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INVITED RESEARCH HIGHLIGHT

Male Aging

Plasma miRNA expression profile in the diagnosis of late-onset hypogonadism

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Researchers reporting in the Nature journal *Scientific Reports*[1] have used next generation sequencing and quantitative reverse transcriptase PCR (RT-PCR) technology to profile plasma microRNA (miRNA) expression in cohorts of men with and without late-onset hypogonadism (LOH). The study proposes a panel of three miRNAs as novel biomarkers to aid in the diagnosis of LOH.

LOH describes a syndrome in aging men of symptomatic biochemical hypogonadism in the absence of identifiable pathology of the gonadal axis. The syndrome has been controversial for a range of reasons. First, mild biochemical hypogonadism is highly prevalent in aging male populations, as are symptoms potentially attributable to hypogonadism, but the association between symptoms and testosterone (T) levels is weak.[2] Second, symptoms which can be the result of hypogonadism are common and nonspecific. There is no definitive biological consequence of hypogonadism in men analogous to the cessation of menses in women. Third, longitudinal observational studies have demonstrated that the age-associated decline in T is better correlated with the accumulation of obesity and comorbidities than with chronological age itself.[3] In the presence of good health, the decline in T after age 40 is minimal.[4] Finally, whether the low T that characterizes LOH is a cause of symptoms or merely a bystanding marker of comorbidity has not been established.[5]

Using derivation and validation cohorts from the European Male Aging Study (EMAS), a set of criteria for the diagnosis of LOH was developed based on symptoms with the strongest associations with T levels and the T thresholds below which those symptoms become increasingly prevalent. This definition requires the presence of 3 sexual symptoms (reduced libido, reduced frequency of morning erections, and reduced sexual thoughts) combined with a repeated total T concentration <8 nmol l^{-1} and a calculated free T <220 pmol l^{-1}.[2] Yet meeting these minimum criteria does not prove that men will benefit from exogenous T. Indeed, some improvements in sexual symptoms aside, evidence that T treatment is beneficial in men meeting the criteria for LOH is limited,[6] and there have been safety concerns.[7] If a subgroup of men with LOH could be identified as having symptoms or end-organ deficits likely attributable to hypogonadism and potentially remediable with its correction, T replacement could be better targeted.

The study by Chen and coworkers represents a novel approach to this problem.[1] The implied hypothesis was that in otherwise healthy Chinese men aged 40–65 years, LOH would be associated with a plasma miRNA expression profile that was different from normal and that this profile could be used as a diagnostic tool to distinguish individual men with LOH from those without.

miRNAs are small noncoding RNA molecules that function as an epigenetic control mechanism by preventing the translation of target mRNA.[8] MicroRNAs can be encoded within introns of host genes encoding proteins or within introns or exons of noncoding but transcribed regions of the genome.[9] miRNAs are transcribed as immature "primary miRNAs" that are processed into mature miRNAs, single-stranded molecules, 18–22 nucleotides in length. In the cytoplasm, they form a protein-RNA complex known as RISC (RNA-induced silencing complex). The miRNA component directs the RISC to complementary target mRNAs based on antisense binding with the 3' untranslated region of mRNA. The result is either degradation of the mRNA strand or translational repression without degradation.[10] There are 2588 human mature miRNAs listed in the miRBase database although only a subset of these entries are designated as "high confidence."[11]

miRNAs have wide-ranging biological roles in tissue development, homeostasis, and disease including in embryonic development,[12] immune responses,[13] and oncogenesis.[14] Potential therapeutic roles for miRNAs are being explored in cancers such as glioblastoma and pancreatic adenocarcinoma.[14] The advent of high-throughput sequencing technology combined with quantitative PCR has allowed the miRNA transcriptome in different physiological and disease states to be characterized. These miRNA signatures are showing promise as biomarkers for a range of diseases.

miRNA expression profiles can be generated from tissue biopsies, isolated from peripheral blood leukocytes or measured in plasma. Plasma miRNAs are found bound to protein or within vesicles, making them resistant to degradation by plasma RNases. They have been shown to be stable in the circulation, and because plasma miRNAs are derived from many tissues and correlate with changes in gene expression, they are attractive as potential biomarkers for disease.[15] To date, miRNA expression profiles have shown potential as biomarkers of malignancies,[14] sepsis,[16] lupus,[13] and many other conditions.

In the present study, researchers conducted random sampling of the age-stratified general male population in six regions of China. A total

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of 6898 men were recruited by invitation and underwent health assessments including a validated Chinese version of the Ageing Males Symptoms Scale, anthropometry, and blood tests for sex hormone levels by immunoassay. The diagnosis of LOH was made based on EMAS criteria[2] after exclusion of comorbidities (gonadal axis disorders, cancer, metabolic syndrome, type 2 diabetes, cardiovascular disease, and "other diseases") and the use of medications that could affect the metabolism of sex hormones. From an unspecified number of men aged 40–65 meeting the EMAS criteria for LOH, 36 men were selected along with 36 controls matched 1:1 for age, BMI, living area, smoking, and alcohol intake.

