A Polycistronic MicroRNA Cluster, miR-17-92, Is Overexpressed in Human Lung Cancers and Enhances Cell Proliferation

Yoji Hayashita,1,4 Hirota Osada,1 Yoshio Tatematsu,1 Hideki Yamada,1,2 Kiyoshi Yanagisawa,1,2 Shuta Tomida,1,3 Yasushi Yatabe,1 Katsunobu Kawahara,1 Yoshitaka Sekido,1 and Takashi Takahashi1,2

1Division of Molecular Oncology, Aichi Cancer Center Research Institute; 2Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine; 3Departments of Anatomic and Molecular Diagnostic Pathology, Aichi Cancer Center Hospital, Nagoya, Japan; and 4Department of Oncological Science (Surgery II), Oita University Faculty of Medicine, Oita, Japan

Abstract

MicroRNAs (miRNAs) are small noncoding RNAs, thought to be involved in physiologic and developmental processes by negatively regulating expression of target genes. We have previously reported frequent down-regulation of the let-7 miRNA family in lung cancers and, in the present study, assessed alteration in a panel of 19 lung cancer cell lines. As a result, we found for the first time that the miR-17-92 cluster, which comprises seven miRNAs and resides in intron 3 of the C13orf25 gene at 13q31.3, is markedly overexpressed in lung cancers, especially with small-cell lung cancer histology. Southern blot analysis revealed the presence of increased gene copy numbers of the miRNA cluster in a fraction of lung cancer cell lines with overexpression. In addition, we were able to show predominant localization of C13orf25 transcripts within the nucleus and introduction of the expression construct of the miR-17-92 cluster, but not the putative open reading frame of C13orf25, enhancing lung cancer cell growth. These findings clearly suggest that marked overexpression of the miR-17-92 cluster with occasional gene amplification may play a role in the development of lung cancers, especially in their most aggressive form, small-cell lung cancer, and that the C13orf25 gene may well be serving as a vehicle in this regard. (Cancer Res 2005; 65(21): 9628-32)

Introduction

Lung cancer is the leading cause of cancer-related deaths in Japan as in many other economically developed countries and is characterized by frequent mutations, amplifications, and epigenetic changes in various cancer-related genes (1). Emerging evidence suggests the potential involvement of altered regulation of microRNAs (miRNAs) in the pathogenesis of a limited range of human cancers (2–5). miRNAs are small noncoding RNAs that are processed from nascent primary transcripts (pri-miRNA) by dsRNA-specific endonucleases, Drosha and Dicer, which are known to negatively regulate gene expression mainly through interactions with 3′-untranslated regions of their target genes (6). Although the precise functions of individual miRNAs have not yet been characterized, these RNA species are thought to play roles in many physiologic and developmental processes. We have previously reported frequent down-regulation of members of the let-7 miRNA family in lung cancers in association with a poor postoperative prognosis (7) whereas down-regulation of Dicer also seems to be associated with a shortened survival (8).

In the present study, we examined a panel of 19 lung cancer cell lines for the presence of alterations in 21 miRNAs, which we preselected based on the potential significance of their putative TargetScan algorithm–predicted target genes (9) in terms of cancer development. We here documented the first evidence of frequent and marked overexpression, with occasional gene amplification, of clustered miRNAs (miR-17-92) within intron 3 of the C13orf25 gene at 13q31.3 in lung cancers, especially examples with small-cell lung cancer histology. We further showed the stimulatory activity of this miRNA cluster in lung cancer cell growth and discussed the possibility that the C13orf25 gene may well be serving as a vehicle for the expression of miR-17-92.

Materials and Methods

Cell lines and tissue samples. All 19 lung cancer cell lines and two immortalized lung epithelial cell lines, HPL1D (10) and BEAS-2B (11), were maintained as previously described. All RNA samples were prepared by the acid phenol extraction procedure except for primary small-cell lung cancer specimens, which underwent DNA ultracentrifugation.

Northern blot analysis. Northern blot analysis of miRNAs was done using 10 μg of RNA as previously described (7). Northern blot analysis of C13orf25 was done according to a standard procedure using a 524-bp PCR-amplified cDNA containing all the exons. For subcellular localization analysis, cell nuclei were collected by brief centrifugation after cell lysis with 0.5% NP40 and the supernatant was harvested as the cytoplasmic fraction.

