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Using miRNA as Biomarkers to Evaluate the Alcohol-Induced Oxidative Stress

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1. Introduction

Oxidative stress is responsible for a variety of degenerative processes in many human diseases, as either cause or effect. At present, some biomarkers of oxidative stress have been used to determine an individual's oxidative status in relation to disease conditions. However, their accuracy, sensitivity, or specificity needs to be improved. The development of novel biomarkers for oxidative stress is urgent.

Micro RNAs (miRNAs) are highly conserved regulatory molecules expressed in eukaryotic cells. They are short non-coding RNAs that regulate gene expression by binding to target mRNAs, which leads to reduced protein synthesis and sometimes decreased steady-state mRNA levels. Although hundreds of miRNAs have been identified, much less is known about their biological function. There are evidences that miRNAs affect pathways fundamental to metabolic control in higher organisms such as adipocyte and skeletal muscle differentiation. Also, some miRNAs are implicated in lipid, amino acid, and glucose homeostasis. Thus, miRNA abnormalities may contribute to common metabolic and systemic diseases where oxidative stress plays a key role in their pathogenesis. Indeed, there are evidences indicated that miRNAs are able to modulate the cellular response to oxidative stress both *in vitro* and *in vivo*. Therefore, miRNA may be novel biomarkers for oxidative stress.

We hypothesize that miRNAs may be biomarkers for oxidative stress because: (1) since miRNA are post-transcriptional gene regulators, they may be able to function as 'quick responders' to oxidative stress. For example, upon exposure to stress, miRNA may rapidly localize to P-bodies or stress granules to regulate key genes involved in the oxidative stress response. After the stress is mitigated, miRNA inhibition may be promptly abated, allowing

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[*] Correponding Author

commencement of translation and expeditive restoration of cells back to their normal state; (2) since miRNAs regulate numerous targets, they have the capacity to powerfully and efficiently coordinate a stress response involving numerous genes; (3) owing to their small size and high stability, miRNAs may be less susceptible to certain types of stress, like genotoxic insults. Hence, their ability to modulate stress response would be less likely to be compromised under oxidative-stress. Given the recent development in the field of miRNA research, we predict that miRNA will be promised biomarkers for oxidative stress.

To test our hypothesis, we chose to study Alcoholic Liver Disease (ALD) as an oxidative stress model because: (1) it has high morbidity and mortality with no satisfactory therapy; (2) we showed that oxidative damage is the major mechanism for ALD; (3) we have established and validate that ALD model in rats is a good model for studies of oxidative stress. Our studies demonstrated that: (1). Nitric oxide-induced oxidative stress is required for alcohol-induced gut leakiness and liver damage in this model; (2) The miRNA expression profile was identified by miRNA microarray analysis. The miRNAs signatures were validated by TaqMan real time PCR assay. Our research results demonstrated that the differentially expressed miRNAs are the sensitive and specific biomarkers for alcohol-induced oxidative stress. (3) We showed that oats supplementation, a diet with strong anti-oxidative effect that is widely used in diets to prevent many diseases associated with oxidative damage, prevents ALD in rats by preventing alcohol-induced oxidative tissue damage.

Numerous markers of oxidative stress and antioxidant status have been evaluated, but there has been little systematic effort to validate sensitive and specific biomarkers for oxidative damage in animal models. The application of miRNA as new biomarkers will lead to: a) identification susceptible individuals who are at risk for oxidative stress and would thus benefit from interventions that provide antioxidants; b) novel strategies to prevent and treat oxidative injury.

2. Biomarkers for oxidative stress in human disease

Oxidative/nitrosative stress, such as alcohol-induced increase of reactive oxygen/nitrogen species (ROS/NOS), is now recognized to be a common cause or a prominent feature of many acute and chronic inflammatory diseases(Dalle-Donne et al., 2006). However, up to date, there are not ideal biomarkers and/or methods available to assess oxidative stress status in human diseases. Thus, we are exploring and searching for new biomarkers of oxidative stress, such as miRNA, to objectively measure and evaluate the role of miRNA in the alcohol-induced oxidative stress.

Biomarkers may provide information on three progressive levels of disease outcome(Dalle-Donne et al., 2006): (*a*) as measurable endpoints of damage of biomolecules such as lipids and proteins; (*b*) as functional markers of, for example, cognitive function; and (*c*) as endpoints related to specific disease. In many cases, oxidative stress is an early and common pathphysiological process. Thus, it is very important to find a series of biomarkers to early detect the alcohol-induced oxidative stress. Studying the association between a biomarker and alcohol-induced oxidative stress could benefit for early detection and, therefore, prevention of diseases associated with oxidative stress.

