Meeting Report

Cancer Research

Twenty-Second Annual Pezcoller Symposium: RNA Biology and Cancer

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Abstract

The 22nd annual Pezcoller Symposium titled "RNA Biology and Cancer" was held in Trento, Italy, on June 10–12, 2010. The program of the symposium was developed by Drs. Rene Bernards, Witold Filipowicz, and David Livingston; they cochaired the meeting in cooperation with Dr. Enrico Mihich. The topics discussed included the opportunities offered by small RNA as tools for cancer drug development, the role of noncoding RNAs, the biochemistry of small RNAs, the function of micro RNA in cancer, and RNAs as diagnostics and therapeutics in cancer. *Cancer Res; 70(24); 10034–7. ©2010 AACR.*

Findings Presented

The 22nd annual Pezcoller Symposium titled "RNA Biology and Cancer" was held in Trento, Italy, on June 10–12, 2010. Carlo Croce (Ohio State University) stated that alterations in the expression of micro RNA (miRNA) genes contribute to human malignancies pathogenesis. Malignant cells depend on dysregulated expression of miRNA genes, which control multiple protein-coding oncogenes or tumor suppressor genes. For example, in the indolent form of chronic lymphocytic leukemia (CLL), the expression of miRNA-15 and -16 is downregulated, unblocking the expression of its target, the antiapoptotic oncogene *Bcl-2*. Coadministration of miRNA-15 and -16 can be advocated for CLL therapy. The same miRNA can behave as a tumor suppressor gene and oncogene depending on the cell type and microenvironment.

Rene Bernards (The Netherlands Cancer Institute) discussed the use of gain and loss of function genetic screens to clarify the mechanisms of drug resistance and sensitivity. Functional approaches can help to predict responsiveness to targeted anticancer drugs. These drugs are often highly selective for the cancer cells harboring activated target pathways. Genetic screens can not only predict how individual patients respond to these drugs but may also facilitate the development of drugs acting synergystically with established drugs to prevent or overcome resistance. Once drug response biomarkers are identified in relevant cell lines, related genes expression is correlated with clinical response. Thus, Bernards showed that resistance to retinoic acid (RA) in neuroblastoma (NB) is not caused by RA receptor (RAR) mutations but rather by loss of expression of the tumor suppressor gene *NF1*, which activates RAS RAF-MEK signaling, which leads to the loss of expression of *ZNF423*, a crucial c oactivator of the RAR in NB. Importantly, inhibition of MEK by a small molecule drug restored responsiveness to RA in NB cells having low level of *NF1* expression, suggesting a potential strategy to overcome RA resistance in NB.

Alan Ashworth (Breakthrough Research Center) outlined the use of high- and medium-throughput RNAi screens to uncover new therapeutic targets for cancer and biomarkers in patients who might respond to specific treatments. The integration of genomic, gene expression, and RNAi data to generate functional maps of cancer cell lines was discussed. Mismatched DNA repair (MMR) was studied; MMR deficiency was accompanied by increased polymerase expression and increased 8-oxoguananine in nucleus. Methotrexate (MTX) was selective for MSH2-deficient cells. Clinical trials of MTX in MSH2-deficient colorectal cancer are underway.

Lars Zender (Hannover Medical School) discussed *in vivo* RNAi screens dissecting signaling pathways in liver regeneration and cancer. Oncogenic *Nras* (NrasG12V) efficiently triggers hepatocellular carcinomas (HCC) derived from $p53^{-/-}$ liver progenitor cells; however, almost no tumors occur when NrasG12V is delivered into $p53^{-/-}$ hepatocytes. In contrast, aggressive HCCs develop when oncogenic Nras is delivered into $p19^{Arf-/-}$ hepatocytes. To identify mediators of p53-independent tumor suppressive functions of $p19^{Arf}$ in the mouse liver, an *in vivo* RNAi screen was set up and several new candidate genes mediating p53-independent tumor suppressive functions of $p19^{Arf}$ in the mouse liver were identified. Furthermore, a new mouse model was developed that allows performing *in vivo* RNAi screens for molecular modulators of liver regeneration. Mouse livers were stably repopulated with an shRNAmir library consisting of 631 constructs, and after repopulation, mouse livers were treated with CCL_4 to induce chronic liver damage. Several shRNAs showed strong enrichment or depletion during liver regeneration and therefore

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Note: The participant list for this meeting is available as supplementary data at Cancer Research Online (http://cancerres.aacrjournals.org/).

