network-search algorithm that selects the set of miRNA target genes as starting points to generate the network around starting points within one path, composed of all kinds of molecular interactions, including direct activation/inactivation, transcriptional activation/repression, and the complex formation. By importing the list of Entrez Gene IDs, KeyMolnet automatically provides corresponding molecules and a minimum set of intervening molecules as a node on networks. The generated network was compared side by side with human canonical networks described above. The algorithm that counts the number of overlapping molecules and/or molecular relations between the extracted network and the canonical network identifies the canonical network showing the most statistically significant contribution to the extracted network. This algorithm is essentially based on that of the GO::TermFinder (Boyle et al. 2004). The significance in the similarity between the extracted network and the canonical network is scored following the formula, where O = the number of overlapping molecules and molecular relations for the pathway or overlapping molecules alone for the disease and the pathological event between the extracted network and the canonical network, V = the number of molecules and/or molecular relations located in the extracted network, C = the number of molecules and/or molecular relations located in the canonical network, T = the number of total molecules and/or molecular relations of KeyMolnet, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score (p)}) \qquad \text{Score (p)} = \sum_{x=O}^{Min(C,V)} f(x) \qquad f(x) = {}_{C}C_{x} \cdot {}_{T-C}C_{V-x} / {}_{T}C_{V}$$

2.3 Molecular Network of MicroRNA Targetome

Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted the targets from 532 miRNAs (43.5%) (Satoh and Tabunoki 2011). Among the 532 miRNAs, 273 miRNAs contained a set of highly reliable targets showing the miTG score $\geq$ 20. Among 273 miRNAs having reliable targets, KeyMolnet successfully extracted valid molecular networks of targetome from 232 miRNAs. They are comprised of 19% of total human miRNAs (miRNAome). Then, the generated network was compared side by side with human canonical

networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events. We found that not all 232 miRNAs contained all three categories of canonical networks because several miRNAs comprised only small numbers of targets. When top three pathways, diseases, and pathological events were individually totalized, the most relevant pathway was 'transcriptional regulation by RB/E2F' (n = 39; 6.8% of total), followed by 'TGF-beta family signaling pathway' (n = 32; 5.6%) and 'transcriptional regulation by POU domain factor' (n = 24; 4.2%), the most relevant disease was 'adult T cell lymphoma/leukemia' (n = 68; 12.1%), followed by 'chronic myelogenous leukemia' (n = 65; 11.5%) and 'hepatocellular carcinoma' (n = 51; 9.1%), and the most relevant pathological event was 'cancer' (n = 97; 24.7%), followed by 'adipogenesis' (n = 46; 11.7%) and 'metastasis' (n = 36; 9.2%) (Fig. 1) (Satoh and Tabunoki 2011).

Next, we identified and characterized the large-scale miRNA targetome networks by uploading targets greater than 100 per individual miRNA onto KeyMolnet (Table 1). Fifty-two miRNAs constructing such a large-scale miRNA target network include let-7, miR-9, 17, 19, 20, 26, 27, 29, 30, 32, 92, 93, 96, 98, 101, 106b, 124, 137, 147, 153, 218, 372, 429, 495, 506, 519, 520, 603, and their closely-related family members. The miRNA targetome established highly complex molecular networks, in which the pathways of 'transcriptional regulation by RB/E2F', 'transcriptional regulation by Ets-domain family', and 'transcriptional regulation by p53', the diseases of 'chronic myelogenous leukemia' and 'viral myocarditis', and the pathological event of 'cancer' were notably accumulated (Table 1) (Satoh and Tabunoki 2011).

3 Biological Implications of MicroRNA Targetome Networks

3.1 Collaborative Regulation by MiRNAs and Transcription Factors

As described above, the present observations revealed that the human miRNA targetome regulated by an individual miRNA generally constitutes the biological network of functionally-associated molecules. Therefore, it is important to gain deeper insights into biological implications of each miRNA targetome network.