The most intuitive goals for a biomarker are to help diagnose symptomatic and presymptomatic disease and to provide surrogate endpoints to demonstrate clinical efficacy of new treatments(Ogino and Wang, 2007). The usefulness of the ideal biomarker of oxidative damage lies in its ability to provide early indication of disease and/or its progression (Fig. 1).
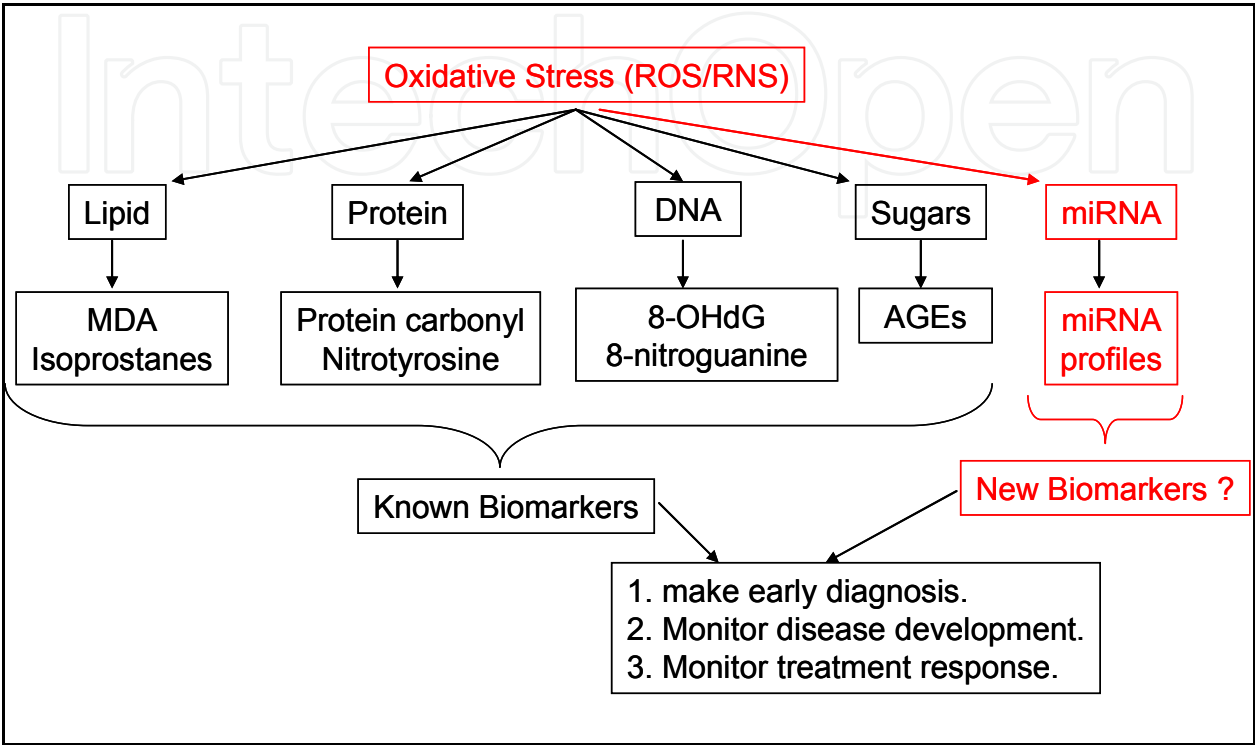

Fig. 1. Biomarkers for oxidative stress

When investigating the status of alcohol-induced oxidative stress, it is unclear what the most appropriate biomarker is and how to measure them. At present, the biomarkers of oxidative stress/damage and the methods used to measure them are different among different study groups. Thus, it is difficult to compare the study findings in different groups to determine which one is the best biomarker to evaluate an individual's oxidative status in relation to alcohol consumption.

In addition, the validity of many biomarkers remains to be established. The biomarkers that have been developed and currently been used to evaluate the oxidative stress have several shortcomings(Dalle-Donne et al., 2006), such as: (*a*) the limited specificity of the assay itself for the product of oxidative/nitrosative damage being measured; (*b*) the fact that the analyte being measured is not a specific product of a specific ROS/RNS; (*c*) the lack of sufficient sensitivity to detect concentrations of the product being measured in healthy individuals, thus not allowing the definition of a reference interval; (*d*) concentrations of the product being measured being influenced by external factors such as the lipid content of the diet; or (*e*) the assay being too invasive for in vivo investigations in humans.

