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Effect of plasma MicroRNA on antihypertensive response to beta blockers in the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) studies

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Abstract

β -blockers show variable efficacy as antihypertensives. Herein, we evaluated plasma miRNAs as biomarkers for defining antihypertensive response to β -blockers. Expression of 22 β -blocker pharmacodynamics-related miRNAs was assessed in baseline plasma samples from 30 responders and 30 non-responders to metoprolol from the PEAR-2 study (Discovery). Logistic regression was performed to identify miRNAs significantly associated with metoprolol response. Those miRNAs were profiled in baseline plasma samples from 25 responders and 25 non-responders to atenolol from the PEAR study (validation). In discovery, miR-101, miR-27a, miR-22, miR-19a, and let-7e were significantly associated with metoprolol response ($P = 0.01, 0.017, 0.025, 0.025, \text{ and } 0.04$, respectively). In validation, miR-19a was significantly associated with atenolol response ($P = 0.038$). Met-analysis between PEAR-2 and PEAR revealed significant association between

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Disclosures

The authors declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejps.2019.02.013>.

miR-19a ($P = 0.004$), miR-101 ($P = 0.006$), and let-7e ($P = 0.012$) and β -blocker response. Hence, miR-19a, miR-101, and let-7e, which regulate β_1 -adrenergic receptor and other β -blocker pharmacodynamics-related genes, may be biomarkers for antihypertensive response to β -blockers.

Keywords

Beta-blocker; microRNA; Antihypertensive response; Biomarker; Hypertension

1. Introduction

Hypertension (HTN) affects around a billion individuals globally and contributes significantly to cardiovascular morbidity and mortality. (Kearney et al., 2005; Mozaffarian et al., 2015) About 50% of the treated hypertensive patients do not have their blood pressure (BP) controlled, (Egan et al., 2010; Mozaffarian et al., 2015) and uncontrolled HTN increases the risk of myocardial infarction, stroke, chronic renal failure, congestive heart failure, and death (Mozaffarian et al., 2015). (β -adrenergic receptor blockers (β -blockers) are commonly used antihypertensive medications, with over 100 million prescriptions annually in the United States. The effects of β -blockers arise from their competitive antagonism of the β_1 -adrenergic receptor (β_1 AR). BP lowering is likely the result of a reduced heart rate and decreased renin release due to β_1 AR antagonism in the juxtaglomerular apparatus in the kidneys. (Hoffman et al., 2001; Laragh, 2001) Thus β -blockers are most effective in those patients who have a renin-angiotensin-aldosterone system (RAAS)-mediated BP elevation. (Turner et al., 2010) Like other antihypertensive drug classes, β -blockers show variability in their efficacy (Materson et al., 1993) and if the goals of precision medicine are to be met, with personalized treatment approaches, a better understanding of the sources of variability is necessary.

MicroRNAs (miRNAs) are highly conserved short sequence (about 18–22 nucleotides) single-stranded RNAs that regulate gene expression mainly by interfering with mRNA translation. (Farh et al., 2005; Pasquinelli et al., 2005) As a group, miRNAs are estimated to target approximately 60% of the human mRNAs, and actively participate in the main biological processes, including cell growth, differentiation, and apoptosis. (Friedman et al., 2009; Kloosterman and Plasterk, 2006) Hence, miRNAs may provide insight into the understanding of complex traits, including disease states and drug responses. (Zhang and Dolan, 2010) Plasma/serum miRNAs show marked stability against RNase activity and other harsh conditions, like extremes of temperature and pH. As such, they have been investigated as minimally invasive biomarkers for several diseases. (Chen et al., 2008; Mitchell et al., 2008) Similarly, they are promising targets for understanding the variable response to drug therapy, and there is interest in integrating miRNAs into pharmacogenomics studies to help advance the goals of precision medicine. (Zhang and Dolan, 2010)

The purpose of this study was to determine the association between the expression of candidate circulating miRNAs and β -blocker response, as assessed in the plasma of responders and non-responders to β -blockers (metoprolol and atenolol). The goal of this

effort was identifying potential biomarkers for defining antihypertensive response to β -blockers that may have clinical utility.