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pinpoint new positive or negative regulators of liver regeneration.

Yi Liu (University of Texas Southwestern Medical Center) discussed small RNAs biogenesis in fungi. In addition to small interfering RNAs (siRNA) and miRNAs, several types of small RNAs play important roles in gene regulation. In *Neurospora*, qiRNA, a type of small RNA induced by DNA damage, was identified. By analyzing small RNA associated with the argonaute protein QDE-2, it was shown that diverse pathways generate miRNA-like small RNAs (milRNA) and Dicer-independent siRNAs in *Neurospora*. milRNAs are processed by at least 4 different mechanisms involving Dicers, QDE-2, the exonuclease QIP, and a novel RNase III domain-containing protein.

Joachim Lingner (Swiss Institute for Experimental Cancer Research) reported on the large noncoding RNA TERRA (telomeric repeat containing RNA) and its function as a natural telomerase inhibitor. TERRA is bound to human telomerase in cell extracts, base-pairing with the template sequence of the telomerase RNA moiety (hTR). In addition, TERRA contacts the telomerase reverse transcriptase (TERT). *In vitro*, TERRA is a very potent inhibitor of human telomerase outcompeting the binding of telomeric DNA. In *Saccharomyces cerevisiae*, genetic experiments support that TERRA inhibits telomerase *in vivo*.

Scott Hammond (University of North Carolina) noted that restoration of some miRNA in cancer cells has anticancer effects. Many miRNAs are increased during embryogenesis. Cluster miR-17-92 is regulated mainly at transcription. Tissue restricted miRNAs and widely expressed miRNAs in the Let-7 and miR-125 families are not expressed in early development but are induced during mid-gestation. These miRNAs are often reduced in cancer. Let-7 biogenesis is regulated posttranscriptionally. A major component of this regulation is via Lin28, whose binding site on the Let-7 stem loop was characterized.

Pier Paolo Pandolfi (Harvard Medical School) discussed a messenger RNA (mRNA) coding-independent function. Given that mRNAs bind to miRNAs, they could possess a regulatory role based on their competition for miRNA binding. The relationship between the mRNAs produced by the *PTEN* tumor suppressor gene and its highly homologous pseudogene *PTENP1* and the critical consequences of this interaction were discussed. *PTENP1* can compete for miRNA binding and regulate cellular levels of *PTEN*. PTENP1 locus is selectively lost in human cancer. These findings attribute a novel biological role to pseudogenes, as they reveal noncoding functions for mRNAs.

Manel Esteller (Spanish National Cancer Center) discussed the disruption of noncoding RNAs in cancer. Possible mechanisms of miRNA deregulation in cancer are failure of miRNA posttranscriptional regulation, CpG island promoter hypermethylation-associated transcriptional silencing, transcriptional repression, and mutational impairment of the TARBP2 miRNA processing gene. Silencing of tumor suppressor miRNAs by CpG island hypermethylation is common in human tumors and was elucidated by treating lymph node metastatic cells with a DNA demethylating agent, followed by hybridization to an expression microarray. miR-148a, miR-34b/c, and miR-9 were found to undergo hypermethylation-associated silencing in cancer cells. The reintroduction of miR-148a and miR-34b/c in cancer cells inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with downregulation of miRNA oncogenic target genes. miR-148a, miR-34b/c, and miR-9 hypermethylation was associated with lymph node metastasis. Silencing of tumor suppressor miRNAs contributes to the development of human cancer metastasis.

Witold Filipowicz (Friedrich Miescher Institute for Biomedical Research) outlined the mechanisms of miRNA repression and metabolism in mammalian cells. Mature miRNAs are incorporated into RNP complexes, miRNPs, which silence mRNA targets. GW182 family proteins are important factors for miRNA repression in metazoa. They interact with Ago proteins and form part of P-bodies implicated in translational repression and mRNA degradation. The C-terminal fragments of GW182 proteins are potent mediators of translational repression and mRNA decay. Like mammalian GM182 protein, *Drosophila* dGW182 causes both inhibition of translation and mRNA deadenylation. In collaboration with Botond Roska, miRNAs regulated by different light levels, independent of circadian time, were identified in the mouse retina. Many retinal miRNAs were found to undergo rapid turnover, and miRNAs of the miR-182/183/96 cluster and miR-204/211 were found to undergo transcriptional upregulation in response to light. Rapid turnover is also characteristic of miRNAs expressed in nonretinal neurons.