Hierarchical clustering. We used the CLUSTER and TREEVIEW programs for hierarchical clustering and visualization of data sets as previously described (7).

Real-time reverse transcription-PCR. Quantitative real-time reverse transcription-PCR (RT-PCR) was done using total RNAs, which were pretreated with DNase (2 units/μg; Promega, Madison, WI) for 1 hour at 37°C as previously described.

Southern blot analysis. Five-microgram aliquots of genomic DNA were digested with SacI or EcoRV and subjected to Southern blot analysis (7). The entire miRNA regions as well as the whole C13orf25 gene residing within each of the corresponding restriction fragments were detected with PCR-amplified pri-miRNA probes. Cαβ, a T-cell receptor β-chain cDNA fragment, served as a loading control. Fold increase of copy number was presented as the signal ratio between pri-miRNA and Cαβ signals, normalized to that of HPL1D, which was set as 1.

Expression constructs and assays for measuring cell proliferation. A 1,740-bp EcoRI-HpaI fragment containing the miR-17-92 cluster was

Note: Y. Hayashita and H. Osada contributed equally to this work.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Shuta Tomida, Takashi Takahashi, Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Phone: 81-52-744-2454; Fax: 81-52-744-2437; E-mail: tak@med.nagoya-u.ac.jp.

©2005 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-2352

Cancer Res 2005; 65: (21). November 1, 2005

9628 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on July 24, 2017. © 2005 American Association for Cancer Research.
cloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) carrying a gene conferring puromycin resistance. A 99-bp open reading frame (ORF) of C13orf25 was PCR amplified using cDNA of normal lung tissue and also cloned into the same vector. PCR-amplified primary forms of miR-18, miR-19a, and miR-20 were individually cloned into pH1-RNA-puro (7). The A549 lung adenocarcinoma cell line was transfected with either of the vectors using Lipofectamine 2000 (Invitrogen) and selected with puromycin (2 μg/ml for 2 days and 0.5 μg/ml thereafter). Ten days after transfection, cell proliferation was measured with a colorimetric assay reagent, Tetra-Color One (Seikagaku Co., Tokyo, Japan). In addition, luciferase reporter activity was measured as a reflection of cell proliferation without any selection agent. A549 cells were cotransfected in triplicate with each miRNA expression vector along with a one-tenth amount of pRL-TK luciferase reporter (Promega). The day after transfection, each transfected dish was divided into four and the reporter activity was then measured each day for up to 4 days.

Results

Identification of miR-17-92 cluster overexpression preferentially in small-cell lung cancer. We first selected 21 miRNA genes as of possible interest based on target prediction by TargetScan (9) and searched for alterations by Northern blot analysis, resulting in the identification of marked overexpression of miR-19a, miR-20, miR-106a, and miR-106b (Fig. 1A). Unsupervised hierarchical clustering analysis using CLUSTER and TREEVIEW software highlighted overexpression of these miRNAs in a specific cluster mainly found in small-cell lung cancer cell lines (Fig. 1B). These miRNAs are components of three paralogous clusters including miR-17-92 at 13q31.3, miR-106a-92 at Xq26.2, and miR-106b-25 at 7q22 with extensive sequence homologies (Fig. 2A). Northern blot analysis showed the miR-20 probe to be relatively specific, exhibiting weak cross-hybridization with miR-106a but not with miR-106b (Fig. 2B), whereas the miR-106b probe cross-hybridized with both miR-20 and miR-106a and could only be distinguished based on the difference in size. Expression levels of primary forms of the miRNAs were therefore measured with real-time RT-PCR using primers with no cross reactivity. The analysis of pri-miR-20 showed a pattern of expression similar to that with Northern blot analysis (Pearson’s correlation coefficient $r = 0.798$, $P < 0.0001$; Fig. 2C), in contrast to poor concordance for miR-106b ($r = 0.272$, $P = 0.224$). The primary form of miR-106a could not be clearly amplified in these cell lines despite the use of three independent sets of primers and various PCR conditions, strongly suggesting that signals detected with the miR-106a probe on Northern blot analysis were actually caused by cross-hybridization. We thus concluded that miR-20 was overexpressed in lung cancer cell lines, and further analyzed the status of other miRNAs belonging to the miR-17-92 cluster in which miR-20 resides. Expression of miR-17-3p and miR-18, additional components of this miRNA cluster, was examined by Northern blot analysis, taking advantage of the fact that miR-17-3p has no homologous miRNA in the human genome and miR-18 has the lowest similarity among the miR-20–related miRNAs. Both miR-18 and miR-17-3p showed overexpression with patterns quite similar to those of miR-20 and miR-19a, indicating overexpression of the miR-17-92 cluster in lung cancer cell lines (Fig. 2D).