2. Material and methods

2.1. Study samples

This study included 110 plasma samples collected during an anti-hypertensive drug-free period (baseline) from two multi-center clinical trials: Pharmacogenomic Evaluation of Antihypertensive Responses-2 (PEAR-2) (Trial registration: [ClinicalTrials.Gov](https://clinicaltrials.gov/ct2/show/study/NCT01203852), [NCT01203852](https://clinicaltrials.gov/ct2/show/study/NCT01203852)) and Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) (Trial registration: [ClinicalTrials.Gov](https://clinicaltrials.gov/ct2/show/study/NCT00246519), [NCT00246519](https://clinicaltrials.gov/ct2/show/study/NCT00246519)). (Johnson et al., 2009) The two trials complied with the Declaration of Helsinki and were approved by the Institutional Review Board at each participating institution. All subjects provided voluntary, written informed consent prior to being screened for participation.

The European American (EAs) participants in PEAR-2 ($n = 189$) were ranked according to the response to the β -blocker metoprolol. The response was measured as the change in DBP after β -blocker therapy as compared to baseline. The discovery phase included two groups: “responders” and “non-responders”. The “responders” group consisted of the 30 patients who showed the largest decrease in DBP after metoprolol therapy as compared to baseline. The “non-responders” group consisted of the 30 patients who showed the smallest decrease in DBP after metoprolol therapy as compared to baseline. According to our power calculation for the discovery phase, we found that with 30 subjects per group we will have a power of 81.5% to detect a two-fold change in miRNA expression levels between responders vs. non-responders, assuming an alpha of 0.0025, with a two-sided test.

For the validation phase, similar to the discovery phase, EA participants from PEAR ($n = 234$) were ranked according to their response to the β -blocker atenolol, with the top and bottom 25 patients constituting the “responders” and “non-responders” groups of the validation phase, respectively (Fig. 1). Based on our power calculation for the validation phase, having 25 subjects per group provides a power of 84.3% to detect a two-fold change in miRNA expression levels, assuming an alpha level of 0.01, with a two-sided test.

2.2. Study design

As shown in Fig. 1, the discovery phase included the expression analysis of 22 candidate miRNAs (selection process is described below) in the plasma samples of “responders” and “non-responders” to metoprolol from PEAR-2, using quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). The miRNAs with significant differences in the expression levels between metoprolol responders vs. non-responders were then analyzed in the validation phase for their differential expression in the plasma samples of “responders” and “non-responders” to atenolol from the other independent study (PEAR), using qRT-PCR.

2.3. Selection of candidate miRNAs

A list of genes related to β -blocker pharmacodynamics was compiled by a review of the literature and relevant databases (Griffiths-Jones et al., 2006; Kanehisa and Goto, 2000) (Supplementary Table S1). Then, the publically available miRNA target prediction databases (Supplementary Table S2) were used to select a set of miRNAs that target the selected genes. From this set, the study list of candidate miRNAs was based on the following criteria: (1) more than one piece of evidence that the candidate miRNA targets the gene of interest; (2) evidence of the expression of the candidate miRNA in plasma; (3) availability of a reliable quantification assay for the candidate miRNA; (4) no more than one miRNA from the same family (in order to avoid bias of using co-regulated candidate miRNAs). The 22 candidate miRNAs used in this study are shown in Supplementary Table S1.