So far, several oxidative stress biomarkers have been used in clinics to assess patient's reaction to oxidative stress, their accuracy, sensitivity, or specificity need to be improved

(Dalle-Donne et al., 2006; Ogino and Wang, 2007). The identification of novel biomarkers is urgently needed. miRNA may hold great promise as a biomarker for oxidative stress.

3. miRNA may be biomarkers for oxidative stress

miRNAs are highly conserved regulatory molecules expressed in eukaryotic cells. They are short noncoding RNAs that regulate gene expression by binding to target mRNAs, which leads to reduced protein synthesis and sometimes decreased steady-state mRNA levels(Ambros, 2004; Carthew, 2006; Carthew and Sontheimer, 2009; Li and Carthew, 2005). Although hundreds of miRNAs have been identified, much less is known about their biological function. There is evidence that miRNAs affect pathways fundamental to metabolic control in higher organisms such as adipocyte and skeletal muscle differentiation. Also, some miRNAs are implicated in lipid, amino acid, and glucose homeostasis. Thus miRNA abnormalities may contribute to common metabolic diseases and there may be novel therapeutic opportunities based on miRNA targeting. Indeed, the expression of certain genes can depend more on levels of regulatory miRNAs than on levels of mRNAs. miRNAs act through a mechanism similar to that of short interfering RNAs (siRNA). The expression of miRNA target genes can be fine-tuned in animals by altering the concentrations or identities of miRNAs within cells.

There are many evidences indicated that miRNAs are able to modulate the cellular response to oxidative stress both in vitro and in vivo(Babar et al., 2008). We hypothesize that miRNAs may be biomarkers for oxidative stress due to following several reasons. First, since miRNA are post-transcriptional gene regulators, they may be able to function as 'quick responders' to oxidative stress. For example, upon exposure to stress, miRNA may rapidly localize to P-bodies or stress granules to regulate key genes involved in the oxidative stress response. After the stress is mitigated, miRNA inhibition may be promptly abated, allowing commencement of translation and expeditive restoration of cells back to their normal state. Second, since miRNA regulate numerous targets, they have the capacity to powerfully and efficiently coordinate a stress response involving numerous genes. Third, owing to their small size and high stability, miRNA may be less susceptible to certain types of stress, such as genotoxic insults. Hence, their ability to modulate stress response would be less likely to be compromised under high-stress condition. Given the recent development in the field of miRNA research, we predict that miRNA will be promised biomarkers for oxidative stress.

4. Animal model of Alcoholic Liver Disease (ALD) is a good model for studying the alcohol-induced oxidative damage

ALD is one of the most common and serious complications of heavy drinking. It is a major health problem in the US, consuming 15% of total health care dollars, and associated with 20% mortality(Maher, 2002). However, the mechanisms linking Ethanol (EtOH) consumption to ALD are not completely understood.

Our in vitro, in vivo animal, and ex-vivo human studies provided compelling evidence for the central involvement of iNOS activation in EtOH-induced gut leakiness. Indeed,

several of our studies (Banan et al., 2000a; Banan et al., 2000b; Banan et al., 2007; Keshavarzian et al., 2001; Keshavarzian and Fields, 2000; Keshavarzian and Fields, 2003; Keshavarzian et al., 1999; Tang et al., 2009a; Tang et al., 2009c) have shown that iNOS activation is required for EtOH-induced gut leakiness. We reported that: **1)** EtOH increases iNOS activity and NO levels in intestinal monolayers and increases monolayer permeability. A specific iNOS inhibitor (L-NIL) prevented EtOH-induced monolayer leakiness; **2)** EtOH no longer can cause leakiness in monolayers incapable of upregulating iNOS (i.e., transfected with dominant negative iNOS antisense); **3)** iNOS is increased in intestinal mucosa of alcoholics with ALD and in alcohol-treated rats with gut leakiness and endotoxemia; **4)** Daily gavage of the specific iNOS inhibitor L-NIL prevented iNOS upregulation and oxidative stress in the intestinal mucosa of alcohol-fed rats and also prevented alcohol-induced gut leakiness; **5)** Daily gavage of Lactobacillus GG or supplementation of the diet with oats prevented nitration of intestinal mucosal proteins, oxidative stress, and gut leakiness in alcohol-fed rats; **6)** A daily, alcohol-containing (Nanji) diet for 4 weeks caused gut leakiness in wild type mice but NOT in iNOS knockout mice. The unanswered question is whether miRNAs are the biomarkers for EtOH-induced oxidative injury in blood, intestinal epithelium, or liver.

