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Serum microRNA profiles among dioxin exposed veterans with monoclonal gammopathy of undetermined significance

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Abstract

We previously reported an increased risk for monoclonal gammopathy of undetermined significance (MGUS), a precursor of multiple myeloma (MM), among Vietnam veterans exposed to Agent Orange and its contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Dysregulated expression of certain microRNAs (miRNAs) has been demonstrated in MGUS and MM. Given the important role of miRNAs in cellular homeostasis, we sought to determine if there was an association between serum levels of selected miRNAs and TCDD in 47 MGUS cases identified in our previous study, using serum specimens and exposure data archived by the Air Force Health Study (AFHS). We measured 13 miRNA levels (let-7a, let-7i, miR-16, miR-20a, miR-21, miR-34a, miR-106b, miR-146a, miR-181a, miR-192, miR-205, miR-335, and miR-361) in serum stored during the 2002 AFHS follow-up and evaluated their relationship to lipid-adjusted serum TCDD levels in 1987 determined by the AFHS. miR-34a showed the strongest association with

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TCDD (coefficient 2.184, p-value 0.02); after age-adjustment, this positive association was more pronounced (coefficient 2.294, p-value 0.008). In contrast, the other 12 miRNAs had absolute values of age-adjusted coefficient estimates below 1.16 and p-values greater than 0.18. The observed strong positive relationship between high body burdens of TCDD and miR-34a, a tumor suppressor regulated by p53, in this MGUS population warrants clarification of the TCDD-miR-34a relationship and its role in the pathogenesis of MGUS and risk for MM.

Keywords

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); dioxin; microRNA; miR-34a; monoclonal gammopathy of undetermined significance (MGUS)

Introduction

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic premalignant disorder of clonal plasma cells that progresses to multiple myeloma or other lymphoid cancer at a rate of approximately 1% per year (Dispenzieri et al. 2010; Kyle et al. 2018). Virtually all cases of multiple myeloma are preceded by MGUS (Landgren et al. 2009; Weiss et al. 2009). The prevalence of MGUS increases with age (Dispenzieri et al. 2010; Kyle et al. 2006; Landgren et al. 2014) and the age-standardized rate is higher among men (5.1%) compared to women (3.5%) in the U.S. general population aged 50 years and older (Dispenzieri et al. 2010). Multiple myeloma manifests “limited or suggestive evidence” of an association with exposure to herbicides in Vietnam veterans, including Agent Orange and its contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (National Academies of Sciences, Engineering, and Medicine 2018).

In multiple myeloma patients, dysregulated expression of microRNAs (miRNAs) has been previously characterized in bone marrow plasma cells (Lionetti et al. 2009; Pichiorri et al. 2008; Roccaro et al. 2009) with fewer studies reported on circulating miRNAs in the serum (Kubiczkova et al. 2014; Wang et al. 2015). miRNAs are highly conserved small (19–25nt) non-coding RNAs that are involved with post transcriptional regulation of gene expression (Bartel 2004). miRNAs bind to the 3’ untranslated region of target messenger RNA (mRNA), leading to degradation of the mRNA or attenuation of protein translation. miRNAs are important in the regulation of homeostatic processes such as cell proliferation, differentiation, and apoptosis (Calvo et al. 2011; Izumiya et al. 2011; Raveche et al. 2007; Wang et al. 2012). Cell-free miRNAs in blood and body fluids arise from exosomes or micro vesicles released from cells into the circulation or from cell lysis (Zhang et al. 2015). Unlike mRNAs, miRNAs are resistant to RNA degradation and are relatively stable under harsh conditions, including storage temperature and time, and repeated freeze-thaw cycles (Gilad et al. 2008; Grasedieck et al. 2012; Mitchell et al. 2008). Recent studies have explored the potential of circulating miRNAs as biomarkers for various cancers, including hematological cancers (Etheridge et al. 2011; Federico et al. 2019; Grasedieck et al. 2013; Hayes, Peruzzi, and Lawler 2014). However, circulating miRNA in MGUS have not been well characterized.