2.4. RNA isolation and Reverse transcription

The miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA, including miRNA, from 200 μ L plasma samples. The manufacturer's instructions were followed, with one modification in the final step, where the miRNA yield was eluted with 20 μ L RNase-free water instead of 14 μ L. During the isolation process, Spike-In Ce_miR-39 (Qiagen, Valencia, CA, USA) was added in an equal amount to each sample as a candidate normalizer. The isolated miRNA samples were stored at -80°C . The miScript II Reverse Transcription Kit (Qiagen, Valencia, CA, USA) was used to convert miRNA to complementary DNA (cDNA), following the manufacturer's instructions. Reactions were incubated for 60 min at 37°C and then 5 min at 95°C , using thermal cycler (Veriti[®] 96-Well Thermal Cycler, Applied Biosystems, CA, USA). RNase-free water (200 μ L) was used to dilute the 20 μ L reverse transcription reaction cDNA product, stored at -20°C .

2.5. qRT-PCR for miRNA expression

The synthesized cDNA served as the template for qRT-PCR analysis using miRNA-specific miScript miRNA PCR primer assays and the miScript SYBR Green Kit (Qiagen, Valencia, CA, USA). Each 10 μ L qRT-PCR reaction consisted of 1 μ L cDNA, 2 μ L RNase-free water, 1 μ L $10\times$ miScript primer assay, 1 μ L $10\times$ miScript universal primer, and 5 μ L $2\times$ QuantiTect SYBR Green PCR master mix. The qRT-PCR was performed in triplicate, using epMotion[®] 5075 (Eppendorf, Hamburg, Germany) automated pipetting system to decrease pipetting errors. Using QuantStudio[™] 12 K Flex Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA), the cycling conditions started with initial HotStart Taq DNA Polymerase activation step at 95°C for 15 min, then 40 cycles each of three steps (94°C for 15 s, then 55°C for 30 s, and then 70°C for 30 s), and then a dissociation curve stage was added to verify specificity and identity of the PCR products. The Ct (cycle threshold) data were generated based on a manually set threshold for each target and an automatically set baseline, using QuantStudio[™] 12 K Flex Software v1.2.2 (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA).

2.6. qRT-PCR data analysis

The Ct values of 37 were excluded from the calculations as Ct values above this threshold indicate a low amount of target nucleic acid which could represent an infection state or

environmental contamination. Outliers among technical replicates were also excluded. Data were normalized to miR-21, which showed the least variability among 4 tested candidate normalizers (miR-16, miR-223, miR-21, and Ce_miR39) as indicated by the smallest DataAssist™-generated score both in the discovery phase (1.55, 1.25, 1.10, 1.48 respectively) and the validation phase (1.39, 1.28, 1.06, 1.58 respectively). For each candidate miRNA, DataAssist™ calculated the fold change using the comparative CT method (Schmittgen and Livak, 2008) as follows: (1) Average Ct = mean of the technical triplicate; (2) $CT = \text{Average Ct}_{\text{test miRNA}} - \text{Average Ct}_{\text{miR-21}}$; (3) Relative concentration of miRNA = $2^{(-CT)}$; (4) Geometric mean of $2^{(-CT)}$ of the samples in each group; (5) Fold change = geometric mean of $2^{(-CT, \text{Responders})}$ / geometric mean of $2^{(-CT, \text{Non-responders})}$. Data were analyzed using DataAssist™ Software version 3.01. (Xia et al., 2010).

2.7. Statistical analysis

Categorical variables were represented as numbers and percentages. Grubb's test was used to remove significant outliers from normally distributed data. For continuous variables, the normality assumption was tested by Kolmogorov-Smirnov and Shapiro-Wilk tests. Mean ($\pm$ standard deviation) was used to represent normally distributed data, and median (Inter-Quartile Range) was used for non-normally distributed data. For categorical variables, Chi-square test or Fisher's Exact test was used, as appropriate, to compare groups. For differential expression data, Student *t*-test was used for comparing the groups if the data were normally distributed. For non-normally distributed data, the Mann-Whitney *U* test was used. A multiple logistic regression model was used to test the association between the relative quantity of miRNA and β -blocker response, adjusting for age, sex, and baseline DBP. Tests with *P*-value < 0.05 were identified as statistically significant. In the validation phase, the hypotheses were one-sided (i.e., same directional association as discovery), and thus one-sided *P*-values were reported. Statistical Package for the Social Sciences (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A met-analysis was performed between the five miRNA (miR-101, miR-19a, let-72, miR-22, and miR-27a) measured in the discovery phase (in PEAR-2) and the replication phase (in PEAR) using Comprehensive Meta-Analysis Software (Version 3.3.070). Meta-analysis was performed based on the inverse variance method, assuming fixed effects.

