MicroRNAs in Cancer: The 22nd Hiroshima Cancer Seminar/The 4th Japanese Association for RNA Interference Joint International Symposium, 30 August 2012, Grand Prince Hotel Hiroshima

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The joint international symposium of the 22nd Hiroshima Cancer Seminar and the 4th Japanese Association for RNA Interference focused on a pivotal role of microRNAs in carcinogenesis, progression and therapy of human cancer. Mammalian immune regulator MCPIP1 (Zc3h12a) RNase acts as a novel suppressor of microRNA activity and biogenesis, suggesting the involvement of MCPIP1 in the alteration of microRNA biogenesis in tumorigenesis. Gene set enrichment analysis and functional assignment of microRNAs via enrichment analysis enable the prediction of microRNA activities from mRNA expression data by combining rank-based enrichment analysis and weighted evaluation of microRNA–mRNA interactions. MiR-124 and miR-203 function as tumor-suppressor microRNAs silenced by DNA methylation in hepatocellular carcinoma. Stella-induced DNA hypomethylation would confer the pathogenic function of DNA hypomethylation in cancer. Senescence-associated microRNA, miR-22, suppresses tumor growth and metastasis in vivo in a murine breast cancer model, and exosomal senescence-associated microRNA may affect the tumor microenvironment. The therapeutic potential of microRNAs for preventing and treating lung cancer using the KrasLSL-G12D+/p53LSL-R172H/+ mouse model suggests that miR-34 may be useful in sensitizing tumors to other conventional therapeutics. MiR-1 and miR-133a cluster may function as tumor suppressors regulating novel pathways in human cancers. The down-regulation of miR-148a is implicated in invasion of gastric cancer, while high miR-21 expression in colorectal cancer is associated with poor survival. Neutral sphingomyelinase 2 regulates exosomal microRNA secretion and promotes angiogenesis within the tumor microenvironment as well as metastasis; in particular, the exosomal miR-210 secretion by neutral sphingomyelinase 2 confers the formation of the tumor vessel network.

Key words: microRNAs – biomarkers – carcinogenesis – progression – DNA methylation – tumor microenvironment – exosome

The 22nd International Symposium of Hiroshima Cancer Seminar (HCS) in conjunction with the 4th Japanese Association for RNA Interference was held on 30 August 2012 at Grand Prince Hotel Hiroshima. The symposium composed of nine invited speakers, had ~200 participants and made active discussion on a pivotal role of microRNAs (miRNAs) in carcinogenesis, progression and early detection as well as therapeutic strategies of human cancers. Invited speakers include Hiroshi I. Suzuki (University of Tokyo, Tokyo), Johji Inazawa (Tokyo Medical and Dental University, Japan).
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Tokyo, Toru Nakano (Osaka University, Osaka), Mark A.K. (Stanford University), H.T.(Hiroshima University, Hiroshima), Andrea L. Kasinski (Yale University, USA) and Naohiko Seki (Chiba University, Chiba), Naohide Oue (Hiroshima University, Hiroshima) and Nobuyoshi Kosaka (National Cancer Center Research Institute, Tokyo).

OPENING ADDRESS

Eiichi Tahara (HCS Foundation), Chairman of the Organizing Committee and HCS Foundation, gave an opening address. E.T. mentioned a brief background and the purpose of this series of 1-day’s symposia annually organized since the establishment of the HCS Foundation in 1992. This year, the organizing committee focused on the importance of miRNA in the development, tumor microenvironment, diagnostic and therapeutic potential of human cancers. E.T. also introduced the biogenesis of miRNA and its dysfunction in gastric cancer induced by Helicobacter pylori. In addition, he described a complex interplay between miRNAs and gene regulation systems implicated in the invasion, lymphocyte activation, cancer stem cells and angiogenesis in tumor microenvironment.

SPECIAL LECTURES ON miRNAs IN CACRINOGENESIS,

PROGRESSION AND THERAPY OF HUMAN CANCERS

The aim of the symposium was to understand a pivotal role of miRNAs in carcinogenesis, progression and therapy of human cancers.