We previously reported an increased risk for MGUS in Vietnam veterans exposed to Agent Orange and its contaminant TCDD by analyzing serum specimens stored by the Air Force

Health Study (AFHS) (Landgren et al. 2015). In the current case-series study, we explore the relationships between 13 serum miRNAs and TCDD by profiling the TCDD-miRNA associations among 47 MGUS cases identified from our previous study. The 13 miRNAs have been shown to be dysregulated in various cancers including MM or pre-malignant conditions such as MGUS: let-7a, let-7i, miR-16, miR-20a, miR-21, miR-34a, miR-106b, miR-146a, miR-181a, miR-192, miR-205, miR-335, and miR-361 (Bouyssou et al. 2018; Calvo et al. 2011; Kubiczkova et al. 2014; Pichiorri et al. 2008; Sun et al. 2013; Wang et al. 2015). Many of the miRNAs on the panel are known to have tumor suppressor functions, including let-7 family members which target genes regulating cell proliferation and cell signaling such as *MYC* and *RAS* (Balzeau et al 2017). miR-34a is a critical component of the p53 pathway which maintains the fidelity of gene replication in response to cellular stress by several mechanisms including activation of DNA repair, cell cycle arrest, or initiating apoptosis (Rokavec et al 2014; Vogelstein et al 2000). Given the known carcinogenicity of TCDD and the important role of miRNAs in cellular homeostasis, we sought to determine if there was an association between serum levels of selected miRNAs and TCDD in Vietnam veterans who developed MGUS.

Methods

Study Population and Exposure Data

The study base population comprised 958 U.S. veterans who participated in the 2002 follow-up of the AFHS at age 50 years or older and whose serum stored during the follow-up was tested for MGUS in 2013, as described previously (Landgren et al. 2015). Briefly, the AFHS was a prospective cohort study of former U.S. Air Force personnel who participated in aerial herbicide spray missions in Vietnam between 1962 and 1971 (the Ranch Hand cohort). The AFHS also included other Air Force veterans who flew and serviced cargo aircraft in Southeast Asia during the same period but did not spray herbicides (the Comparison cohort); Comparison veterans were matched to Ranch Hand veterans on age, race, and military occupation. All Ranch Hand and Comparison veterans were male. Six physical examinations were conducted until the study ended in 2005. Serum specimens were collected at each examination and stored at −70°C. Lipid-adjusted TCDD concentrations were measured in serum collected at all examinations subsequent to baseline (1982) using high-resolution gas chromatographic/mass spectrometric analysis (Patterson et al. 1987). Because most veterans had a TCDD measurement in 1987, the AFHS set 1987 as a TCDD reference point. For veterans who did not have a 1987 TCDD measurement, the measurement existing in the nearest subsequent examination was chosen. For Ranch Hand veterans whose chosen measurement exceeded 10 parts per trillion (ppt), the 1987 level was extrapolated employing a first-order kinetics model with a constant half-life of 7.6 years (Michalek, Robinson, and Fox 2005). TCDD values below the limit of detection were estimated as the limit of detection divided by the square root of 2 (Hornung and Reed 1990). All AFHS participants had consented to the use of their data and specimens for future research studies. In 2013, we selected 479 Ranch Hand and 479 Comparison veterans by frequency matching to age, and tested their serum specimens stored during the 2002 follow-up for MGUS (Landgren et al. 2015). We identified 49 veterans with MGUS, including 34 Ranch Hand veterans and 15 Comparison veterans, from the 958 veterans (Landgren et al. 2015).

Of the 49 veterans with MGUS, a 2002 serum specimen was available in 47 veterans for miRNA testing. In the present study, we included all 47 veterans. An institutional review board (IRB) of the US Centers for Disease Control and Prevention approved the study protocol. The Institute of Medicine that served as a custodian for AFHS resources approved access to the AFHS data and stored serum specimens for this study.

RNA Isolation

We used the serum specimens that were stored during the AFHS 2002 follow-up. Total RNA was isolated from serum using miRNeasy Mini Kits (Qiagen, Germantown, MD) according to manufacturer's recommendations with some modifications. Briefly, 2.5 μl of 10% sodium dodecyl sulfate solution was added to 50 μl of serum, then 500 μl of QIAzol Lysis Reagent and 500 μl of chloroform were added. Cel-miR-39 (5 μl of 5 nM) was added to the phenol phase as a spike-in control. The mixture was vortexed vigorously and centrifuged at 13,000 rpm for 20 min. The aqueous phase was brought to 600 μl with phosphate-buffered saline, then 600 μl acidic phenol and 120 μl chloroform were added. The mixture was vortexed vigorously and centrifuged at 13,000 rpm for 20 min. The aqueous phase was transferred, and 0.7 volume of isopropanol was added, mixed, and collected into RNeasy mini spin columns. The column was washed with RWT and RPE buffers, and eluted with 20 μl water. RNA concentration was determined using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE).

