0817	0.0796
SAA4	NM_006512	0.6052	P	0.0521	P	0.6040	P	0.0861	0.0863
SAA1	NM_000331	10.6803	P	1.0635	P	10.1069	P	0.0996	0.1052
CCR1	NM_001295	0.0807	P	0.0092	A	0.0858	P	0.1144	0.1077
SAA2	NM_030754	5.8777	P	0.6367	P	5.8671	P	0.1083	0.1085
CIITA	NM_000246	0.0657	P	0.0104	A	0.0925	P	0.1590	0.1129
SPTBN1	NM_003128	0.0835	P	0.0101	A	0.0893	P	0.1210	0.1132
MGP	NM_001190839	0.5979	P	0.0643	P	0.5636	P	0.1075	0.1141
XAF1	NM_017523	0.6196	P	0.0819	P	0.7122	P	0.1322	0.1150
BTBD8	NM_183242	0.0712	P	0.0083	A	0.0659	P	0.1162	0.1255
PLAC8	NM_001130715	0.0686	P	0.0086	A	0.0683	P	0.1259	0.1265
OLFM4	NM_006418	0.0751	P	0.0085	A	0.0667	P	0.1128	0.1269
CLCA2	NM_006536	0.1033	P	0.0105	A	0.0787	P	0.1017	0.1335
CCL2	NM_002982	0.5769	P	0.0771	P	0.5550	P	0.1337	0.1390
SAMD9L	NM_152703	0.7186	P	0.1299	P	0.9136	P	0.1807	0.1421
TRERF1	NM_033502	0.0967	P	0.0131	A	0.0889	P	0.1354	0.1472
PLEKHG4	NM_015432	0.0871	P	0.0157	A	0.1017	P	0.1797	0.1539
ITPK1-AS1	NR_002808	0.0947	P	0.0120	A	0.0760	P	0.1262	0.1572
DEFB127	NM_139074	0.1531	P	0.0088	A	0.0538	P	0.0572	0.1627
TFEC	NM_012252	0.2166	P	0.0095	A	0.0576	P	0.0438	0.1648
MEOX1	NM_004527	0.0839	P	0.0131	A	0.0792	P	0.1559	0.1652
GGT5	NM_001099781	1.6842	P	0.2891	P	1.7144	P	0.1717	0.1687
RPGR	NM_001034853	0.0956	P	0.0179	A	0.1027	P	0.1869	0.1741
CD99L2	BC025729	0.0623	P	0.0109	A	0.0623	P	0.1749	0.1748
CCNG2	NM_004354	0.0846	P	0.0156	A	0.0878	P	0.1848	0.1781
TNFSF15	**NM_005118**	**3.6060**	**P**	**0.6136**	**P**	**3.4121**	**P**	**0.1702**	**0.1798**
IL7R	NM_002185	0.7080	P	0.1181	P	0.6427	P	0.1668	0.1837
HSH2D	NM_032855	0.4028	P	0.0771	P	0.4128	P	0.1915	0.1869
BDKRB1	NM_000710	0.1140	P	0.0108	A	0.0578	P	0.0948	0.1869
SPARC	NM_003118	12.4315	P	2.4446	P	12.9549	P	0.1966	0.1887
HSD17B11	NM_016245	0.1320	P	0.0245	A	0.1294	P	0.1856	0.1894
EBI3	NM_005755	0.0981	P	0.0150	A	0.0789	P	0.1531	0.1904
ADRA1B	NM_000679	1.2211	P	0.2366	P	1.2080	P	0.1938	0.1959

MOCK, empty vector-transduced; miR-31SS, miR-31 sense strand-transduced; miR-31 antisense strand-tranduced NHBE-T cells; accession, gene bank accession no.; flag indicates whether gene expression was present (P) or absent (A).

E746-A750), point mutation L858R], but not by oncogenic BRAF (V600E) or oncogenic PIK3CA (E545K, H1047R) (Fig. 7). The expression of miR-31 was consistently not induced by specific mutants in KRAS that activated MEK, RAL-GDS or PI3K (Fig. 7). Inhibitors for MEK (the BRAF-MEK-ERK pathway) or PI3Kinase (the PIK3CA-mediated pathway) interfered with the induction of miR-31 by oncogenic KRAS.