Hiroshi I. Suzuki opened the symposium by describing that mammalian immune regulator MCPIP1 (Zc3h12a) RNase acts as a novel suppressor of miRNA activity and biogenesis (1). MCPIP1 counteracts Dicer and suppresses miRNA biosynthesis through the cleavage of the terminal loops of precursor miRNAs (pre-miRNAs). Transcriptome analysis showed a potential antagonistic relationship between MCPIP1 and Dicer in human cancer, suggesting the involvement of MCPIP1 in the alteration of miRNA biogenesis in carcinogenesis. Regarding the non-cell-autonomous roles of miRNAs in cancer progression processes, microRNA-135b (miR-135b) participates in nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-driven oncogenicity and plays a non-cell-autonomous role in the pathogenesis of malignant lymphoma subtype. The term of non-cell-autonomous cancer progression processes means the cancer progression processes modulated independently by the regulation of cell-autonomous function of cancer cells themselves.

Interestingly, miR-135b suppressed Th2 master regulators, GATA3 and STAT6, and rendered Th17-like immunophenotype to anaplastic large cell lymphoma (ALCL) cells, thereby promoting paracrine inflammatory response and tumor angiogenesis. These results collectively illuminated unique contribution of oncogenic kinase-linked miRNA to tumorigenesis through the modulation of tumor immunophenotype and microenvironment. In addition, a novel approach for the dissection of mRNA–miRNA network in various cancers was presented. Previous studies have shown that mammalian miRNAs decrease the levels of many target mRNAs and reduce protein production predominantly by the destabilization of target mRNAs. However, it has not yet been fully assessed whether this scheme is widely applicable to more realistic conditions. Excitingly, gene set enrichment analysis and functional assignment of microRNAs via enrichment analysis (GFA) enables the prediction of miRNA activities from mRNA expression data by combining rank-based enrichment analysis and weighted evaluation of miRNA–mRNA interactions. This cooperative approach delineated a better widespread correlation between predicted miRNA activities and miRNA expression levels in cancer transcriptomes, thereby providing proof-of-concept of the mRNA-destabilization scenario. Moreover, the inference of miRNA activity by GFA could be utilized for the selection of prognostic miRNAs in the development of cancer survival prediction models. These results will provide the molecular basis for the development of diagnostic and therapeutic strategies based on small RNA biology.

Johji Inazawa reported tumor-suppressor miRNAs (TS-miRNAs) silenced by DNA methylation using three different approaches as follows: (i) DNA methylation-based screening by combined bisulfite restriction analysis and bisulfate sequencing, (ii) expression-based approach with quantitative PCR assays of 148 miRNAs in a panel of cancer cell lines and the control and (iii) function-based screening with a cell proliferation assay for synthetic miRNA precursor libraries and a series of sequential analyses of DNA methylation and expression. Through the methylation-based screening, miR-124 and miR-203 were identified as TS-miRNAs in hepatocellular carcinoma (HCC). MiR-124 and miR-203 showed frequent tumor-specific methylation, and their expression status was inversely correlated with methylation status. The ectopic expression of miR-124 or miR-203 in HCC cells lacking their expression inhibited cell growth, with the direct down-regulation of possible targets, CDK6, vimentin (VIM), SMYD3 and IQGAP1 or ABCE1, respectively. In addition, epigenetic silencing of miR-137 and miR-193 might play a pivotal role in oral carcinogenesis. Moreover, using the function-based approach, epigenetically silenced TS-miR, miR-218, was found in oral squamous cell carcinoma (2) and miR-152 in endometrial cancer, respectively. Interestingly, those two miRNAs can inactivate rapamycin-insensitive companion of MTOR as their target, inducing the activation of a TOR-Akt signaling pathway. Restoring the function of TS-miR(s) by miRNA replacement may be a promising therapy in cancers.

Toru Nakano described Stella-mediated DNA hypomethylation in transformation.
Stella, also known as PGC7 and Dppa3, is a protein essential for early embryogenesis, as Stella binds to the chromatin containing di-methylated histone H3 lysine 9 (H3K9me2) and controls the subcellular localization of Tet3, which is a critical enzyme for active DNA demethylation in early embryos (3). To examine the molecular function of Stella in more detail, the gene was introduced into NIH3T3 cells. Although it was expected that Stella would have protected the DNA methylation in the somatic cells as well as in the early embryos, global DNA hypomethylation took place oppositely. This hypomethylation is presumably due to the binding of Stella to NP95, a hemi-methylated DNA-binding protein essential for the maintenance of DNA methylation.

In addition, quite unexpectedly, the transformation of 3T3 cells and the enhancement of metastatic ability of B16 melanoma cells were brought about by Stella. The experimental system of Stella-induced DNA hypomethylation would be an excellent model to cast new insights onto the pathogenic function of DNA hypomethylation in cancer.