3. Results

3.1. Baseline characteristics

For the discovery analysis, biological samples and clinical data were obtained from EAs treated with metoprolol in the Pharmacogenomic Evaluation of Antihypertensive Responses-2 (PEAR-2) study. In PEAR-2 EA participants included in this study, there was no statistically significant difference in the distribution of age, baseline systolic blood pressure (SBP), body mass index (BMI), or smoking between responders (*n* = 30) and non-responders (*n* = 30). However, females were overrepresented among the responders.

Additionally, responders had a significantly higher baseline diastolic blood pressure (DBP) than non-responders (Table 1). In the PEAR cohort used for validation, responders to atenolol were more commonly females, significantly younger than non-responders and had

significantly higher baseline DBP. However, baseline SBP, BMI, and smoking showed no statistically significant difference between responders ($n = 25$) and non-responders ($n = 25$) (Table 1). To overcome any confounding effect in our results, we adjusted for age, sex, and baseline DBP in both the discovery and the validation analyses.

3.2. Discovery phase

Twenty-two miRNAs that target β -blocker pharmacodynamics-related genes were originally selected for the study. In the first 20 samples tested (ten responders and ten non-responders), five of the 22 miRNAs showed very low expression in plasma (Ct values > 40). These miRNAs (miR-141, miR-135b, miR-539, miR-138, and miR-182) were excluded. Among the remaining 17 miRNA candidates, miR-27a, miR-19a, miR-22, miR-101, and let-7e were differentially expressed between responders and non-responders (Supplementary Table S3). After adjusting for age, sex, and baseline DBP in a multiple logistic regression model, these five miRNAs continued to be significantly associated with antihypertensive response to metoprolol (Table 2; Supplementary Table S4).

3.3. Validation phase

The five miRNAs (let-7e, miR-19a, miR-101, miR-22, and miR-27a) from the discovery analysis were tested for validation. Out of the five miRNAs, miR-19a showed significant differential expression in the atenolol-exposed cohort with up-regulation in responders versus non-responders, which is the same direction as the discovery phase (one-sided $P = 0.017$). MiR-101 and let-7e showed the same direction of differential expression as the discovery phase, but with non-significant differential expression (one-sided $P = 0.077$ and 0.128 , respectively) (Fig. 2). However, miR-22 and miR-27a did not replicate as they had differential expression opposite to the direction in the discovery phase (Supplementary Tables S3 and S5).

After adjusting for age, sex, and baseline DBP in a multiple logistic regression model, increasing miR-19a expression was the only remaining miRNA with a significant association with antihypertensive response to atenolol [OR (95% CI) = 2.15 (1.06–4.36)]. Although miR-101 and let-7e were non-significant, but they were trending in the same direction of association observed in the discovery analysis [OR (95% CI) = 1.73 (0.76–3.95) and 0.44 (0.18–1.05), respectively] (Table 2). Results from the meta-analysis of the three miRNAs (miR-19a, let-7e, miR-101) from both the discovery (PEAR-2) and validation (PEAR) cohorts revealed a statistically significant association with β -blocker's antihypertensive response in the three miRNAs (Table 2).

4. Discussion

To the best of our knowledge, this is the first study to examine miRNA pharmacogenetics and response to β -blockers. The PEAR and PEAR-2 multi-center clinical trials aimed to discover, replicate, and delineate the genetic determinants of BP response to two anti-hypertensive drug classes, thiazide diuretics, and β -blockers, and the underlying mechanistic basis. Following a candidate miRNA approach, this study showed that miRNAs, which regulate genes involved in β -blocker pharmacodynamics, have the potential to influence

response to β -blockers in the EA hypertensive patients. MiR-19a was significantly associated with response to both metoprolol and atenolol. Importantly, miR-19a was included in the study due to targeting *ADRB1*, which encodes the β_1 -adrenergic receptor protein target of β -blockers. Interestingly, miR-101 and let-7e, which also target *ADRB1*, also showed a significant association with antihypertensive response in the meta-analysis performed between the atenolol cohort and the metoprolol cohort.