Discussion

The findings of an initial study on breast cancer suggested that miR-31 could be a tumor suppressor that especially inhibited the invasive and metastatic spread of neoplastic cells (14). Previous studies have supported this initial observation and identified the potential molecular mechanisms responsible

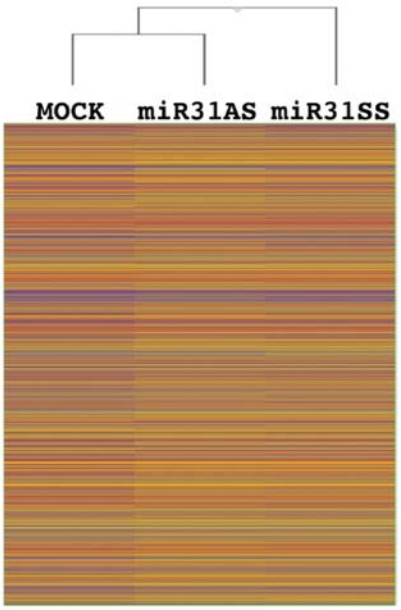

Figure 6. mRNA expression in the empty vector (MOCK)-, the miR-31 sense strand (SS), and the miR-31 antisense strand (AS)-transduced NHBE-T cells was comprehensively evaluated with a gene chip microarray. A hierarchical clustering analysis (Ward's Method) was performed and a dendrogram was described.

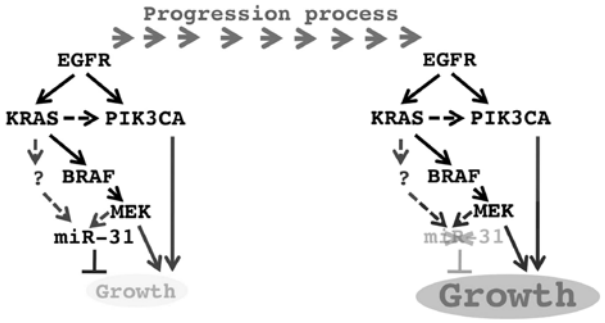

Figure 8. The putative role of miR-31 in carcinogenesis is described in a scheme. Physiologically, miR-31 may be induced by stimuli promoting cell growth in response to tissue damage and may control regenerative reactions (the left panel). In carcinogenesis, autonomous growth stimuli due to oncogenic mutations may also induce miR-31 that may interfere with unlimited growth. If the machinery to induce miR-31 is disrupted, autonomous stimuli for growth is augmented and further promote the progression of carcinogenesis (the right panel).

for the inhibition of invasion and metastasis by miR-31 (15). miR-31 was also shown to be downregulated in gastric cancer and was suggested to function as a tumor suppressor (16). In contrast, previous studies reported that miR-31 was upregulated in colorectal cancer and may also promote the invasive and metastatic spread of neoplastic cells (17,18). Thus, the potential role of miR-31 in carcinogenesis may differ depending on the type of cancer. The expression of miR-31 in lung cancer has generally been reported to be higher in tumor tissue than in the corresponding non-tumorous tissue (19-25). However, miR-31 levels have been shown to vary in primary lung tumors. Some tumors expressed miR-31 at lower levels than the corresponding non-tumorous tissue or did not express it at all. miR-31 expression was markedly reduced or completely lost in some lung cancer cell lines (Fig. 3). This result suggested that miR-31 may be a suppressor in lung carcinogenesis. The restoration of miR-31 in lung cancer cell lines that had lost its expression markedly attenuated their growth activities (Fig. 4). The knockdown of miR-31 expression in cell lines that retained its expression enhanced oncogenic phenotypes such as anchorage-independent growth activity (Fig. 5). These results supported miR-31 being a suppressor. However, a recent study demonstrated the oncogenic role of miR-31 in lung carcinogenesis using experiments with different cell lines