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used for the quantitation of miRNAs. qRT-PCR was performed with a polyadenylation step before reverse transcription as described previously (Luo et al. 2012) with some modifications. Briefly, 7 μl of RNA was poly-adenylated using the poly-(A) polymerase (New England Biolabs, Ipswich, MA) according to manufacturer's recommendations. Reverse transcription was conducted using an anchor primer (Supplementary Table 1). Real-time PCR was performed on the StepOnePlus instrument (Life Technologies, Grand Island, NY) using a forward primer containing mature microRNA sequence and a universal reverse primer and a FAM-ZEN-IABK-labeled TaqMan probe (Integrated DNA Technologies, Coralville, IA) (Supplementary Table 1). Cycle threshold (Ct) values for each miRNA were used to determine the miRNA levels and were normalized to the cel-miR-39 level, which were calculated as $2^{-\Delta Ct}$ (Livak and Schmittgen 2001).

Statistical Methods

We described demographic, clinical, and exposure characteristics of the veterans with MGUS. The differences in the characteristics between the Ranch Hand and Comparison veterans were examined by using Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. The miRNA-age, miRNA-cohort, and miRNA-TCDD relationships were examined by employing univariate rank regression models for each miRNA. The models included a $\log_2$-transformed, normalized miRNA as the dependent variable. For the purpose of rank regression, each sample without detectable amplification for a miRNA was assigned the maximum Ct value for that miRNA observed in the entire study population. The miRNA-TCDD and miRNA-cohort relationships were also examined

after adjusting for the age effect by including age in the multivariate models as a covariate. The strength of each association was assessed by the magnitude of its regression coefficient in combination with the magnitude of its p-value. We defined the strongest association as having the smallest p-value and largest absolute value of the coefficient. No corrections were made for multiple comparisons because there was no a priori hypothesis about the associations with miRNAs. Finally, for miRNA having the strongest TCDD effect, we contrasted the miRNA-TCDD relationships between the two cohorts of veterans with MGUS (i.e., Ranch Hand veterans vs. Comparison veterans) using color-coded scatter plots overlaid with LOESS curves. We used the Rfit package in R Version 3.4.4 for rank regression analysis and SAS Version 9.4 (SAS Institute Inc., Cary, NC) for Windows for the remaining analyses.

Results

Demographic, Clinical, and Exposure Characteristics of the MGUS Cases

Ranch Hand (n=33) and Comparison (n=14) veterans with MGUS were similar in their demographic and clinical characteristics, although the Ranch Hand veterans were slightly younger than the Comparison veterans (median age in 2002, 67 versus 69.5 years, p=0.21) (Table 1). Lipid-adjusted serum TCDD levels were significantly higher among Ranch Hand veterans than in Comparison veterans (median level of 9.77 ppt versus 5.20 ppt, p<0.01), with levels in 48% of Ranch Hand veterans higher than the highest level in Comparison veterans.

The miRNA-Age, miRNA-Cohort, and miRNA-TCDD Relationships

Table 2 shows the univariate rank regression results on the effects of age, TCDD level, and cohort status on serum levels of the 13 miRNAs measured in all 47 veterans with MGUS. Increasing age was associated with decreasing serum levels of 10 miRNAs (let-7a, let-7i, miR-146a, miR-361, miR-106b, miR-16, miR-20a, miR-21, miR-335, and miR-34a), whereas increasing age was associated with increasing serum levels of three miRs (miR-192, miR-181a, and miR-205). As judged by the magnitude of the regression coefficients and the p-values, the impact of a one year increase in age on mean reduction of a $\log_2$-transformed normalized miRNA was strongest for let-7a (coefficient −0.198, p-value 0.003), followed by miR-146a (coefficient −0.175, p-value 0.01), while the age-associated increase was strongest for miR-181a (coefficient 0.210, p-value 0.02).