5. Oats supplementation is an antioxidant

Oats, like many other plant materials, contain numerous constituents vitamins, minerals, essential fatty acids, β-glucan (fermentable fibers), and phytochemicals, including several phenolic compounds. These constituents have been found to possess many types of bioactivity, including antioxidant, antiproliferaton, anti-inflammatory, and detoxification effects, which may contribute to the promotion of good health (Chen et al., 2007).

We hypothesized that oats supplementation protects through its effects on oxidative pathways. We had two primary rationales for our hypothesis. First, it has been generally accepted that oats are of benefit to human health and normal gut growth and function not only because of their nutrient and fiber values, but also, because of their antioxidant and anti-inflammatory activities. Second, several studies have demonstrated the importance of oxidative stress and upregulated iNOS in alcohol-induced tissue injury and organ dysfunction. More specifically, several reports demonstrated the pivotal role of the upregulation of iNOS and oxidative stress in alcohol-induced gut leakiness. For example, our in vitro studies showed that preventing the upregulation of iNOS that is induced by alcohol, using both iNOS inhibitors and dominant negative mutant for iNOS, prevented alcohol-induced disruption of the barrier integrity of intestinal cell monolayers.

Furthermore, we recently showed that inhibition of iNOS by L-NIL reduces EtOH-induced NO overproduction, oxidative tissue injury and gut leakiness in alcohol-treated rats. Our current study, which uses immunohistochemical staining, provides direct evidence that EtOH induces iNOS activation in colonic epithelium and that oats prevent this effect and prevent alcohol-induced intestinal mucosal oxidative stress.

We confirmed our in vitro findings in an animal model of alcoholic steatohepatitis (ASH). We showed that chronic, daily alcohol administration to rats caused gut leakiness. More importantly, we showed that EtOH-induced gut leakiness in rats was associated with

endotoxemia and alcoholic steatohepatitis. Furthermore, we showed that oats supplementation prevents loss of intestinal barrier integrity, endotoxemia and steatohepatitis(Keshavarzian et al., 2001; Tang et al., 2009a). However, the mechanism for the protective effects of oats is unclear.

6. Opportunity and potential impact

Numerous markers of oxidative stress and antioxidant status have been evaluated, but there has been little systematic effort to validate sensitive and specific biomarkers for oxidative damage in animal models.

Development of miRNA as a new biomarker for alcohol-induced oxidative stress is limited by the lack of easy access to the tissues from patient populations. Therefore, the majority of discovery work will need to be carried out in the animal model of ALD. The advantage provided by animal models is the ability to control and define disease stages. We have to correlate these model diseases to the clinical status of actual patient populations. The process of biomarker discovery in animal models will be validated by clinical studies. As the technology develops to allow higher throughput screening of miRNA, these candidate biomarkers can be more easily tested and applied to larger patient populations.

The *long term goal* of our laboratory is to design an effective therapeutic intervention to prevent and treat oxidant-induced disorders. Increasing our understanding of the mechanism of oxidant-induced gut leakiness should lead to identification of optimal targets for development of new preventive and therapeutic strategies for alcohol-induced, endotoxin mediated, tissue damage such as ALD. Our studies should thus lead to development of: **a)** miRNA as biomarkers for identifying susceptible individuals who are at risk for endotoxemia & ALD and would thus benefit from interventions that prevent gut leak; **b)** novel strategies to prevent ALD by preventing gut leakiness and endotoxemia in alcoholics; **c)** novel therapies to treat advanced alcoholic and non-alcoholic liver disease, because gut leakiness can perpetuate the hepatic necroinflammatory cascade (via feedback loops) and thereby contribute to the progression of liver injury. Since our model involves oxidative stress and iNOS, the results could have relevance to other pathological conditions where gut leakiness and oxidative stress play key roles like non-alcoholic liver disease, inflammatory bowel disease & food allergy to name a few.

7. Results

7.1 Relative miRNA expression in rat intestine of ALD model

Fluorescence signals of the hybridized miRXploreTM Microarrays were detected using a laser scanner from Agilent (Agilent Technologies). In figure 2 a representative false-color image of the microarray experiment is shown as an example; Red color indicates that the Hy5 signal intensity is higher than the Hy3 signal intensity. Therefore, the corresponding gene is overexpressed in the Hy5-labeled sample. Green spots, however, indicate that the fluorescence intensity in the control sample is stronger than in the experimental sample. Yellow spots indicate that the signal intensities are equal for both samples. The signal intensities of each spot/miRNA that passed the quality filtering are shown in a double-

logarithmic scale (Fig.3), represented by a dot. X-axis: Hy3 signal intensity, y-axis: Hy5 signal intensity. Dashed diagonal lines define the areas of x-fold differential signal intensities. Approximately 30 miRNAs including miR-212, miR-7, miR-145, and miR-146a are expressed primarily in digestive tract tissues.