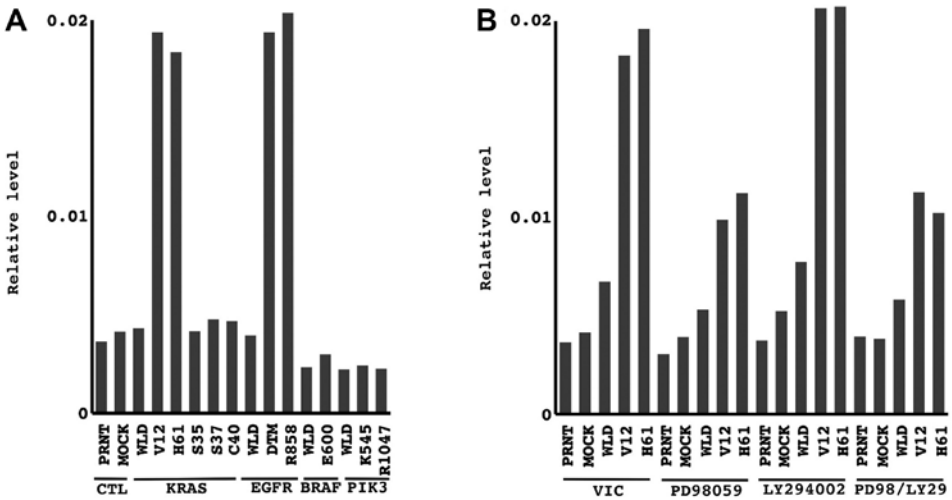

Figure 7. The modulation of miR-31 expression through the essential oncogenic pathway. (A) The level of miR-31 in NHBE-T cells transduced with different mutants of KRAS, EGFR, BRAF and PIK3CA was evaluated. The copy number of miR-31 and U6 snRNA was measured by quantitative RT-PCR. The level of miR-31 normalized to that of U6 snRNA is presented. PRNT, parental NHBE-T; MOCK, empty vector-transduced NHBE-T; WLD, wild-type KRAS-transduced NHBE-T; V12, oncogenic mutant of KRAS/V12-transduced NHBE-T; H61, oncogenic mutant of KRAS/H61-transduced NHBE-T; S35, a mutant of KRAS activating only the MEK pathway-transduced NHBE-T; G37, a mutant of KRAS activating only the Ral-GDS pathway-transduced NHBE-T; C40, a mutant of KRAS activating only the PI3K pathway-transduced NHBE-T. (B) The level of miR-31 in NHBE-T cells treated with the inhibitors for MEK (PD98059, 50 μM), PI3K (LY294002, 50 μM), and their combination (PD98/LY29, 50 μM each) for 24 h was evaluated. CTL, control; VIC, vehicle.

from the ones used in the present study (26). miR-31 may play a pleiotropic role in the development of individual tumors. Further investigations are required to resolve this issue.

In the present study, miR-31 was upregulated not only by oncogenic KRAS, but also by oncogenic EGFR *in vitro* (Fig. 7). Neither oncogenic BRAF nor oncogenic PIK3CA induced its expression (Fig. 7). Similarly, the specific mutant of KRAS that activated the MEK-mediated pathway only and another specific mutant that activated the PI3K-mediated pathway only did not induce the expression of miR-31 (Fig. 7). On the other hand, the MEK inhibitor, but not PI3K inhibitor, attenuated the oncogenic KRAS-induced expression of miR-31 (Fig. 7). These results suggest that miR-31 could be a common player that regulates the KRAS/EGFR-mediated essential oncogenic pathway (Fig. 8). The MEK-mediated pathway may be necessary, but not sufficient for the induction of miR-31 expression (Fig. 8). The PI3K-mediated pathway may not be involved in its regulation (Fig. 8). A novel pathway may be inducing the expression of miR-31 in cooperation with the MEK-mediated pathway.

This comprehensive search for the potential target of miR-31 revealed that the downregulated transcripts included many molecules mediating the cytokine/chemokine signaling pathway (Tables II and III). TNFSF15, in particular, has been published on an online site as the predicted downstream target for miR-31 (http://mirdb.org/miRDB/index.html). TNFSF15, a member of the tumor necrosis factor (TNF) and TNF receptor superfamilies, regulates cell growth and apoptosis in an autocrine manner, and has been reported to suppress cancer cell growth by inhibiting angiogenesis (27). Thus, TNFSF15 could be a factor that participates in miR-31-induced growth modulations. Future studies that focus on the potential downstream targets of miR-31 may provide insight into the molecular mechanisms involved in lung cancer.

The present study comprehensively searched miRNAs, the expressions of which were regulated by oncogenic KRAS, and focused on miR-31 in order to investigate its potential involvement in lung carcinogenesis. The expression of miR-31 was markedly attenuated in lung cancer cell lines. The restoration of miR-31 in lung cancer cell lines that lost its expression attenuated their growth activities. The knockdown of miR-31 expression in cell lines that retained its expression enhanced oncogenic phenotypes. These results suggest that miR-31 may be a suppressor, the loss of which may promote lung carcinogenesis.

Acknowledgements

This study was supported by the Japanese Ministry of Education, Culture, Sports and Science (Tokyo Japan), and by a grant from the Yokohama Medical Facility (Yokohama, Japan). We especially thank Emi Honda and Misa Otara (Kanagawa Prefectural Cardiovascular and Respiratory Center Hospital, Yokohama, Japan) for their assistance.