Increasing serum TCDD levels were associated with increasing serum levels of eight miRNAs (let-7a, miR-146a, miR-361, miR-181a, miR-20a, miR-21, miR-335, and miR-34a), whereas increasing TCDD levels were associated with decreasing serum levels of the other five miRNAs (let-7i, miR-16, miR-106b, miR-192, and miR-205). The TCDD-associated increase was strongest for miR-34a (coefficient 2.184, p-value 0.02), while the TCDD-associated decline was largest for miR-205 (coefficient −1.123, p-value 0.24) (Table 2).

With regard to cohort status, the serum levels of 10 miRNAs (let-7a, let-7i, miR-146a, miR-361, miR-106b, miR-16, miR-20a, miR-21, miR-335, and miR-34a) were higher

among the Ranch hand veterans than in Comparison veterans, with the largest effect seen in let-7i (coefficient 1.309, p-value 0.10) (Table 2).

In multivariate models, after adjusting for the TCDD effect on the age-miRNA relationship, let-7a continued to show the strongest decline with increasing age (coefficient −0.200, p-value <0.001), followed by miR-146a (coefficient −0.176, p-value 0.007). The age-associated increase remained strongest for miR-181a after TCDD adjustment (coefficient 0.219, p-value 0.02) (Table 3). Similarly, after adjusting for the cohort effect on the age-miRNA relationship, the age-related decline remained strongest for let-7a followed by miR-146a, and the age-related rise remained strongest for miR-181a (Table 4).

The age-adjusted TCDD effects on the miRNAs are shown in Table 3. The increasing trend of miRNA levels associated with increasing TCDD levels persisted for the same eight miRNAs after age adjustment; the strongest association between miR-34a and TCDD was more pronounced after age adjustment (coefficient 2.294, p-value 0.008) (Table 3). After adjusting for the age effect on the cohort-miRNA relationships, the cohort effect remained largest for let-7i (Table 4).

Figure 1 shows a scatterplot of the serum miR-34a levels and serum TCDD levels for the 47 veterans with MGUS. Among the Ranch Hand veterans with MGUS, serum miR-34a initially decreased as TCDD increased until the TCDD level reached between 15 ppt and 16 ppt, and then it markedly rose as TCDD increased. Among the Comparison veterans with MGUS, the increase in serum miR-34a began at a lower level of TCDD.

Discussion

Vietnam veterans exposed to Agent Orange and its contaminant TCDD have an increased risk of developing MGUS (Landgren et al. 2015), a premalignant condition with an increased risk of transforming to MM or another malignant plasma-cell or lymphoid disorder (Kyle et al. 2018). TCDD is classified as a human carcinogen (https://monographs.iarc.fr/wp-content/uploads/2018/06/mono100F-27.pdf), and it has been one of the chemicals of interest in the continuing assessment of health effects in Vietnam veterans exposed to herbicides, including Agent Orange (National Academies of Sciences, Engineering, and Medicine 2018). In the most recent National Academy of Medicine committee's assessment, MGUS was added to the specific health outcomes considered to have sufficient evidence of a positive association with Agent Orange exposure (National Academies of Sciences, Engineering, and Medicine 2018).

In this case-series study of 47 male veterans with MGUS who served in Vietnam or other Southeast Asian countries during the Vietnam War, we measured the serum levels of 13 miRNAs associated with a number of cancers including MM or non-malignant conditions such as MGUS (Bouyssou et al. 2018; Calvo et al. 2011; Kubiczkova et al. 2014; Pichiorri et al. 2008; Sun et al. 2013; Wang et al. 2015). In univariate analysis, the strongest associations with increasing age were decreases in the tumor suppressor let-7a and increases in miR-181a. Decreased let-7a level has been reported in plasma samples from early stage colorectal carcinoma (Ghanbari et al. 2015). Let-7a has been shown to target oncogenes and

genes related to tumor migration such as *MYC* and *HMGA2* (Liu et al. 2012; Wu et al. 2015). miR-181a has been found to be upregulated in multiple myeloma cell lines (Liu et al. 2019). miR-181a was also reported to inhibit cutaneous squamous cell carcinoma proliferation by targeting *KRAS* (Neu et al. 2017) and to inhibit proliferation and survival of diffuse large B-cell lymphoma by targeting NF-κB (Kozloski et al. 2016).