Fig. 2. Hy5/Hy3 false-color image after scanning of microarray

The successful development of effective antioxidant therapies remains a key goal, the attainment of which is required to elucidate the role played by accumulation of oxidized molecules in clinical picture of disease associated with oxidative stress. The use of miRNA as a biomarker provides a logical scientific basis for major intervention trials of antioxidants; such trials could in turn eventually validate or disprove the biomarker concept.
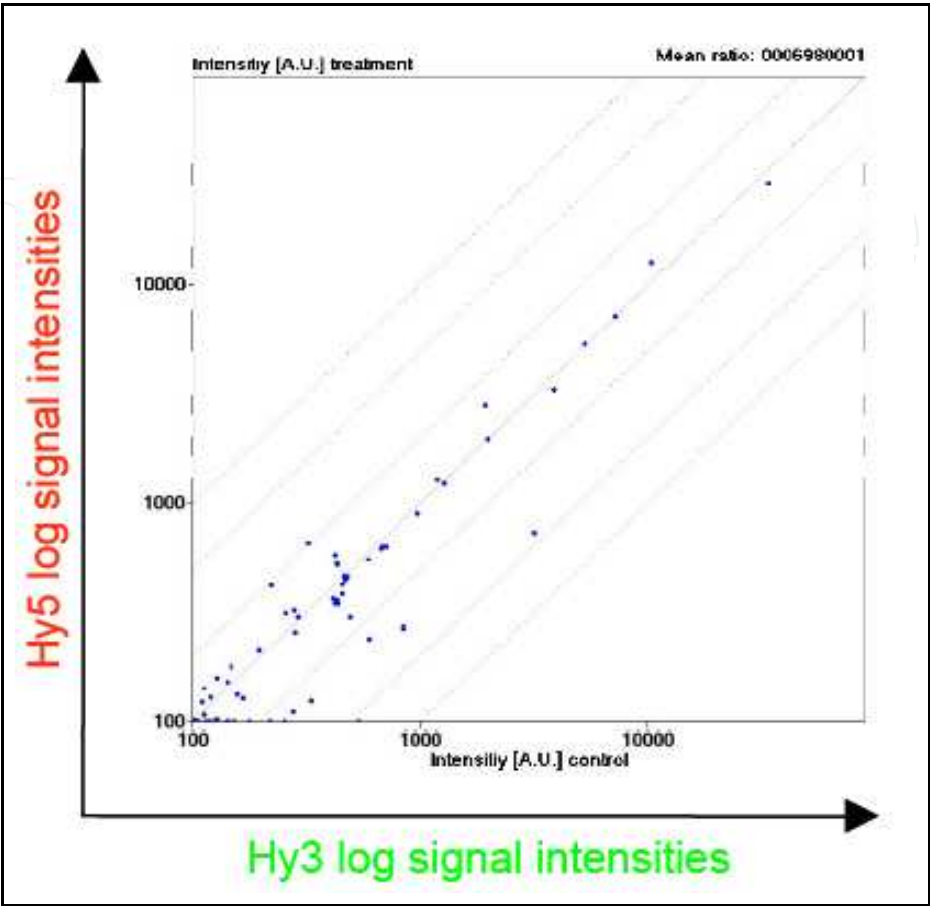
Fig. 3. Duble-log scatter plot

7.2 miR-212 expression in animal model of ALD

To translate our in vitro observation to an in vivo model to establish the key role of miRNA-212 in EtOH-induced gut leakiness, we turned to use an animal model of alcohol-induced gut leakiness. This model has been established and validated in our previous studies. Recently, to determine whether gut leakiness and endotoxemia are one of the key co-factors for development of alcoholic steatohepatitis (ASH), we studied time courses for development of gut hyperpermeability, endotoxemia, and liver injury and showed that gut leakiness and endotoxemia occurred several weeks prior to development of ASH. These data support the notion that gut leakiness causes endotoxemia, which leads to alcoholic steatohepatitis and serious ALD.