It is evident that individual miRNAs can target mRNAs from multiple genes and regulate their translation. (Ganesan et al., 2013; van Rooij et al., 2008) Using Ingenuity Pathway Analysis (IPA[®], QIAGEN Redwood City, www.qiagen.com/ingenuity), miR-19a targets many genes, some of which are related to the cardiac β -adrenergic signaling pathway, such as the beta-adrenergic receptor kinase *βARK2* (*GRK3*), beta catalytic subunit of cAMP-dependent protein kinase A (*PRKACB*), adenylyl cyclase (*ADCY7* and *ADCY9*), and phosphodiesterase (*PDE4A* and *PDE7B*). Moreover, miRNA-gene prediction databases (Supplementary Table S2) were used to determine whether other β -blocker pharmacodynamics-related genes listed in Supplementary Table S1, in addition to *ADRB1*, are targets of miR-19a. Interestingly, miR-19a also targets the cardiac β -adrenergic signaling pathway-related G protein-coupled receptor kinase five gene (*GRK5*) and the RAAS-related nuclear receptor subfamily 3, group C, member two-gene (*NR3C2*). The observation that miR-19a targets multiple genes associated with β -blocker pharmacodynamics suggests that the association of miR-19a with antihypertensive response to β -blocker may not be due only to targeting and regulating *ADRB1*, but due to an interplay between a group of genes in the pathway.

As a member of the oncogenic miRNA cluster (miR17–92), miR-19a is associated with different cancer types, including gliomas, (Jia et al., 2013) gastric cancer, (Qin et al., 2013) and cervical carcinoma. (Xu et al., 2012) Also, miR-19a showed an association with inflammation-related diseases, including asthma (Simpson et al., 2014) and acute myocardial infarction. (Zhong et al., 2014) In the field of pharmacogenomics, miR-19a showed a potential as a biomarker for the prediction of response to the first-line chemotherapy regimen FOLFOX (Folinic acid, Fluorouracil, Oxaliplatin) in advanced colorectal cancer. (Chen et al., 2013) However, to our knowledge, this is the first study documenting an association of miR-19a expression with β -blocker therapy response in hypertension.

This study has a few limitations. First, the sample size is relatively small, although this is offset to some degree by use of the extreme phenotype approach. Second, the number of both the target genes and the selected candidate miRNAs was relatively small, and they were selected through a candidate approach. Nonetheless, we were able to identify and replicate a miRNA associated with antihypertensive response to two different β -blockers in two unique cohorts. Lastly, we found a difference in age, sex and baseline BP between the responders and the non-responders groups tested in this study. However, we adjusted for age, sex, and baseline BP in all the regression models we ran to overcome any confounding observations. Future studies may evaluate the association between sex hormone miRNAs and beta-blocker response to gain a better understanding of the sex difference in response that was observed.

5. Conclusions

The advantages of using miRNAs circulating in plasma/serum as biomarkers include that their collection requires a less invasive procedure than tissues, they are stable against harsh conditions like degradation by RNases, and are amenable to quantification by the reliable and sensitive qRT-PCR method. In this study, among a list of tested plasma miRNAs that target genes related to β -blockers pharmacodynamics, miR-19a, as well as miR-101 and let-7e, may be useful in defining the antihypertensive response to β -blockers in EAs; hence, advancing precision medicine approaches in treating EA hypertensive patients. Future replication of the findings from this study in large well-designed studies is still needed to validate the importance of miRNA 19a as a potential biomarker of β -blocker's antihypertensive response in the clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A flow chart representing study design. miRNAs that were significantly differentially expressed between responders and non-responders in the discovery phase were included in the validation phase. BP: Blood Pressure; PEAR-2: Pharmacogenomic Evaluation of Antihypertensive Responses-2 clinical trial; PEAR: Pharmacogenomic Evaluation of Antihypertensive Responses clinical trial; qRT-PCR: Quantitative Reverse Transcription Polymerase Chain reaction.