TCDD levels were associated with increasing levels of eight miRNAs and decreasing levels of five miRNAs, but the increase in miR-34a was the strongest effect observed. Cohort status was not strongly associated with any of the 13 miRNAs. These findings persisted after adjusting for the age effect: notably, the strong association between serum miR-34a and TCDD levels (coefficient 2.294, p-value 0.008). In a graphical examination without age adjustment, the positive relationship between serum miR-34a and TCDD was most prominent above 15 ppt, suggesting that high levels of TCDD may result in cellular stress that activates the p53 tumor suppressor pathway. Future investigations using functional assays may be helpful in elucidating the miR-34a and TCDD relationship in oncogenesis.

The biologic effects of TCDD are mediated through the aryl hydrocarbon receptor (AhR) which is required for optimal B-cell proliferation (Villa et al. 2017) and is involved in many critical intracellular signaling pathways. TCDD can induce AhR expression and activation, which in turn regulates expression of several genes affecting B-cell proliferation and survival, such as *CD27*, *HIC1* and *MTSS1L* (Kovalova et al. 2017). Dysregulation of AhR may contribute to events such as tumor initiation, promotion, and progression (Feng, Cao, and Wang 2013). In particular, a recent study using mouse models demonstrated that TCDD induces blood cell abnormalities and plasma cell neoplasms resembling multiple myeloma (Wang et al. 2019). In the Vk*Myc mouse, TCDD induced Akt and p53 phosphorylation and activation in both the spleen and bone marrow, and p53 target genes encoding *Puma* and *p21* were upregulated by TCDD treatment (Wang et al. 2019). Moreover, wild-type C57BL/6J mice exposed to TCDD developed monoclonal IgG bands analogous to MGUS.

miR-34a is a known p53 target which suppresses tumor development by inhibiting *MYC* oncogene expression (Zhang, Zheng, and Pei 2014). The increased levels of serum miR-34a in our study population may have resulted from high body burdens of TCDD over a prolonged period, leading to activation of p53. In a 20-year follow-up after an industrial accident involving TCDD exposure in Seveso, Italy, TCDD plasma levels were associated with a reduction in AhR expression in unstimulated peripheral blood mononuclear cells (Landi et al. 2003). This finding suggests that long-term exposure to TCDD may disturb gene expression regulated by AhR pathway.

Dysregulated miR-34a expression has been associated with multiple cancers (Jansson and Lund 2012; Slabakova et al. 2017). Some tumors have decreased levels of miR-34a associated with loss of p53 (Corney et al. 2010; Gallardo et al. 2009). However, increased serum levels of miR-34a were reported in breast cancer patients with advanced tumor stages (Roth et al. 2010). Most relevant to this study, miR-34a has been previously reported to be upregulated in serum of MM patients in comparison to controls (Kubiczkova et al. 2014). In addition to *MYC*, other mRNA targets of miR34a include *IL6R* (Mishra and Dingli 2019),

BCL2, CCND1, FOXP1, HDAC1, E2F3, CDKN2C, SIRT1, and *LEF1* (Sethupathy, Corda, and Hatzigeorgiou 2006).

The strength of this study includes the uniqueness of the data. The base population is well defined, despite the relatively small sample size. Other strengths include the wide range of exposure levels in the study subjects, the availability of objective TCDD measurements, the long-term follow-up, and the use of archived biological specimens and associated covariate information. However, the cellular sources of miRNAs we measured in serum are undetermined. Other limitations include the lack of balance in terms of MGUS status (present, absent) and cohort status (Ranch Hand, Comparison), which prevented us from fully addressing multivariate aspects of the relationship between the MGUS, TCDD, and miR-34a.