M.A.K. introduced parameters influencing the generation and loading of siRNAs from transcriptionally derived duplex RNAs. In order to optimize the shRNA activity, he studied the parameters that affect miRNA/shRNA processing and duplex RNA loading into RNA-induced silencing complex (RISC) in mammalian cells. The mammalian RISC contains a single-stranded RNA derived from a duplex miRNA/siRNA and one-of-four Argonaute (Ago) proteins. Ago loading is the process of duplex RNA association followed by the removal of the inactive passenger strand RNA. He established that shRNAs are loaded into mammalianagos in two stepwise processes, physical association and activation. Although RNA duplexes processed from shRNAs bind to Ago in cells with similar affinity, the degree by which the complexes are activated (coupled with the removal of the passenger strand) correlates with the thermodynamic instability of RNA duplexes rather than the structure of the RNA, as was previously demonstrated in Drosophila (4). He has begun to further dissect the process of RISC loading by studying the function of the evolutionary conserved PAZ (Piwi/Ago/Zwille) domain of Ago proteins. These genetic, cellular and biochemical studies establish that this domain plays an important role in RISC maturation. Taken together, these results provide insights into new shRNA designs for RNA interference-based therapeutics.

Hidetoshi Tahara talked on senescent-associated miRNAs and extracellular vesicles in aging and cancer. Cellular senescence confers an important mechanism of tumor suppression, and thus dysregulation of senescence-associated miRNAs (SA-miRNAs) may promote tumor formation in vivo. Among identified putative several SA-miRNAs, miR-22 is significantly up-regulated in senescent fibroblasts and down-regulated in various cancer cell lines (5). Importantly, miR-22-induced cellular senescence accompanied by enlarged morphology, senescence-associated β-Gal activity (SA-β-Gal) and senescent-associated heterochromatin foci formation. Surprisingly, therapeutic miR-22 delivery significantly suppresses tumor growth and metastasis in vivo in a murine breast cancer model. In fact, miR-22 down-regulates several putative target genes such as CDK6, SP-1 and SIRT1 by 3’UTR assay and western blotting. Interestingly, while these three putative target genes are involved in senescent phenotype, miR-22 activates senescence-associated secretory phenotype (SASP), which regulates chemokine and inflammatory cytokine signals in senescent cells. Recently, extracellular vesicles such as exosome-like vesicles, which are 50–100 nm in size including miRNA, also secrete from mammalian cells. These exosome-like vesicles can interact with neighboring cells as well as other cells in tissue through communication with body fluids such as blood. Although SASP is well characterized in cellular senescence, little is known about exosome in cellular senescence. Therefore, he examined exosome secretion and exosome-miRNA profiling using miRNA array analysis and then found the significant increased secretion of exosomes in senescent fibroblast cells. Interestingly, little secretion of extracellular exosomes containing SA-miRNAs such as miR-22, miR-30 and miR-34a is found in senescent cells, whereas significant secretion of exosomes is found in senescent cells. In addition, these senescent-associated exosomes may also influence the tumor microenvironment. Taken together, these results indicate that the alterations of intracellular senescence-associated miRNA expression accompanied with the alteration of secretory factors, including exosomes and SASP during cellular senescence, are key regulators of aging, and may affect tumor microenvironments.

Andrea L. Kasinski presented the therapeutic potential of miRNAs for preventing and treating lung cancer using the Kras\textsuperscript{LSL-G12D/+};p53\textsuperscript{LSL-R172H/+} mouse model, which is characterized by one of the most accurate representations of human lung cancer generated thus far. In the model, both Kras and p53 transgenes are induced spatially and temporally following recombination when exposed to cre-recombinase, which was delivered intratracheally to the lung using lentiviral-cre. Following transgene activation, adenocarcinomas were evident as early as 10 weeks with severe lung inflammation presenting at 22 weeks. Epithelial cells from these tumors were generated and shown to be capable of supporting growth in soft agar assays, invading based on transwell migration assays and forming palpable tumors in nude mice. The levels of miR-34 were evaluated in both the cell lines and lung tumor tissue obtained from Kras\textsuperscript{+};p53\textsuperscript{+} mice. While the three miR-34 family members showed some variability in their expression levels, globally total precursor and mature miR-34 levels were decreased substantially while oncogenic miRNAs such as miR-21 and miR-155 were elevated. To identify whether miR-34 replacement might be a viable option in vivo, miR-34a was first introduced into the Kras\textsuperscript{+};p53\textsuperscript{+} mutant cells using replication incompetent miR-34a expressing lentiviral particles, which upon integrating into the genome of transduced cells results in sustained high pre-miR-34a expression. When compared with control-transfected cells, miR-34-treated lines were reduced in their
proliferation, ability to form colonies in two dimensions, capability to recover from a scratch wound and invasion potential. Molecularly, the miR-34 targets, Bcl-2 and Met were down-regulated following transient transfection with miR-34a. Based on these promising results, two series of in vivo experiments were presented. First, the contribution of miR-34 to prevent tumor formation in Kras\(^{LSL-G12D/+; \ p53^{LSL-R172H/+}}\) mice was evaluated. Animals that were treated with lentivirus of miR-34 at the same time as pre-induced recombination of transgenes showed little-to-no evidence of tumorigenesis 19 weeks post-transgene activation/treatment, while control animals had multiple nodules that represented \(\sim 8\%\) of the total lung area. The second series of in vivo experiments evaluated the ability of miR-34 to function as a treatment for preformed tumors using lentivirus delivery. Although miR-34 was unable to reduce the size of the preformed tumors in these animals, it did prevent further tumor growth. These data support the use of miR-34 as a tumor preventive mechanism and suggest that miR-34 may be useful in sensitizing tumors to other conventional therapeutics (6).