The protooncogene c-myb is a key transcription factor for development of normal hematopoietic cells and neoplasms. Recent studies showed that miR-15a targets c-myb, while c-myb binds to the promoter of miR-15a, providing an autoregulatory feedback loop in human hematopoietic cells (Chung et al. 2008; Zhao et al. 2009). Consistent with these studies, we found 'transcriptional regulation by myb' as the most relevant pathway to the miR-15a targetome network (the score = 602; the score p-value = 7.39E-182) (Fig. 2) (Satoh and Tabunki 2011). These results suggest a scenario that in the miR-15a targetome network, miR-15a synchronously downregulates both c-myb itself and downstream genes transcriptionally regulated by c-myb, resulting in more effective inactivation of the complex molecular network governed by the hub gene c-myb. Thus, a collaborative regulation of gene expression operates at both transcriptional and posttranscriptional levels, which involves coordinated regulation by miRNAs and transcription factors. Therefore, disruption of fine balance of the coordination could lead to development of cancers.

The retinoblastoma protein Rb/transcription factor E2F pathway acts as a gatekeeper for G1/S transition in the cell cycle. The Rb/E2F-regulated G1 checkpoint control is often disrupted in cancer cells. A recent study showed that miR-106b is directly involved in posttranscriptional regulation of E2F1 (Petrocca et al. 2008). E2F1 activates transcription of miR-106b, while miR-106b targets E2F1, serving as a negative feedback loop in gastric cancer cells. Consistent with these findings, we identified 'transcriptional regulation by Rb/E2F' as the most relevant pathway to the miR-106b targetome network (the score = 854; the score p-value = 7.21E-258) (Fig. 3) (Table 1) (Satoh and Tabunoki 2011). Again, it is possible that in the miR-106b

targetome network, miR-106b simultaneously downregulates both E2F family transcription factors and downstream genes transcriptionally regulated by E2Fs, resulting in efficient inactivation of the complex molecular network governed by the hub molecules E2Fs. The relationship between miR-106b and Rb/E2F would serve as another example of coordinated regulation of gene expression by miRNAs and transcription factors.

3.2 Human MiRNAs Act as a Central Regulator of Oncogenesis

A recent study by miRNA expression profiling of thousands of human tissue samples showed that diverse miRNAs constitute a complex network composed of coordinately regulated miRNA subnetworks in both normal and cancer tissues, and they are often disorganized in solid tumors and leukemias (Volinia et al. 2010). During development of cancers, various sets of miRNAs act as either oncogenes named oncomir or tumor suppressors termed anti-oncomir, or both, by targeting key molecules and their networks involved in apoptosis, cell cycle, cell adhesion and migration, chromosome stability, and DNA repair (Blenkiron and Miska 2007; Garzon et al. 2010). Many miRNA gene loci are clustered in cancer-associated genomic regions (Calin et al. 2004). Furthermore, miRNA expression signatures clearly discriminate different types of cancers with distinct clinical prognoses (Lu et al. 2005). All of these observations support the general view that miRNAs act as a central regulator of oncogenesis (Blenkiron and Miska 2007; Garzon et al. 2010).

To prevent oncogenesis in the cells exposed to stressful insults, the transcription factor p53 acts as the guardian of the genome by regulating a battery of target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Deregulation of p53 function is closely associated with oncogenesis (Rivlin et al. 2011). We found 'transcriptional regulation by p53' as the most relevant pathway to the target network of all let-7 family members except for let-7d (Table 1) (Satoh and Tabunoki 2011). It is worthy to note that the tumor suppressor p53 regulates the expression of various components of the miRNA-processing machinery, such as Drosha, DGCR8, Dicer, and TARBP2, all of which have p53-reponsive elements in their promoters (Boominathan 2010). Furthermore, Dicer and TARBP2, along with

p53, serve as a target of the let-7 family miRNAs, suggesting a close link between p53 and let-7 in miRNA biogenesis (Boominathan 2010). The let-7 family regulates the expression of a critical oncogene RAS in human cells (Johnson et al. 2005), and the expression of let-7 family members was greatly reduced in certain cancer cells (Takamizawa et al. 2004).

The micropthalmia associated transcription factor (MITF), a basic helix-loop-helix zipper (bHLH-Zip) transcription factor, acts as not only a master regulator of melanocyte differentiation but also an oncogene promoting survival of melanoma. Recent studies indicate that MITF is a direct target of both miR-137 and miR-148b (Bemis et al. 2008; Haflidadóttir et al. 2010). Again, we identified 'transcriptional regulation by MITF family' as the most relevant pathway to both miR-137 (the score = 339; the score p-value = 1.19E-102) (Table 1) and miR-148b (the score = 40; the score p-value = 3.91E-142) targetome networks (Satoh and Tabunoki 2011).