In this animal model, rats were given daily EtOH (6 g/kg, by gastric gavage) for 10 weeks. The miRNAs expression levels in the intestine mucosa were assayed by miRNA microarray analysis (Miltenyi Biotec, Auburn CA). The data show that miR-212 expression in EtOH treated group was up regulated by 1.5 fold compared with control group (**Fig.4**).
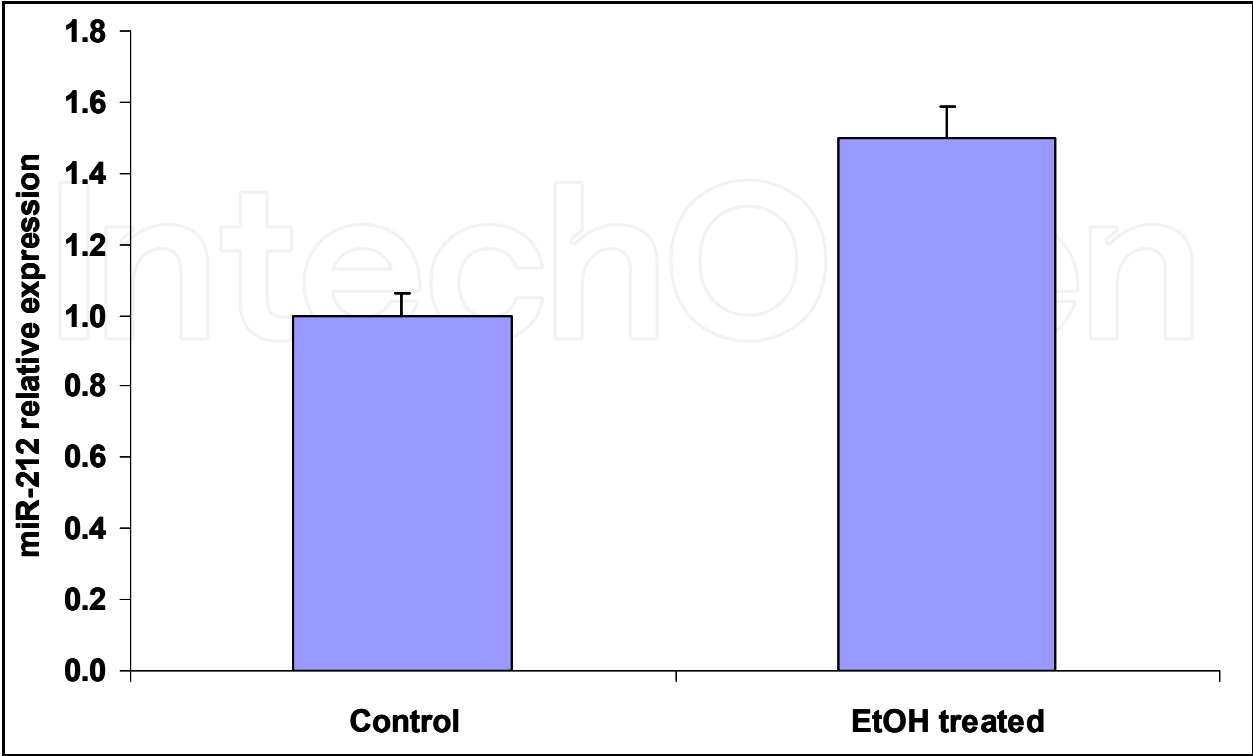
Fig. 4. miR-212 expression levels in intestinal mucosa were increased in rats of ALD model. The rats were fed with EtOH (6 g/kg, by gastric gavage) for 10 weeks. The miR-212 expression levels were assayed by miRNA microarray analysis

8. Our recent publications related to the role of miRNA in the alcohol-induced oxidative stress (Keshavarzian et al., 2009; Tang et al., 2008; Tang et al., 2009a; Tang et al., 2009c)

8.1 Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease (Tang et al., 2008)

Alcoholic liver disease (ALD) is commonly associated with intestinal barrier dysfunction. Alcohol-induced dysregulation of tight junction proteins such as zonula occluden 1 (ZO-1) in intestinal epithelial cells, plays an important role in regulation of intestinal permeability. MicroRNAs (miRNAs) are recently discovered small noncoding RNAs that can regulate gene expression by targeting mRNAs and triggering either translational repression or RNA degradation. ZO-1 is predicted to be a target gene of one such miRNA, miR-212. We previously showed that miR-212 levels in colon biopsy samples in ALD patients were higher than in healthy controls; while ZO-1 protein levels were lower. Here, we studied the mechanisms for the involvement of miR-212 in alcohol-induced gut leakiness. We showed that miR-212 is highly expressed in intestinal tissues using a TaqMan microRNA real time PCR assay. Alcohol-induced miR-212 over-expression is accompanied by reductions in ZO-1 protein expression, disruption of tight junction protein (ZO-1), and increased permeability of Caco-2 cell monolayers. Alcohol-induced