Naohiko Seki introduced the expression profiles of tumor suppressive miRNAs in several types of human cancers including head neck cancer, lung cancer, bladder cancer, renal cell cancer and prostate cancer. Excitingly, the expression levels of miR-1 and miR-133a were significantly down-regulated in various cancers (7). In addition, miR-1-1/133a-2 and miR-1-2/miR-133a-1 are clustered on different chromosomal regions in the human genome, 20q13.33 and 18q11.2, respectively. Restoration of miR-1 or miR-133a in cancer cells revealed significant inhibition of proliferation, migration and invasion, suggesting that both the miRNAs may function as tumor suppressors regulating several oncogenic pathways in human cancers.

Naohide Oue addressed on alterations of miRNA expression in gastrointestinal cancer. miRNA expression was analyzed in 20 gastric cancer (GC) cases by miRNA-PCR array. By comparing miRNA expression profiles, the down-regulation of miR-148a frequently took place in GC. Array analysis revealed that the expression of several invasion-associated genes, such as MMP1 and MIA, was repressed in miR-148a-transfected GC cells, and the invasion ability of miR-148a-transfected GC cells was 40% less than that of the negative control siRNA-transfected GC cells. In looking at whether the expression levels of miR-21 can predict the prognosis for patients with colorectal cancer (CRC), high miR-21 expression in tumors was associated with poor survival, independent of clinical covariates, including TNM staging. In patients who received adjuvant chemotherapy, high miR-21 expression was significantly associated with poor therapeutic outcomes. These results indicate that miRNAs play important roles in cancer cell invasion and provide a great deal of information on biomarkers to identify patients with poor prognosis.

Nobutoshi Kosaka described exosomal miRNA as a novel humoral factor for cancer development. Recent evidence indicates that exosomal miRNAs play critical roles in mediating cell–cell communication, specifically between immune cells, endothelial cells and cancer cells. He recently found that miRNAs are released through neutral sphingomyelinase 2 (nSMase2)-regulated secretory machinery and that these secretory minas by exosome are transferable and functional in recipient cells. The established breast cancer cell lines stably overexpressing siRNA specific for mouse nSMase2 showed that preventing nSMase2 expression abrogated the metastatic ability of cancer cells to target lung tissues, whereas administration of exosomes isolated from metastatic cancer cells rescued this phenomenon. Interestingly, the number of endothelial cells observed in inoculated tumors was proportional to the expression level of nSMase2 in cancer cells. In fact, exosomes derived from a metastatic cancer cell line enhanced the capillary formation of endothelial cells in vitro, which provides evidence of a permissive niche for outgoing tumor cells. In addition, the expression profile of exosomal miRNAs obtained from metastatic cancer cells demonstrated that a set of angiogenic miRNAs were highly concentrated in these exosomes. One of them, miR-210, was up-regulated under the hypoxic condition, leading to the enrichment of miR-210 in exosome. This miR-210-enriched exosome enhanced the migration and capillary formation through the suppression of specific target gene, which resulted in enhanced angiogenesis, suggesting that exosomal miRNAs secretion confers the formation of tumor vessel network, thereby promoting the metastasis. These findings provide the evidence that the intercellular network between cancer cells and their environmental cells via secretory miRNAs exported by exosome is essential for cancer development and metastasis (8).

Conflict of interest statement
None declared.

References