Zinc finger transcription factors ZEB1 and ZEB2 act as a transcriptional repressor of E-cadherin. A recent study showed that the expression of miR-200b, which targets both ZEB1 and ZEB2, was downregulated in the cells that undergo TGF-beta-induced epithelial-mesenchymal transition (EMT), and was lost in invasive breast cancer cells (Gregory et al. 2008). EMT is a morphological marker of tumor progression, characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility and invasiveness. We identified 'transcriptional regulation by ZEB' as the third-rank significant pathway (the score = 155; the score p-value = 1.88E-47) (Fig. 4) and 'EMT' as the third-rank significant pathological event relevant to the miR-200b targetome network (the score = 61; the score p-value = 4.15E-19) (Satoh and Tabunoki 2011).

Thus, various miRNAs positively and negatively regulates diverse gene networks associated closely with promotion and prevention of oncogenesis.

4 Concluding Remarks

A single miRNA concurrently downregulates hundreds of target mRNAs by binding to the corresponding 3'UTR of mRNA via either perfect or imperfect sequence complementarity (Selbach et al. 2008). Such fuzzy miRNA-mRNA interactions are responsible for the redundancy of miRNA-regulated targets and their networks. We have addressed the question whether the human miRNA targetome regulated by an individual miRNA constitutes the biological network of functionally-associated molecules or reflect a random set of functionally-independent genes. First, Diana-microT 3.0 identified highly reliable targets from 273 miRNAs out of 1,223 all human miRNAs. Then, KeyMolnet successfully extracted molecular networks from 232 miRNAs, comprising of approximately 20% of the whole human miRNAome. We found that the miRNA targetome regulated by an individual miRNA generally constitutes the biological network of functionally-associated molecules in human cells (Satoh and Tabunoki 2011). Being consistent with our observations, a recent study showed that interacting proteins in the human PPI network tend to share restricted miRNA target-site types than random pairs (Liang and Li 2007). Interestingly, a computational method named mirBridge that assesses enrichment of functional sites for a given miRNA in the annotated gene set showed that many miRNAs coordinately regulate multiple components of signaling pathways and protein complexes (Tsang et al. 2010).

We identified a coordinated regulation of gene expression by transcription factors and miRNAs at transcriptional and posttranscriptional levels in cancer-associated miRNA targetome networks. In mammalian genomes, gene regulatory networks, consisting of positive and negative transcriptional coregulation of miRNAs and their targets, play a crucial role in enhancement of the robustness of gene regulation (Tsang et al. 2007). The protooncogene c-myc directly activates transcription of E2F1, but at the same time limits its translation by upregulating expression of miR-17-5p and miR-20a, both of which negatively regulate E2F1 (O'Donnell et al. 2005). Importantly, a recent study showed that the genes with more transcription factor-binding sites have a higher probability of being targeted by miRNAs and

have more miRNA-binding sites (Cui et al. 2007).

We found that the most relevant pathological event in the whole human miRNA targetome is 'cancer', when top three pathological events were overall cumulated. Furthermore, the highly relevant diseases include 'adult T cell lymphoma/leukemia', 'chronic myelogenous leukemia', and 'hepatocellular carcinoma'. These observations support the general view that the human microRNAome plays a specialized role in regulation of oncogenesis. Therefore, the miRNA-based therapy designed to simultaneously target multiple cancer-associated networks and pathways might serve as the most effective approach to suppressing the oncogenic potential of a wide range of cancers.