Identification of gene amplification of the miR-17-92 cluster region in lung cancers. The miR-17-92 cluster is located within intron 3 of the C13orf25 gene, which has been reported to be amplified in lymphomas (12). Detection of its overexpression in lung cancers prompted us to perform Southern blot analysis using PCR-prepared pri-miRNA probes and a control C13β probe to determine

Figure 1. Search for miRNAs with altered expression in lung cancers. A, Northern blot analysis of miRNAs in lung cancer cell lines. Note marked overexpression of miR-19a, miR-20, miR-106a, and miR-106b. Normal Lung, a mixture of RNAs from 11 normal lung tissues; BEAS-2B and HPL1D, two immortalized human epithelial cell lines representing proximal and distal airway cells, respectively. B, unsupervised hierarchical clustering analysis highlighting overexpression of miR-19a, miR-20, miR-106a, and miR-106b mainly in small-cell lung cancer cell lines (red). Blue, normal lung tissues and the two immortalized human epithelial cell lines, BEAS-2B and HPL1D.
Evidence of involvement of the miR-17-92 cluster but not of coding capability of C13orf25. It is possible that C13orf25 could be the primary target for gene amplification in lung cancers because this is where the miR-17-92 cluster resides (Fig. 4A). Northern blot analysis of C13orf25 showed an expression pattern similar to that of the miR-17-92 cluster as expected (Fig. 4B). The function of the C13orf25 gene is still ambiguous because of its very limited protein-encoding capacity and poor sequence conservation, even within mammals. To clarify whether C13orf25 transcripts may work as protein-coding miRNAs or as vehicle for miRNAs forming the miR-17-92 cluster, we first studied subcellular localization of C13orf25 transcripts by Northern blot analysis using RNAs derived from five primary small-cell lung cancer specimens (Fig. 3C).
Overexpression of miR-17-92 in Lung Cancers

Enhanced proliferation of lung cancer cells on introduction of the miR-17-92 cluster. A, schematic diagram of the structure of the C13orf25 gene and expression constructs corresponding to two RNA species, polycistronic miR-17-92 and C13orf25-ORF-containing mRNA. B, Northern blot analysis. C13orf25 transcripts (arrowheads) are clearly detected in lung cancer cells with high expression in PC1, ACC-LC-48, and SK-LC-2 expressing abundant miR-17-92. C, Northern blot analysis using RNAs derived from nuclear (N) and cytoplasmic (C) fractions. Note that the great majority of the C13orf25 transcripts (arrowheads) are localized within the nucleus. D, Northern blot analysis showing abundant miR-20 expression in transfectants receiving the miR-17-92 expression construct. VC, empty vector. E, cell proliferation assay of miR-17-92 transfectants. Cell proliferation of A549 transfected with either miR-17-92 or C13orf25 measured using a colorimetric assay 10 days after transfection and subsequent puromycin selection. Note clear enhancement of cell proliferation by about 2-fold with miR-17-92, but not with C13orf25. Note lack of any promoting effects of transfection with individual miRNAs. F, luciferase reporter assay of miR-17-92 and C13orf25 transfectants. Stimulatory effects on cell proliferation are evident after cotransfection with miR-17-92 but not with C13orf25. Cell proliferation was measured by luciferase activity at 10 days after transfection. Note lack of any promoting effects of individual miRNAs.

Discussion

In this study, we have shown for the first time that the miR-17-92 cluster, composed of seven miRNAs and residing in intron 3 of the C13orf25 gene at 13q31.3, is markedly and frequently overexpressed in lung cancers, with occasional gene amplification, especially in those with small-cell lung cancer histology. Furthermore, evidence was obtained that introduction of miR-17-92, but not the putative coding region of C13orf25, can enhance lung cancer cell growth.