Conclusions

Serum levels of miR-34a were strongly associated with lipid-adjusted TCDD levels in veterans with MGUS who served in Vietnam or other Southeast Asian countries during the Vietnam War. No strong relationship was seen between TCDD and 12 other miRNAs. These results suggest that high TCDD body burdens over a prolonged period activate p53, a direct regulator of miR-34a. This activation could be related to induction of the AhR pathway. The biological plausibility of this possible mechanism warrants further studies to clarify the TCDD-miR-34a relationship and its role in the pathogenesis of MGUS and risk of progression to MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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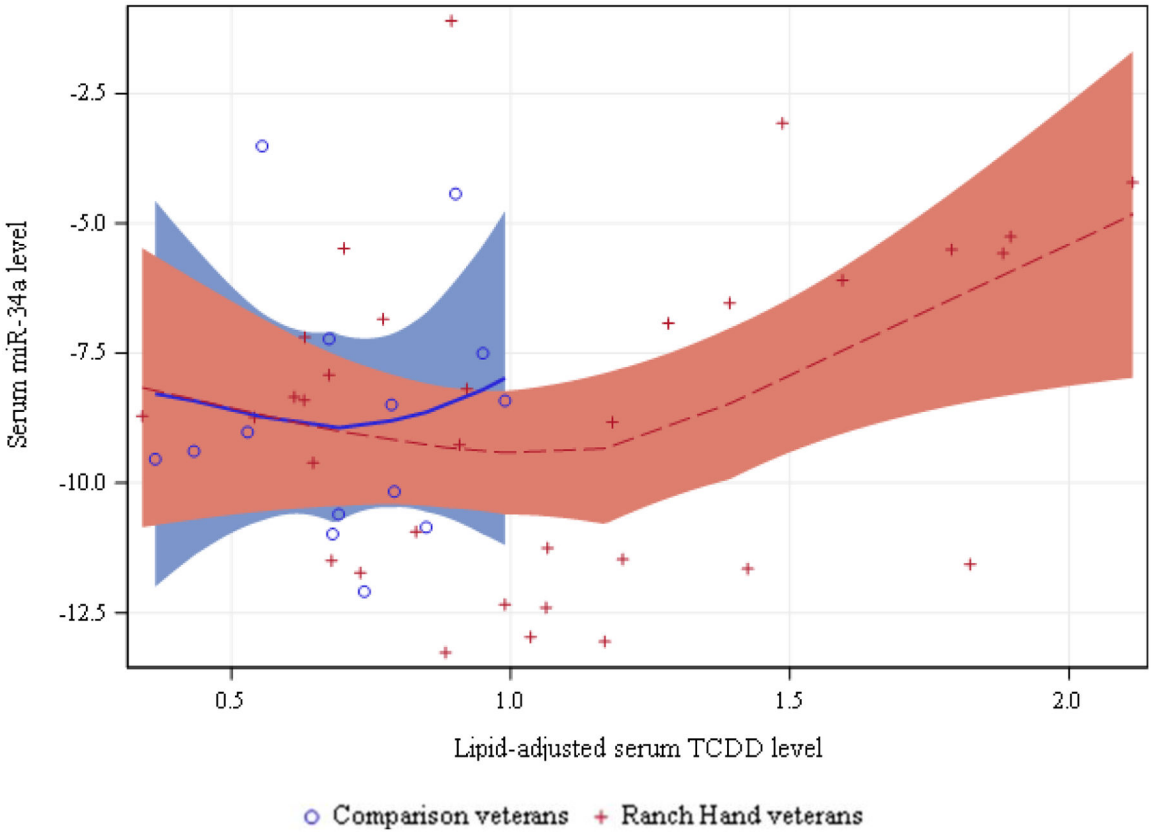

Figure 1.

Scatter plot of serum TCDD and serum miR-34a levels overlaid with LOESS curves and 95% confidence limits, contrasting Ranch Hand (blue) versus Comparison (red) veterans. We used the serum specimens stored during the 2002 follow-up and lipid-adjusted TCDD levels (parts per trillion) in 1987 determined by the Air Force Health Study as described in the Methods. Serum miR-34a levels were measured using quantitative real-time PCR and were normalized to spike-in cel-miR-39 values. The normalized values were $\log_2$-transformed for the plot.

Table 1.