5 Acknowledgements

The author thanks Dr. Hiroko Tabunoki and Ms. Midori Ohta for their invaluable help. This work was supported by grants from the Research on Intractable Diseases (H21-Nanchi-Ippan-201; H22-Nanchi-Ippan-136), the Ministry of Health, Labour and Welfare (MHLW), Japan and the High-Tech Research Center Project (S0801043) and the Grant-in-Aid (C22500322), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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Figure legends

Fig. 1. The pathways, diseases, and pathological events relevant to 232 miRNA targetome networks. Among 1,223 human miRNAs examined, Diana-microT 3.0 identified the set of reliable targets from 273 miRNAs. Among them, KeyMolnet extracted molecular networks from 232 miRNAs. The generated network was compared side by side with human canonical networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events to identify the canonical network showing the most statistically significant contribution to the extracted network. After top three pathways, diseases, and pathological events were individually totalized, the cumulated numbers of top 10 of **(a)** pathway, **(b)** disease, and **(c)** pathological event categories are expressed as a bar graph. The figure is cited from our recent study (Satoh and Tabunoki 2011).

Fig. 2. Molecular network of miR-15a targetome. By the neighboring network-search algorithm, KeyMolnet illustrated a highly complex network of miR-15a targetome, which has the most statistically significant relationship with the pathway of 'transcriptional regulation by myb'. Red nodes represent miR-15a direct target molecules predicted by Diana-microT 3.0, whereas white nodes exhibit additional nodes extracted automatically from the core contents of KeyMolnet to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). The transcription factor myb is highlighted by a blue circle. The figure is cited from our recent study (Satoh and Tabunoki 2011).

Fig. 3. Molecular network of miR-106b targetome. By the neighboring network-search algorithm, KeyMolnet illustrated a highly complex network of miR-106b targetome, which has the most statistically significant relationship with the pathway of 'transcriptional regulation by

Rb/E2F'. Red nodes represent miR-106b direct target molecules predicted by Diana-microT 3.0, whereas white nodes exhibit additional nodes extracted automatically from the core contents of KeyMolnet to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). The transcription factor E2F family is highlighted by a blue circle. The figure is cited from our recent study (Satoh and Tabunoki 2011).

Fig. 4. Molecular network of miR-200b targetome. By the neighboring network-search algorithm, KeyMolnet illustrated a highly complex network of miR-200b targetome, which has the third-rank significant relationship with the pathway of 'transcriptional regulation by ZEB'. Red nodes represent miR-200b direct target molecules predicted by Diana-microT 3.0, whereas white nodes exhibit additional nodes extracted automatically from the core contents of KeyMolnet to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). The transcription factors ZEB1 and ZEB2 are highlighted by a blue circle.

Table 1. Large-Scale Human MicroRNA Targetome Networks

MicroRNA	Number of Targets	Molecules in KeyMolnet Networks	Top Pathway	Score	p-Value	Top Disease	Score	p-Value	Top Pathological Event	Score	p-Value
hsa-let-7a	244	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7b	242	1016	Transcriptional regulation by p53	594	1.83E-179	Viral myocarditis	113	9.32E-35	Cancer	206	7.66E-63
hsa-let-7c	243	1020	Transcriptional regulation by p53	593	2.49E-179	Viral myocarditis	113	1.11E-34	Cancer	206	1.10E-62
hsa-let-7d	145	885	Transcriptional regulation by RB/E2F	836	2.18E-252	Chronic myelogenous leukemia	72	1.95E-22	Cancer	130	9.68E-40
hsa-let-7e	236	1111	Transcriptional regulation by p53	575	8.90E-174	Viral myocarditis	116	1.20E-35	Cancer	175	1.86E-53
hsa-let-7f	244	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7g	245	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7i	245	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-miR-9	352	1115	Transcriptional regulation by PPARa	340	5.28E-103	Hepatocellular carcinoma	72	1.69E-22	Cancer	171	3.50E-52
hsa-miR-17	195	961	Transcriptional regulation by RB/E2F	971	3.27E-293	Chronic myelogenous leukemia	92	2.83E-28	Cancer	181	3.58E-55
hsa-miR-19a	226	1094	Transcriptional regulation by RB/E2F	760	2.10E-229	Chronic myelogenous leukemia	113	1.26E-34	Cancer	253	7.04E-77
hsa-miR-19b	225	1094	Transcriptional regulation by RB/E2F	760	2.10E-229	Chronic myelogenous leukemia	113	1.26E-34	Cancer	253	7.04E-77
hsa-miR-20a	165	1038	Transcriptional regulation by RB/E2F	856	1.64E-258	Chronic myelogenous leukemia	87	6.09E-27	Cancer	85	3.33E-26
hsa-miR-20b	198	981	Transcriptional regulation by RB/E2F	962	2.35E-290	Chronic myelogenous leukemia	98	3.39E-30	Cancer	183	6.98E-56
hsa-miR-26a	148	672	Transcriptional regulation by RB/E2F	919	1.76E-277	Chronic myelogenous leukemia	107	6.15E-33	Cancer	181	3.20E-55
hsa-miR-26b	148	672	Transcriptional regulation by RB/E2F	919	1.76E-277	Chronic myelogenous leukemia	107	6.15E-33	Cancer	181	3.20E-55
hsa-miR-27a	229	1192	Transcriptional regulation by CREB	1022	2.23E-308	Chronic myelogenous leukemia	95	1.96E-29	Cancer	194	3.05E-59
hsa-miR-27b	261	1337	Transcriptional regulation by CREB	1022	2.23E-308	Chronic myelogenous leukemia	94	4.51E-29	Cancer	211	4.11E-64
hsa-miR-29a	119	543	Transcriptional regulation by Ets-domain family	430	4.36E-130	Glioma	85	3.46E-26	Cancer	139	1.41E-42
hsa-miR-29b	118	578	Transcriptional regulation by Ets-domain family	422	1.15E-127	Glioma	82	1.55E-25	Cancer	146	1.44E-44