miR-212 over-expression correlated with alcohol-induced disruption of monolayer integrity. To demonstrate that miR-212 acts directly at the ZO-1 3'UTR, we inserted the miR-212 target site of ZO-1 3'UTR into luciferase reporter construct and transfected it into Caco-2 cells. The expression of luciferase was significantly decreased when cotransfected with miR-212. This suppression was relieved by a single base mutantion in the UTR binding site. To see if miR-212 regulates ZO-1 levels, we did both overexpression studies using miR-212 precursors and inhibition studies using miR-212-specific antisense oligonucleotide inhibitors (anti-miR-212). miR-212 over-expression significantly inhibited ZO-1 protein expression. Knocking down of miR-212 expression in Caco-2 cells using anti-miR-212 inhibited alcohol-induced hyperpermeability by 50% (p<0.05). Our studies suggest a novel mechanism for alcohol-induced gut leakiness. Alcohol induces miR-212 over-expression which disrupts intestinal barrier integrity by inhibiting ZO-1 translation. This cascade could lead to dysfunction of tight junction and increase intestinal permeability. This mechanism provides a potential therapeutic target for preventing the leaky gut in patients with ALD.

8.2 Oxidative stress is required for alcohol-induced gut leakiness and liver damage (ALD model)(Keshavarzian et al., 2009)

Thus ALD model is excellent model for studying the biomarkers of oxdative stress. Time courses for development of gut hyperpermeability, nitric oxide production, oxidative injury to the gut, endotoxemia, and liver injury were assessed in these ALD medol. Liver fat and serum transaminase increased after 2 weeks, but evidence of liver cell injury and inflammation occurred after 8 weeks. Gut leakiness, intestinal oxidative injury, and endotoxemia occurred in weeks 2-4 and progressed thereafter. Our data support the hypothesis that oxidative stress is a key co-factor (trigger) for ALD.

8.3 Nitric oxide mediated oxidative injury is required for ALD (Tang et al., 2009c)

We hypothesized that iNOS inhibitors (L-NAME, L-NIL) in vivo will inhibit the above cascade and liver injury in an animal model of alcoholic steatohepatitis (ASH). Male Sprague-Dawley rats were gavaged daily with alcohol (6 g/kg/day) or dextrose for 10 weeks ± L-NAME, L-NIL or vehicle. Systemic and intestinal NO levels were measured by nitrites and nitrates in urine and tissue samples, oxidative damage to the intestinal mucosa by protein carbonyl and nitrotyrosine, intestinal permeability by urinary sugar tests, and liver injury by histological inflammation scores, liver fat, and myeloperoxidase activity. The results showed that alcohol caused tissue oxidation, gut leakiness, endotoxemia and ASH. L-NIL and L-NAME, but not the D-enantiomers, attenuated all steps in the alcohol-induced cascade including NO overproduction, oxidative tissue damage, gut leakiness, endotoxemia, hepatic inflammation and liver injury. *Conclusions:* The mechanism we reported for alcohol-induced intestinal barrier disruption in vitro – NO overproduction, oxidative tissue damage, leaky gut, endotoxemia and liver injury – appears to be relevant in vivo in an animal model of alcohol-induced liver injury. That iNOS inhibitors attenuated all steps of this cascade suggests that prevention of this cascade in alcoholics will protect the liver against the injurious effects of chronic alcohol and that iNOS may be a useful target for prevention of ALD.

8.4 Oats supplementation prevents alcohol-induced oxidative tissue damage in rats (Tang et al., 2009b)

We previously reported that oats supplementation prevents gut leakiness and alcoholic steatohepatitis in our rat model of ALD. Since oxidative stress is implicated in the pathogenesis of both alcohol-induced gut leakiness and ASH, and since oats have anti-oxidant properties, we tested the hypothesis that oats protect by preventing alcohol-induced oxidative damage to the intestine. Oxidative stress and injury were assessed by measuring colonic mucosal iNOS (by immunohistochemistry), nitric oxide, (colorimetric assay) and protein carbonylation and nitrotyrosination (immunoblotting). Colonic barrier integrity was determined by assessing the integrity of the actin cytoskeleton (immunohistochemistry) and the integrity of tight junctions (electron microscopy). Oats supplementation prevented alcohol-induced upregulation of iNOS, nitric oxide overproduction in the colonic mucosa, and increases in protein carbonyl and nitrotyrosine levels. This protection was associated with prevention of EtOH-induced disorganization of the actin cytoskeleton and disruption of tight junctions. We conclude that oats supplementation attenuates EtOH-induced disruption of intestinal barrier integrity, at least in part, by inhibiting EtOH-induced increases in oxidative stress and oxidative tissue damage. This inhibition prevents alcohol-induced disruption of the cytoskeleton and tight junctions. This study suggests that oats may be a useful therapeutic agent – a nutriceutical – for the prevention of alcohol-induced oxidative stress and organ dysfunction.