Characteristics of Ranch Hand and Comparison Veterans with MGUS

Characteristics	MGUS Group[a], N (%)		*P* Value[b]
	Ranch Hand (n=33)	Comparison (n=14)	
Age in 2002, Median [Q1, Q3], y	67.0 [62, 72]	69.5 [67, 70]	0.21
55–59	5 (15.15)	0 (0)	
60–64	8 (24.24)	1 (7.14)	
65–69	10 (30.30)	6 (42.86)	
70–74	7 (21.21)	6 (42.86)	
75–89	3 (9.09)	1 (7.14)	
Men	33 (100)	14 (100)	
Race			
Black	4 (12.12)	0 (0)	0.30
Non-black	29 (87.88)	14 (100)	
Body mass index, Median [Q1, Q3][c]	28.5 [25.3, 30.5]	29.1 [27.1, 31.3]	
Normal	6 (18.18)	0 (0)	
Overweight	14 (42.42)	9 64.29	0.20
Obese	13 (39.39)	5 35.71	
Received treatment for cancer within 5 years[c]	2 (6.06)	0 (0)	
Tested positive for HIV infection[c]	0 (0)	0 (0)	
Occupation group			
Officer	15 (45.45)	10 (71.43)	
Enlisted flyer	8 (24.24)	1 (7.14)	0.25
Enlisted ground	10 (30.30)	3 (21.43)	
Lifetime cigarette smoking history (pack-years)[c]	18.0 [1.2, 38]	14.6 [0.9, 21.5]	0.29
Lifetime alcohol history (drink-years)[c]	20.7 [7.0, 44.5]	20.0 [13.2, 53.1]	0.76
Lipid-adjusted TCDD, Median [Q1, Q3], ppt[d]	9.77 [5.0, 24.7]	5.20 [3.6, 7.1]	<0.01

Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; Q1, the first quartile; Q3, the third quartile; HIV, human immunodeficiency virus; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; ppt, parts per trillion

[a] Includes 47 of the 49 MGUS cases identified in 2013 from the Ranch Hand cohort (n=479) and Comparison cohort (n=479) (Landgren et al. 2015) using the serum specimens stored during the 2002 physical examination by the Air Force Health Study. Two cases (1 Ranch Hand and 1 Comparison) whose specimen volume was not sufficient for the miRNA assay were excluded.

[b] Kruskal-Wallis test for continuous variables; Fisher's exact test for categorical variables.

[c] Data were obtained during the 2002 physical examination by the Air Force Health Study.

[d] Lipid-adjusted TCDD concentrations (ppt) in 1987 was determined by the Air Force Health Study as described in the Methods.

Table 2.

Univariate Rank Regression Coefficient Estimates of Age, TCDD, and Cohort Status for 13 miRNAs among 47 Veterans with MGUS

miRNA[a]	Age[b]		TCDD[c]		Cohort[d]	
	Estimate (SE)	P Value	Estimate (SE)	P Value	Estimate (SE)	P Value
let-7a	−0.198 (0.062)	0.003	1.208 (1.132)	0.29	0.625 (1.073)	0.56
let-7i	−0.066 (0.060)	0.28	−0.054 (0.894)	0.95	1.309 (0.786)	0.10
miR-146a	−0.175 (0.066)	0.01	0.610 (1.061)	0.57	0.346 (0.995)	0.73
miR-192	0.110 (0.069)	0.12	−0.396 (1.079)	0.72	−0.530 (1.076)	0.63
miR-361	−0.081 (0.063)	0.21	0.359 (1.031)	0.73	0.516 (0.895)	0.57
miR-181a	0.210 (0.089)	0.02	0.565 (1.280)	0.66	−0.224 (1.226)	0.86
miR-106b	−0.107 (0.054)	0.06	−0.422 (0.841)	0.62	0.658 (0.816)	0.42
miR-16	−0.106 (0.062)	0.10	−0.162 (0.910)	0.86	0.450 (0.882)	0.61
miR-205	0.138 (0.065)	0.04	−1.123 (0.948)	0.24	−0.537 (0.914)	0.56
miR-20a	−0.116 (0.060)	0.06	0.543 (0.903)	0.55	0.065 (0.800)	0.94
miR-21	−0.096 (0.067)	0.16	1.015 (0.975)	0.30	0.893 (0.965)	0.36
miR-335	−0.109 (0.082)	0.19	1.331 (1.098)	0.23	0.663 (1.033)	0.52
miR-34a	−0.003 (0.077)	0.97	2.184 (0.877)	0.02	0.043 (1.047)	0.97

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; miRNA, microRNA; MGUS, monoclonal gammopathy of unknown significance; SE, Standard error

[a] Serum miRNA levels were measured using quantitative real-time PCR and normalized to spike-in cel-miR-39 values. The models included a $\log_2$-transformed, normalized miRNA as the dependent variable.