hsa-miR-29c	118	543	Transcriptional regulation by Ets-domain family	430	4.36E-130	Glioma	85	3.46E-26	Cancer	139	1.41E-42
hsa-miR-30a	455	1494	Transcriptional regulation by RB/E2F	777	9.43E-235	Chronic myelogenous leukemia	86	1.11E-26	Cancer	195	2.39E-59
hsa-miR-30b	455	1480	Transcriptional regulation by RB/E2F	781	1.08E-235	Chronic myelogenous leukemia	87	7.01E-27	Cancer	188	1.92E-57
hsa-miR-30c	454	1495	Transcriptional regulation by RB/E2F	778	6.13E-235	Chronic myelogenous leukemia	86	1.15E-26	Cancer	191	3.63E-58
hsa-miR-30d	452	1491	Transcriptional regulation by RB/E2F	778	7.28E-235	Chronic myelogenous leukemia	86	1.01E-26	Cancer	195	1.96E-59
hsa-miR-30e	455	1481	Transcriptional regulation by RB/E2F	780	1.29E-235	Chronic myelogenous leukemia	87	7.25E-27	Cancer	188	2.05E-57
hsa-miR-32	261	905	Transcriptional regulation by RB/E2F	842	2.74E-254	Gastric cancer	80	8.85E-25	Cancer	157	4.19E-48
hsa-miR-92a	219	642	Transcriptional regulation by MEF2	335	1.51E-101	Viral myocarditis	59	1.62E-18	Epithelial-mesenchymal transition	83	7.76E-26
hsa-miR-92b	258	701	Transcriptional regulation by MEF2	328	1.59E-99	Viral myocarditis	60	1.23E-18	Cancer	94	3.97E-29
hsa-miR-93	195	958	Transcriptional regulation by RB/E2F	972	2.37E-293	Chronic myelogenous leukemia	92	2.47E-28	Cancer	181	2.77E-55
hsa-miR-96	142	688	Transcriptional regulation by Ets-domain family	407	3.42E-123	Viral myocarditis	36	1.06E-11	Cancer	106	1.37E-32
hsa-miR-98	162	671	Transcriptional regulation by Myb	549	4.73E-166	Viral myocarditis	85	2.66E-26	Cancer	126	1.42E-38
hsa-miR-101	188	806	Transcriptional regulation by AP-1	492	1.10E-148	Hepatocellular carcinoma	70	6.40E-22	Cancer	127	4.26E-39
hsa-miR-106b	164	1028	Transcriptional regulation by RB/E2F	854	7.21E-258	Chronic myelogenous leukemia	87	5.48E-27	Cancer	85	2.93E-26
hsa-miR-124	285	1346	Transcriptional regulation by RB/E2F	756	3.57E-228	Chronic myelogenous leukemia	83	9.34E-26	Cancer	185	1.90E-56
hsa-miR-137	288	941	Transcriptional regulation by MITF family	339	1.19E-102	Adult T cell lymphoma/leukemia	66	1.30E-20	Cancer	179	1.00E-54
hsa-miR-147	199	867	Transcriptional regulation by RB/E2F	805	4.06E-243	Chronic myelogenous leukemia	113	6.60E-35	Cancer	132	2.57E-40
hsa-miR-153	154	1019	Transcriptional regulation by Myb	507	2.35E-153	Multiple myeloma	60	6.44E-19	Cancer	174	4.31E-53
hsa-miR-218	155	830	Transcriptional regulation by AP-1	344	2.28E-104	Hepatocellular carcinoma	69	1.63E-21	Cancer	136	1.52E-41
hsa-miR-372	101	562	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	85	1.90E-26	Cancer	144	2.75E-44