Reuven Agami (The Netherlands Cancer Institute) discussed miRNA and RNA binding proteins (RBP) in cancer, with particular emphasis on miRNA 221 regulation of p27 and the function of p53. RBPs potentially control the biogenesis, stability, activity of miRNAs, and their accessibility to target mRNAs. A large-scale screen to identify RBPs that controls miRNA accessibility to target mRNAs has led to the identification of RBPs required for cancer cells proliferation and for optimal p53 function.

Frank Slack (Yale University) discussed the possibility of targeting miRNAs as a therapy for human cancer. miR-21 is almost ubiquitously overexpressed in human cancer, and the prediction is that *mir-21* is an oncogene (oncomiR). Slack showed that mice overexpressing the *mir-21* oncomiR develop an aggressive disease reminiscent of pre-B cell lymphoma and die of the disease. The lymphoid cells are malignant and can cause tumors in immunocompromised mice. When diseased mice have the expression of miR-21 terminated, the tumors completely regress. Tumor regression involves a combination of apoptosis and proliferation arrest. Tumors thus show evidence of miR-21 dependence, the so-called "oncomiR addiction." These studies support efforts to target oncomiRs as an anticancer therapy.

Gunther Hartmann (University of Bonn) outlined the mechanisms of immunogenic tumor cells apoptosis induced by RIG-I. RNA with a triphosphate group at the 5′ end (3pRNA) is the ligand for RIG-I, and the crystal structure of 3pRNA bound to the CTD domain of RIG-I was resolved. RIG-I-RNA ligand interaction not only activates type I IFN but also induces inflammasome activation and proapoptotic signaling.

RIG-I ligands are promising candidates for therapy of viral infection and cancer. siRNAs containing triphosphate groups at the 5′ ends (3p-siRNA) were developed for melanoma therapy and to silence bcl-2 and activated the cytosolic helicase RIG-I. Treatment with bcl-2–specific 3p-siRNA elicited strong activity in a metastatic melanoma model. RIG-I–induced apoptosis of tumor cells synergized with apoptosis induced by siRNA-mediated bcl-2 silencing. *In vivo*, these mechanisms provoked massive apoptosis in lung metastases. The therapeutic activity of 3p-siRNA *in vivo* required NK cells, type I IFN, and silencing of bcl-2. Thus, 3p-siRNA represents a novel single-molecule–based combinatorial approach for tumor therapy.

Irene Bozzoni (University of Rome La Sapienza) discussed the pathogenesis of Duchenne muscular dystrophy (DMD) and ways to treat this disease that is caused by mutations in the dystrophin gene. The disruption of the dystrophin-associated protein complex at the muscle membrane leads to the disease pathogenesis. Exon skipping restores dystrophin expression and confers benefit in animal models. Taking advantage of a controlled rescue of dystrophin synthesis through exon skipping in *mdx* mice, molecular circuitries, important for muscle differentiation and tissue integrity, were found to be directly controlled by dystrophin through epigenetic modulation of a specific class of miRNAs. Several circuitries controlled by the these miRNAs, such as the one linking miR-1 to the G6PD enzyme and the redox state of cell, or miR-29 to extracellular proteins and the fibrotic process, explain some of the DMD pathogenetic traits.

Eva Hernando's (New York University Langone Medical Center) laboratory is studying the role of miRNAs in melanoma progression. She showed that miR-182-96-183 overexpression, which promotes melanoma metastasis, not only can result from genomic amplification but can also be due to epigenetic dysregulation. Using miRNA arrays, her laboratory has also identified another miRNA cluster (miR-30b/30d) as overexpressed in metastatic melanoma, with higher levels correlating with increased stage and recurrence rate in primary tumors. Ectopic expression of miR-30b or miR-30d enhanced the invasive capacity of melanoma cells *in vitro* and their metastatic potential *in vivo*. Transcriptional analyses revealed that this cluster exerts these effects by modulating direct and indirect targets involved in cell adhesion and immune modulation. Her laboratory is currently exploring the therapeutic potential of targeting miRNAs, using anti-miR oligonucleotides against a mouse model of melanoma liver metastasis.