Fig. 2.

Relative expression of the microRNAs that showed the same direction of differential expression in both in the discovery phase and the validation phase. Bar chart represents the fold change of the differentially expressed miRNA (miR-19a, miR-101, and let-7e) between responders (R) and non-responders (NR) to the antihypertensive effect of the β -blocker metoprolol in the discovery phase and atenolol in the validation phase. Numbers represent P-value of Mann-Whitney *U* test.

Table 1

Characteristics of the study populations.

	Discovery cohort (n = 60)			Validation cohort (n = 50)		
	Responders (n = 30)	Non-responders (n = 30)	P-value	Responders (n = 25)	Non-responders (n = 25)	P-value
Age (years), median (IQR)	51 (13.75)	49.5 (13.25)	0.668	46 (18)	54 (9.5)	0.012
Females, n (%)	20 (67%)	11 (37%)	0.02	19 (76%)	11 (44%)	0.021
Baseline SBP (mmHg), mean (SD)	147.03 (10.03)	148.7 (12.2)	0.564	148.06 (10.01)	146.32 (9.29)	0.528
Baseline DBP (mmHg), mean(SD)	95.78 (6.53)	92.45 (5.26)	0.034	96.87 (6.09)	92.23 (5.02)	0.005
DBP (mm Hg), median (IQR)	-17.27 (2.18)	-0.04 (3.72)	< 0.001	-20.83 (5.13)	-0.7 (2.73)	< 0.001
BMI (Kg/m ²), mean (SD)	31.22 (5.39)	30.97 (3.79)	0.832	29.25 (6.27) ^a	29.01 (6.05) ^a	0.861
Smoking status						
Ever smoker, n (%)	7 (23%)	12 (40%)	0.165	8 (32%)	10 (40%)	0.556
Current smoker, n (%)	2 (7%)	2 (7%)	1	1 (4%)	4 (16%)	0.349
Former smoker, n (%)	5 (17%)	10 (33%)	0.136	7 (28%)	6 (24%)	0.747

IQR: Inter-quartile range; SD: Standard deviation; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; DBP: change from baseline in DBP in responders and non-responders to metoprolol and atenolol in the discovery and replication phase, respectively; BMI: Body mass index.

^aNumbers represent median (IQR) as the data are not normally distributed.

Table 2

Adjusted regression analysis and meta-analysis of the microRNAs that showed a significant association with antihypertensive response to metoprolol in the discovery phase.

miRNA	Discovery (PEAR-2) Adjusted OR (95% CI) ^a	Validation (PEAR) Adjusted OR (95% CI) ^a	Meta-analysis Adjusted OR (95% CI) ^a
19a	2.18 (1.106–4.32)	2.15 (1.06–4.36)	2.17 (1.28–3.68)
Let-7e	0.48 (0.23–0.97)	0.44 (0.18–1.05)	0.47 (0.26–0.85)
101	2.54 (1.25–5.17)	1.73 (0.76–3.95)	2.23 (1.25–3.96)
27a	3.32 (1.24–8.89)	0.95 (0.31–2.95)	NA
22	2.44 (1.12–5.32)	0.47 (0.21–1.03)	NA

NA: Not available. The meta-analysis was not done as the candidate miRNA showed opposite direction of differential expression between responders and non-responders in the discovery phase and the validation phase.

PEAR-2: Pharmacogenomic Evaluation of Antihypertensive Responses-2 clinical trial; PEAR: Pharmacogenomic Evaluation of Antihypertensive Responses clinical trial.

^a Adjusted odds ratio for age, gender, and baseline diastolic blood pressure.