It is interesting to note that the predicted targets for the miR-17-92 cluster include tumor suppressor genes PTEN and RB2. Recent bioinformatic studies on the algorithm for the prediction of miRNA targets added numerous genes to the list of potentially down-regulated target genes (13, 14), nominating more than 600 for miR-19a and miR-20. Consistent with this, it was recently shown through microarray analysis that miRNAs can indeed affect expression levels of an enormous number of genes (15). Therefore, future studies directly addressing the actual targets for the miR-17-92 cluster in lung cancers will be of interest to better understand how this alteration might contribute to development of this fatal cancer as well as to shed light on the molecular mechanisms of miRNA function. Our finding of preferential overexpression of the miR-17-92 cluster in lung cancers with small-cell lung cancer histology, a subtype of lung cancer with prominent neuroendocrine feature, warrants study on its regulation and potential involvement from the cell differentiation point of view. Whereas significant enhancement of lung cancer cell growth was evident on introduction of the miR-17-92 cluster, we have not seen any clear growth stimulatory effects after transfection with individual components of this cluster in an ongoing study being conducted with miR-18, miR-19a, and miR-20 expression constructs. Further investigations are certainly required for clarification but it is possible that the whole structure of the miR-17-92 cluster might be necessary for appropriate processing and optimization of dosages of each component to ensure growth stimulatory effects.

Just before submission of this article, He et al. (16) reported overexpression of the miR-17-92 cluster in B-cell lymphomas, and introduction of miR-17-92 into hematopoietic stem cells in Eμ-myc transgenic mice was shown to significantly accelerate...
formation of lymphoid malignancies. That their reported lack of alteration in colon cancer may be a reflection of the cell differentiation in this tissue, given our findings for preferential occurrence in small-cell lung cancer, is an interesting possibility (16). Further studies on other epithelial malignancies including carcinoids and small cell cancers of various origins are clearly necessary. A recently discovered connection between the miR-17-92 cluster and the c-myc oncogene (17) is of special note because members of the myc gene family have been shown to be frequently amplified and/or overexpressed in small-cell lung cancers. Interestingly, our previous studies on the myc gene family in lung cancers revealed that among the three small-cell lung cancer cell lines (ACC-LC-48, ACC-LC-172, and SK-LC-2) with marked over-expression of the miR-17-92 cluster, ACC-LC-172 features gene amplification and overexpression of c-myc (18, 19), and L-myc is markedly overexpressed in ACC-LC-48 carrying gene amplification, whereas SK-LC-2 has modest overexpression of L-myc without gene amplification. Our findings suggest that L-myc might also induce the expression of miR-17-92. In our preliminary RT-PCR analysis of other cell lines, overexpression of miR-17-92 without gene amplification seemed to be associated with up-regulation of at least one member of the myc gene family (Supplementary Figure).

Thus, two potential mechanisms, which lead to overexpression of the miR-17-92 cluster, may be operative in lung cancers—gene amplification of the miRNA cluster itself and increased expression of the myc gene family, with or without gene amplification.

We previously reported down-regulation of members of the let-7 miRNA family in association with a poor prognosis in lung cancers (7). The RAS oncogene has subsequently been shown to be a target gene for the let-7 miRNA family (20), suggesting potential functional consequences of altered let-7 expression in lung cancer development. Global expression profiling analysis using transfectants of the miR-17-92 cluster is now indicated to elucidate underlying molecular mechanisms as well as consequences of altered expression. We envisage that advances through such studies should ultimately provide clues to better understanding how cell growth and differentiation are controlled and eventually lead to development of novel cancer therapeutics for lung malignancies.

Acknowledgments

Received 7/5/2005; revised 8/24/2005; accepted 9/15/2005.

Grant support: Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Grants-in-Aid for Scientific Research (B) and (C) from the Japan Society for the Promotion of Science, and a Grant-in-Aid for the Second Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Curtis C. Harris (National Cancer Institute, Bethesda, MD) for the BEAS-2B cell line, Dr. Masao Seto (Aichi Cancer Center, Nagoya, Japan) for the T-cell receptor β-chain cDNA probe, and Dr. Takashi Miura (Faculty of Medicine, Oita University, Oita, Japan) for his encouragement throughout this study.

References

A Polycistronic MicroRNA Cluster, miR-17-92, Is Overexpressed in Human Lung Cancers and Enhances Cell Proliferation

Yoji Hayashita, Hirotaka Osada, Yoshio Tatematsu, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/21/9628

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2005/11/03/65.21.9628.DC1

Cited articles
This article cites 20 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/21/9628.full#ref-list-1

Citing articles
This article has been cited by 100 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/21/9628.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.