hsa-miR-429	123	634	Transcriptional regulation by RB/E2F	918	2.45E-277	Chronic myelogenous leukemia	76	1.71E-23	Cancer	130	5.28E-40
hsa-miR-495	156	601	Transcriptional regulation by Ets-domain family	431	2.14E-130	Rheumatoid arthritis	77	5.90E-24	Adipogenesis	79	1.32E-24
hsa-miR-506	394	1536	Transcriptional regulation by Ets-domain family	317	4.69E-96	Viral myocarditis	99	1.73E-30	Cancer	172	1.43E-52
hsa-miR-519a	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-519b-3p	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-519c-3p	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-520a-3p	184	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520b	182	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520c-3p	182	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	93	9.28E-29	Cancer	145	1.77E-44
hsa-miR-520d-3p	183	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520e	184	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-603	252	1150	Transcriptional regulation by Ets-domain family	344	3.26E-104	Multiple myeloma	84	4.36E-26	Cancer	161	4.24E-49

Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted reliable targets from 273 miRNAs. Among them, KeyMolnet extracted molecular networks from 232 miRNAs. The generated network was compared side by side with human canonical networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events. The canonical pathways, diseases, and pathological events with the most statistically significant contribution to the extracted network are shown. The table contains only the large-scale miRNA targetome networks generated by importing targets greater than 100 per individual miRNA into KeyMolnet. The table is cited from our recent study (Satoh and Tabunoki 2011).

中山書店

シリーズ　アクチュアル脳・神経疾患の臨床

最新アプローチ　多発性硬化症と視神経脊髄炎

第 1 章　多発性硬化症の病態と診断

K. 病因・病態をめぐって

2. 網羅的遺伝子発現解析からみた病因・病態

佐藤　準一

Immunopathogenesis of Multiple Sclerosis Clarified by Global Gene Expression Analysis

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Point

- 多発性硬化症(multiple sclerosis: MS)では、遺伝要因と環境要因の複雑な相互作用により誘導された自己抗原反応性の Th1 細胞や Th17 細胞が炎症性脱髄を惹起し、臨床的病理学的に多様な病態を呈する。
- ヒトゲノムが解読され、DNA マイクロアレイや次世代シークエンサーを用いて、個々の細胞における全遺伝子の発現情報を網羅的に解析出来るようになった。
- 近年、ゲノムワイド関連解析(genome-wide association study: GWAS)により、MS 発症のリスクアレルが多数同定された。
- MS と健常者、再発期と寛解期、インターフェロンベータレスポンダーとノンレスポンダーを比較したトランスクリプトーム解析により、各病態に特徴的な遺伝子が多数同定された。
- 生体を複雑なシステムとして捉える観点からすると、分子ネットワークを詳細に解析することにより、論理的な仮説に裏付けられた創薬標的分子を効率的に同定することが出来る。

MSの病態多様性

MS は主として若年成人に好発し、中枢神経系白質に炎症性脱髄巣が多発し、様々な神経症状が再発を繰り返す難病である。MS では遺伝要因と環境要因の複雑な相互作用により、自己抗原反応性を示す Th1 細胞や Th17 細胞が誘導される[1]。これらの細胞は血液脳関門を通過して脳や脊髄に浸潤し、マクロファージやミクログリアを活性化して、炎症性サイトカインの産生を誘導し、脱髄と軸索傷害を惹起する。現在最も一般的な治療法として、再発期にステロイドパルス、寛解期にインターフェロンベータ(interferon-beta: IFNB)の継続的投与が行われている。しかしながら IFNB ノンレスポンダーも多い。MS は臨床経過から再発寛解型(relapsing-remitting MS: RRMS), 2 次進行型(secondary-progressive MS: SPMS), 1 次進行型(primary-progressive MS: PPMS)に分類される。病理学的には T 細胞の浸潤、抗体の沈着、オリゴデンドロサイトのアポトーシスの所見に基づき、4 病型に分類されている[2]。このような MS の病態多様性が、治療難航の一因となっている。また現在まで、髄鞘や軸索の再生促進薬はなく、新規の標的分子に対する画期的な創薬が待望されている。