In the discovery phase, pooled plasma from 10 LOH patients was compared to that from 10 controls. RNA was isolated and molecules 18–30 nucleotides in length were selected. RT-PCR and then PCR amplification of cDNA products were performed before purification of products by electrophoresis and quantitation by real-time PCR. PCR products were then sequenced using next generation sequencing technology. By aligning to the reference genome in GenBank, sequences corresponding to miRNA were documented. The vast majority of sequences aligned to regions annotated as other species of short RNA and these were not included in further analysis. Thus, an miRNA expression profile was developed for the LOH plasma (828 miRNAs) and the control plasma (1101 miRNAs). There was differential expression of 239 miRNAs. Sixteen were considered to have significantly different expression based on stringent criteria.

The 16 putative biomarkers were then verified using an independent method, namely direct measurement with quantitative RT-PCR using the same pooled plasma samples. Expression ratios between LOH plasma and control plasma were normalized using the mean ratios for three small RNAs, commonly used as endogenous controls. Of the original 16 miRNAs, five emerged from this analysis as having robust, repeatable, and significant fold differences in expression between LOH and control plasma.

In the validation phase, the differential expression of these five miRNAs was investigated in the remaining 22 participant pairs. Expression levels were directly measured in each individual's plasma by quantitative RT-PCR. This analysis confirmed significant differences in expression of only three miRNAs, namely, miR-125a-5p, miR-361-5p, and miR-133a-3p. Each of these was downregulated in LOH patients compared with controls. Combining these in a panel with optimized expression cutoffs yielded an area under the ROC curve of 0.835 (95% CI 0.717–0.952) for the diagnosis of LOH (sensitivity of 68.2% and specificity of 86.4%).

Differential expression of miRNAs suggests differences in posttranscriptional gene regulation in men with LOH. Chen and colleagues discuss the biological plausibility for downregulation of miR-125a-5p, miR-361-5p, and miR-133a-3p in LOH, but the known biological roles of these miRNAs do not readily suggest a role in LOH.[1] However, an individual miRNA may regulate hundreds of target genes,[10] and the range of genes regulated by many miRNAs is incompletely defined. miR-361-5p expression was negatively associated with T level while miR-133a-3p was positively associated with symptoms.

At present, T and calculated free T are the only biomarkers for hypogonadism in aging men. Men who have compatible symptoms, repeatedly low T and evidence of disease affecting the gonadal axis, can be readily diagnosed with classic hypogonadism, and treated with replacement T. In aging men, when LOH is being contemplated, the common clinical scenario is a mildly low T, possibly compatible symptoms and the presence of comorbidities and medications which themselves may be responsible for gonadal axis suppression and symptoms. The minimum diagnostic criteria for LOH developed from the EMAS cohort provides some guidance but does not prove that the symptoms are due to low T, nor does it imply that T treatment will be beneficial. Clinical examination for evidence of hypogonadism such as sarcopenia, visceral adiposity, and assessment for osteopenia may add diagnostic information, but these findings are complicated by the same confounding, lack of specificity, and reverse causality considerations as serum T.

Assuming accessibility of an assay and further validation in larger, more diverse cohorts, would this new panel of biomarkers assist in making the diagnosis of LOH? It would be helpful to define whether these downregulated miRNAs are a cause or an effect of reduced androgen signaling, or less helpfully, whether they are markers of comorbid conditions such as obesity. In the validation cohort, men with LOH had a higher prevalence of obesity, smoking, and alcohol consumption compared to controls, raising the possibility of confounding from these, but also from other unmeasured variables. Notably, the predictive value of each of the three miRNAs did remain in multivariate logistic regression analysis on variables including age, smoking status, alcohol intake, and BMI. It would be informative to test the hypothesis that men with asymptomatic mild biochemical hypogonadism and those with sexual symptoms but eugonadism would be categorized as normal by these biomarkers.

Concerning generalizability, the validation cohort used in this study (n = 22 pairs of Chinese men) was younger than European men in the EMAS cohort used to validate the LOH definition (n = 1609)[2] owing to the exclusion of men older than 65. They also had a higher prevalence of smoking and alcohol consumption than in EMAS. The EMAS cohort had prevalent heart disease (15%), diabetes (8.4%), and cancer (5.2%), which were comorbidities that were excluded from this study.

Finally, T was measured by gas chromatography-mass spectrometry (GC-MS) in EMAS and by immunoassay in this study. Immunoassay is inaccurate and may overestimate low concentrations of total T with reference to GC-MS[17] which would have resulted in the inclusion of men with comparatively higher testosterone levels in the LOH cohort. It is unclear whether men in this study had repeat measurements of testosterone, and if not, this too would have tended to include eugonadal men in the LOH group.

This study is the first to examine the plasma miRNA transcriptome in LOH. Generalizability concerns aside, it raises the possibility that just as in better-defined disease states such as cancer, there is a distinct miRNA expression profile in LOH. This expression profile was accurate in this population at distinguishing men with clinically defined LOH from controls. The real utility of a new biomarker for LOH would be if it were able to identify a subgroup of men who are more likely to benefit from treatment with T, given the current conflicting evidence about the risk-benefit ratio of widespread treatment. The current study was not designed to assess this, but it may prompt further research involving miRNAs as biomarkers in LOH.

COMPETING INTERESTS

Both authors declared no competing interests.

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