9. Conclusion

Our research results demonstrated that the differentially expressed miRNAs are the sensitive and specific biomarkers for alcohol-induced oxidative stress. MiRNAs are potential biomarkers to accurately measure the degree of oxidative stress, early detect the indication of disease, and evaluate the effectiveness of antioxidant therapy (fig.5). The validation of miRNAs as biomarkers for alcohol-induced oxidative stress requires further studying. The key steps for validation of miRNA as suitable biomarkers for alcohol-induced oxidative stress are summarized in Fig.6. The application of miRNA as new biomarkers will lead to: a) identification susceptible individuals who are at risk for oxidative stress and would thus benefit from interventions that provide antioxidants; b) novel strategies to prevent and treat oxidative injury.
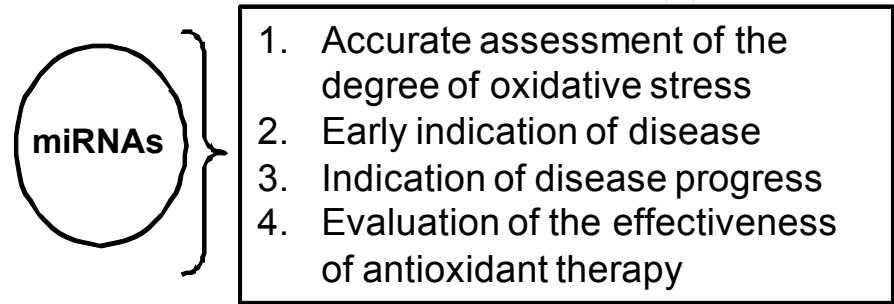

Fig. 5. Potential uses of miRNA as biomarkers for oxidative stress

> 1. Selection of miRNAs as biomarkers for oxidative stress
> 2. Measuring miRNAs (microarray and real time PCR)
> 3. Verification of miRNAs as biomarkers in suitable animal model (ALD)
> 4. Test the sensitivity, simplicity, and specificity of miRNAs for oxidative stress-induced injury
> 5. Validation of miRNAs as biomarkers for oxidative stress using antioxidants (oats)

Fig. 6. Key steps for validation of miRNAs as suitable biomarkers of oxidative stress

10. Future direction

In last two decades, there has been great progress in development of biomarkers of oxidative stress that may eventually be useful in disease prevention. The challenges for future miRNA studies are (1) to validate available biomarkers for oxidative stress in animal and human studies based on their specificity, stability for storage, reproducibility, causal relation with disease, and response to antioxidant intervention; (2) to examine the basal lebels of oxidative damage in healthy subjects; and (3) to assess the long-term effect of antioxidants, such as oats, on oxidative damage by well-designed, randomized, controlled trials in human and as well as to examine the consistency of the findings among various studies. The identification of miRNAs as biomarkers of oxidative damage, if validated, may open the way for the development of early detection and prevention strategies for oxidative stress-associated human diseases.

11. Acknowledgement

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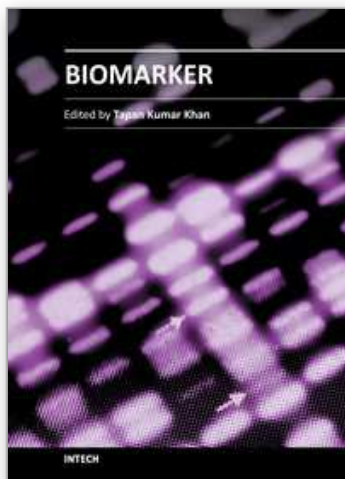
Biomarker

Edited by Prof. Tapan Khan

Clinicians, scientists, and health care professionals use biomarkers or biological markers as a measure of a person's present health condition or response to interventions. An ideal -biomarker should have the following criteria: (I) ability to detect fundamental features of the disease, (II) ability to differentiate from other closely related diseases, (III) ability to detect early stages and stages of progression, (IV) the method should be highly reliable, easy to perform and inexpensive, and (V) sample sources should be easily accessible from body. Most of the chapters in this book follow the basic principle of biomarkers.

How to reference

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