[b] Rank regression model: miRNA=Age.

[c] Rank regression model: miRNA= $\log_{10}$(TCDD).

[d] Rank regression model: miRNA=Cohort (Ranch Hand versus Comparison).

Table 3.

Multivariate Rank Regression Coefficient Estimates of Age and TCDD for 13 miRNAs among 47 Veterans with MGUS

miRNA[a]	Age[b]		TCDD[b]	
	Estimate (SE)	P Value	Estimate (SE)	P Value
let-7a	−0.200 (0.055)	<0.001	1.058 (0.787)	0.19
let-7i	−0.064 (0.060)	0.29	−0.080 (0.866)	0.93
miR-146a	−0.176 (0.063)	0.007	0.896 (0.900)	0.32
miR-192	0.106 (0.071)	0.14	−0.040 (1.020)	0.97
miR-361	−0.081 (0.067)	0.23	0.163 (0.959)	0.87
miR-181a	0.219 (0.090)	0.02	0.946 (1.299)	0.47
miR-106b	−0.101 (0.055)	0.07	−0.416 (0.786)	0.60
miR-16	−0.106 (0.064)	0.11	−0.176 (0.920)	0.85
miR-205	0.129 (0.071)	0.08	−0.859 (1.028)	0.41
miR-20a	−0.110 (0.061)	0.08	0.471 (0.883)	0.60
miR-21	−0.103 (0.063)	0.11	0.948 (0.908)	0.30
miR-335	−0.107 (0.077)	0.17	1.159 (1.113)	0.30
miR-34a	0.014 (0.057)	0.81	2.294 (0.820)	0.008

Abbreviations: miRNA, microRNA; MGUS, monoclonal gammopathy of unknown significance; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; SE, Standard error

[a]Serum miRNA levels were measured using quantitative real-time PCR and normalized to spike-in cel-miR-39 values. The models included a $\log_2$-transformed, normalized miRNA as the dependent variable.

[b]Rank regression model: miRNA=age + $\log_{10}$(TCDD).

Table 4.

Multivariate Rank Regression Coefficient Estimates of Age and Cohort for 13 miRNAs among 47 Veterans with MGUS

miRNA[a]	Age[b]		Cohort[b]	
	Estimate (SE)	P Value	Estimate (SE)	P Value
let-7a	−0.201 (0.066)	0.004	0.268 (0.879)	0.76
let-7i	−0.063 (0.056)	0.27	1.365 (0.754)	0.08
miR-146a	−0.168 (0.068)	0.02	0.254 (0.910)	0.78
miR-192	0.107 (0.070)	0.14	−0.207 (0.942)	0.83
miR-361	−0.079 (0.064)	0.22	0.296 (0.861)	0.73
miR-181a	0.217 (0.094)	0.03	0.272 (1.265)	0.83
miR-106b	−0.109 (0.054)	0.05	0.535 (0.723)	0.46
miR-16	−0.107 (0.065)	0.11	0.304 (0.871)	0.73
miR-205	0.143 (0.068)	0.04	−0.317 (0.910)	0.73
miR-20a	−0.125 (0.058)	0.04	−0.248 (0.782)	0.75
miR-21	−0.088 (0.066)	0.19	0.561 (0.882)	0.53
miR-335	−0.109 (0.088)	0.22	0.379 (1.179)	0.75
miR-34a	−0.003 (0.079)	0.97	0.039 (1.062)	0.97

Abbreviations: miRNA, microRNA; MGUS, monoclonal gammopathy of unknown significance; SE, Standard error

[a]Serum miRNA levels were measured using quantitative real-time PCR and normalized to spike-in cel-miR-39 values. The models included a $\log_2$-transformed, normalized miRNA as the dependent variable.

[b]Rank regression model: miRNA=age + Cohort (Ranch Hand versus Comparison veterans)