References

1. Hoffman PC, Cohen EE, Masters GA, *et al*: Carboplatin plus vinorelbine with concomitant radiation therapy in advanced non-small cell lung cancer: a phase I study. Lung Cancer 38: 65-71, 2002.
2. Spira A and Ettinger DS: Multidisciplinary management of lung cancer. N Engl J Med 350: 379-392, 2004.
3. Okudela K, Woo T and Kitamura H: KRAS gene mutations in lung cancer: particulars established and issues unresolved. Pathol Int 60: 651-660, 2010.
4. Woo T, Okudela K, Yazawa T, *et al*: Prognostic value of KRAS mutations and Ki-67 expression in stage I lung adenocarcinomas. Lung Cancer 65: 355-362, 2009.
5. Okudela K, Yazawa T, Ishii J, *et al*: Down-regulation of FXYD3 expression in human lung cancers: its mechanism and potential role in carcinogenesis. Am J Pathol 175: 2646-2656, 2009.
6. Pao W and Girard N: New driver mutations in non-small-cell lung cancer. Lancet Oncol 12: 175-180, 2011.
7. van Eijk R, Licht J, Schrumpf M, *et al*: Rapid *KRAS*, *EGFR*, *BRAF* and *PIK3CA* mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PLoS One 6: e17791, 2011.
8. Xu J, He J, Yang H, *et al*: Somatic mutation analysis of EGFR, KRAS, BRAF and PIK3CA in 861 patients with non-small cell lung cancer. Cancer Biomark 10: 63-69, 2011-2012.
9. Cozens AL, Yezzi MJ, Kunzelmann K, *et al*: CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol 10: 38-47, 1994.
10. Yazawa T, Kamma H, Fujiwara M, *et al*: Lack of class II transactivator causes severe deficiency of HLA-DR expression in small cell lung cancer. J Pathol 187: 191-199, 1999.
11. Okudela K, Yazawa T, Suzuki T, Sugimura H and Kitamura H: Role of 3'-phosphoinositides in oncogenic KRAS-induced modulation of shape and motility of airway epithelial cells. Pathol Int 59: 28-37, 2009.
12. Okudela K, Suzuki M, Kageyama S, *et al*: *PIK3CA* mutation and amplification in human lung cancer. Pathol Int 57: 664-671, 2007.
13. Okudela K, Yazawa T, Woo T, *et al*: Down-regulation of DUSP6 expression in lung cancer: its mechanism and potential role in carcinogenesis. Am J Pathol 175: 867-881, 2009.
14. Valastyan S, Reinhardt F, Benaich N, *et al*: A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell 137: 1032-1046, 2009.
15. Augoff K, Das M, Bialkowska K, McCue B, Plow EF and Sossey-Alaoui K: miR-31 is a broad regulator of β1-integrin expression and function in cancer cells. Mol Cancer Res 9: 1500-1508, 2011.
16. Zhang Y, Guo J, Li D, *et al*: Down-regulation of miR-31 expression in gastric cancer tissues and its clinical significance. Med Oncol 27: 685-689, 2010.
17. Cottonham CL, Kaneko S and Xu L: miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. J Biol Chem 285: 35293-35302, 2010.
18. Slaby O, Svoboda M, Fabian P, *et al*: Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology 72: 397-402, 2007.
19. Guan P, Yin Z, Li X, Wu W and Zhou B: Meta-analysis of human lung cancer microRNA expression profiling studies comparing cancer tissues with normal tissues. J Exp Clin Cancer Res 31: 54, 2012.
20. Jang JS, Jeon HS, Sun Z, *et al*: Increased miR-708 expression in NSCLC and its association with poor survival in lung adenocarcinoma from never smokers. Clin Cancer Res 18: 3658-3667, 2012.
21. Gao W, Yu Y, Cao H, *et al*: Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis. Biomed Pharmacother 64: 399-408, 2010.
22. Võsa U, Vooder T, Kolde R, *et al*: Identification of miR-374a as a prognostic marker for survival in patients with early-stage nonsmall cell lung cancer. Genes Chromosomes Cancer 50: 812-822, 2011.
23. Xing L, Todd NW, Yu L, Fang H and Jiang F: Early detection of squamous cell lung cancer in sputum by a panel of microRNA markers. Mod Pathol 23: 1157-1164, 2010.
24. Yu L, Todd NW, Xing L, *et al*: Early detection of lung adenocarcinoma in sputum by a panel of microRNA markers. Int J Cancer 127: 2870-2878, 2010.
25. Tan X, Qin W, Zhang L, *et al*: A 5-microRNA signature for lung squamous cell carcinoma diagnosis and hsa-miR-31 for prognosis. Clin Cancer Res 17: 6802-6811, 2011.
26. Meng W, Ye Z, Cui R, *et al*: MicroRNA-31 predicts the presence of lymph node metastases and survival in lung adenocarcinoma patients. Clin Cancer Res 19: 5423-5433, 2013.
27. Duan L, Yang G, Zhang R, Feng L and Xu C: Advancement in the research on vascular endothelial growth inhibitor (VEGI). Target Oncol 7: 87-90, 2012.