ポストゲノム時代の創薬研究

2003 年にヒトゲノムプロジェクトが完了し、全塩基配列が解読され、DNA マイクロアレイを用いて、個々の細胞における全遺伝子の発現情報(トランスクリプトーム)を網羅的に解析出来るようになった。さらに最近では、次世代シークエンサー(next-generation sequencing technology: NGS)を用いて、発現量の低い遺伝子も一括して解析可能となっている。また質量分析装置を用いて、細胞のタンパク質(プロテオーム)や代謝物(メタボローム)の網羅的解析も行われている。このようなポストゲノム時代を迎え、創薬研究の中心はオミックス(omics)研究に足場を置くゲノム創薬へとパラダイムシフトした。さらに薬理ゲノミクスは急成長を遂げ、遺伝子多型から薬物応答性の個人差を予測可能となり、テーラメイド医療(personalized medicine)の樹立に道が開かれた。システム生物学(systems biology)の観点からは、ヒトは大規模な分子ネットワークで精密に構築された複雑系であり、多くの難病がシステム固有の防御機構であるロバストネス(robustness)の破綻に起因していると考えられている[3]。従って難病の病態

解明のためには、バイオインフォマティクスの手法を駆使したゲノムワイドの分子ネットワーク解析が必須の研究手段となりつつある[4]。

網羅的発現解析から分子ネットワーク解析へ

現在、DNA マイクロアレイや次世代シークエンサーを用いて、個々の細胞における数万遺伝子の発現情報を短時間で網羅的に解析出来る。多数のサンプルの遺伝子発現情報を迅速に比較解析出来るマイクロアレイは、臨床所見や画像のみでは鑑別困難な疾患の補助診断ツール、腫瘍の悪性度や予後の予測、薬物応答や副作用の予測、治療効果の判定など幅広い臨床の場で利用されている[5]。DNA マイクロアレイは、プローブと呼ばれるオリゴヌクレオチドをスライドグラスやシリカビーズの基盤上に固定するスタンフォード方式と、基盤上で直接オリゴヌクレオチドを合成する®GeneChip(Affymetrix)に大別される。発現解析アレイの他には、スプライスバリアントの網羅的解析が可能なエクソンアレイ、全ゲノムにおける遺伝子多型マッピングや染色体コピー数を解析出来るジェノタイピングアレイ、Chromatin immunoprecipitation (ChIP) on Chip 解析に用いるゲノムタイリングアレイがある。

マイクロアレイでは、比較の対象なる遺伝子発現レベルが異なる 2 種類以上の細胞や組織(例えば正常細胞と癌細胞、治療前後の細胞など)から total RNA または mRNA を抽出し、cDNA, cRNA に変換して蛍光色素で標識後に、フラグメントに切断してハイブリダイゼーションを行う(図 1)[4]。1 色法では 1 サンプルに対して 1 アレイを使用し、アレイ間の発現レベルを比較する。同じ実験条件で、レプリケートとしてアレイを 2-3 枚使用する。アレイを専用スキャナーでスキャン後に、シグナル強度を正規化(normalization)して、サンプル間の遺伝子発現プロフィールを統計学的に比較解析する。マイクロアレイでは一度に非常に多数の遺伝子の発現レベルを解析するため、遺伝子毎に t 検定で評価すると、多数の偽陽性遺伝子を拾ってしまう。通常は多重検定を行い Bonferroni の補正を付加するか、偽陽性率(false discovery rate: FDR)を考慮する。最終的に、比較するサンプル間で、有意な発現差異を呈している遺伝子群(differentially expressed genes: DEG)を抽出し、定量的 PCR を行って発現レベルを検証する。