Anna Krichevsky (Brigham and Women's Hospital) discussed the functions of miRNA in glioblastoma (GBM). miR-21 is upregulated in GBM. It regulates genes associated with cell cycle, apoptosis, migration, and invasiveness, including RECK and TIMP3, tumor suppressors and inhibitors of matrix metalloproteinases (MMP). miR-21 inhibition with antisense oligonucleotides reduces MMP activities *in vitro* and in human glioma in nude mice. miR-296 is upregulated in primary tumor endothelial cells from human brain tumors compared with normal brain endothelial cells and in endothelial cells cocultured with glioma cells *in vitro*. By targeting hepatocyte growth factor–regulated tyrosine kinase substrate mRNA, miR-296 controls growth factor receptors on endothelial cells and thus tumor-associated angiogenesis.

Gunter Meister (Max-Planck Institute of Biochemistry) outlined the role of miRNAs in GBM pathogenesis. miR-9 and miR-9* are abundant in GBM stem cells. Inhibition of miR-9/9* promotes neuronal differentiation, suggesting that miR-9/9* inhibits differentiation of GBM stem cells. A miR-9/9* target calmodulin-binding transcription activator 1 is a novel tumor suppressor in GBMs that correlates with patient survival.

Dalia Cohen (Rosetta Genomics) discussed the use of miRNA in therapeutics in HCC and in diagnostics of lung cancer. Hsa-miR-191 was found to be a potential drug target for HCC and its inhibition caused a decrease in cell proliferation and apoptosis *in vitro* and a reduction in an orthotopic liver tumor xenograft. This miRNA was found to be a key regulator of cancer-related pathways. miRNAs can be used for differential diagnosis and drug monitoring. In patients with lung cancer, miR-205 was found to be highly expressed in squamous cell carcinoma and low in nonsquamous cell carcinoma. This miRNA differential expression in non–small cell lung carcinoma is the basis of a diagnostic test differentiating between squamous versus nonsquamouns

Sakari Kauppinen (Santaris Pharma a/s) presented progress in locked nucleic acid (LAN)-based miRNA targeting for therapeutics. LNAs comprise bicyclic, high-affinity RNA analogues, in which the ribose ring in the sugar-phosphate backbone is locked in an RNA-like, C3′-endo-conformation by the introduction of a 2′-O,4′-C methylene bridge. Single-stranded, LNA-modified oligonucleotides have high binding affinity to complementary miRNAs. miRNA knockdown was achieved using 7- to 8-nucleotide LNA-modified phosphorothioate oligonucleotides, termed tiny LNAs, complementary to miRNA seed region. Transfection of tiny LNAs into cells results in successful inhibition of miRNA seed families with concomitant upregulation of direct targets. Tiny LNAs are taken up by several tissues, but they do not pass the blood–brain barrier. These data support the utility of tiny LNAs in exploring the functions of miRNA families, with important implications for the development of therapeutic strategies aiming at pharmacologic inhibition of disease-associated miRNAs. Kauppinen also presented an update on the therapeutic development of LNA-anti-miR-122 for the treatment of hepatitis C virus infection.

Luigi Naldini (San Raffaele Telethon Institute for Gene Therapy) discussed the possibility of exploiting miRNA regulation in gene therapy. By incorporating target sites for a specific miRNA into a gene transfer vector, its expression becomes susceptible to regulation in cells where that miRNA is expressed. miR-130a and miR-126 were expressed in mouse and human HSC and early progenitors. miR-126 expression was maintained in human cord blood progenitors during *in vitro* culture, and this allowed isolation of cells capable of long-term engraftment in immunodeficient mice. By making the vector responsive to miR-126 regulation, transgene expression was suppressed in HSC while sustained at high levels in mature cells. This vector design allowed for the first time

successful gene therapy in a mouse model of globoid cell leukodystrophy.

Summary

As discussed, a large number of different miRNAs have important roles in determining the development and maintenance of different types of cancer and other diseases. They offer, among others, opportunities for diagnosis, therapeutic intervention, selection of appropriate patients for treatment, and monitoring treatment effectiveness. The discussions held at this symposium should stimulate further progress in each of these directions and should provide further insights into the mechanisms of miRNA action.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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