Non-coding RNA Research 10 (2025) 35–40

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

LncRNA NEAT1 and miRNA 101 as potential diagnostic biomarkers in patients with alopecia areata

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ARTICLE INFO

Keywords:
Alopecia areata
Autoimmunity
NEAT1
MiR-101

ABSTRACT

Background: Alopecia areata (AA) commonly displays as non-scarring, irregular hair loss. Experimental and clinical research have specifically implicated autoimmunity and genetics in the disruption of anagen hair follicles. AA patients' scalp lesions and peripheral blood mononuclear cells (PBMCs) exhibited an immune state imbalance. Numerous studies attempt to establish a connection between the occurrence and prognosis of AA and the epigenetic modulation of gene expression by long noncoding RNA (lncRNA) and microRNA (miRNA). The current study aimed to examine the serum levels of nuclear enriched abundant transcript 1 (NEAT1) and its target miRNA101 (miR-101) in AA and investigate the ability to use them as diagnostic biomarkers in the disease.

Methods: Seventy-two AA patients were included in this prospective cohort study. Demographics, patient history, laboratory characteristics, and treatments were recorded. The miR-101 and NEAT1 levels were evaluated.

Results: Serum NEAT1 levels were lower in AA patients, but there was no significant difference. However, there was no substantial disparity in NEAT1 level regarding other disease characteristics. There was a substantial positive association between NEAT1 and miR-101 levels among cases. On the other hand, the results showed a markedly low mean of miR-101 levels among patients, but the miR-101 marker shows no significant difference regarding different disease characteristics. The specificity and sensitivity test for the miR-101 marker shows a significant specificity of 60 % and sensitivity of 75 % with a p-value of 0.001 and a cut-off value of 0.897.

Conclusions: The current research determined that miR-101 works as a diagnostic biomarker for AA.

1. Introduction

Alopecia areata (AA) typically manifests as non-scarring, sporadic hair loss [1]. Alopecia universalis, alopecia totalis, or ophiasis hair loss are characteristics of severe forms of alopecia [2,3]. The global incidence of AA varies from 0.1 % to 0.2 %. Approximately 1.7 % of the entire population is at risk of developing autoimmune disorders as AA throughout their lifetime [4]. AA is linked to a higher risk of mental health issues and cardiovascular disorders, in addition to its obvious cosmetic effects [5]. Experimental and clinical investigations have implicated autoimmunity and genetics, specifically concerning the destruction of anagen hair follicles [6,7]. AA patients' scalp lesions and

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https://doi.org/10.1016/j.ncrna.2024.08.005
Received 23 April 2024; Received in revised form 29 June 2024; Accepted 14 August 2024
Available online 14 August 2024

peripheral blood mononuclear cells (PBMCs) exhibited an immune state imbalance [8]. Moreover, AA is a complex genetic autoimmune cutaneous condition for which its molecular foundation is unknown [9]. Immunological homeostasis is modulated by regulatory T cells (Treg), which avert autoimmune disorders. The control of Treg activity is intricate, and a number of non-coding RNAs (ncRNAs), apart from transcription factors, cytokines, and receptors, have been demonstrated to impact T cell development and functionality [10]. Though the aetiology of AA is complicated, dysregulation of Th1, Th2, and Th17 cytokines has been documented. Investigations have revealed varying relationships for several of these cytokines [11].

Noncoding RNAs (ncRNAs), including lncRNAs (>200 nucleotides) and small ncRNAs like miRNAs (~20 nucleotides), serve an essential role in the control of diverse physiological processes [12]. In addition, ncRNAs can operate as natural miRNA sponges or competing endogenous RNAs (ceRNAs); they interact and modulate one another by battling for attachment to shared microRNAs [13,14]. Numerous pieces of research have demonstrated that these ncRNAs are abnormally expressed and are essential in controlling numerous multigenetic disorders in humans, such as neurological conditions, malignancies, and autoimmune disorders [15–17]. Several studies demonstrated that the PBMCs of individuals with autoimmune conditions like myasthenia gravis and systemic lupus erythematosus (SLE) displayed altered expressions of lncRNAs that can serve as diagnostic biomarkers [18–20]. A recent study uncovered that one thousand lncRNAs throughout hair follicle growth are differentially expressed in dermal papilla cells [21].

Long noncoding RNA nuclear paraspeckle assembly transcript 1 (lnc-NEAT1) is thought to regulate autoimmunity and inflammation [22–25]. In particular, lnc-NEAT1 can target miR-125a and miR-21 to regulate inflammation [22]. In addition, lnc-NEAT1 stimulates fibroblast-like synoviocyte proliferation and the creation of inflammatory cytokines in rheumatoid arthritis by regulating miR-204-5p [24]. Through regulating exosome-mediated macrophage polarization, lnc-NEAT1 modifies the inflammatory process in inflammatory bowel disorders [26]. Additionally, lnc-NEAT1 is involved in the clinical field of autoimmune disorders. For instance, lnc-NEAT1 expression is dysregulated among individuals with SLE relative to healthy individuals [25].

MicroRNAs are a subclass of short non-coding RNAs that are crucial for post-transcriptional gene control and have evolved to be conserved throughout evolution. It has been reported that a number of miRNAs are essential for T cell differentiation, maturation, and activation [27]. MiR-101 was additionally identified to play a role in various cancer-related biological events, like angiogenesis, drug resistance, proliferation, metastasis, apoptosis, and invasion [28]. However, miR-101 is associated with non-malignant diseases like multiple system atrophy (MSA) [29], HBV-associated chronic hepatitis [30], gestational diabetes mellitus (GDM) [31], and acute kidney injury (AKI) [32]. The implication of miRNAs in AA pathogensis and their influences on inflammatory marker release were confirmed by previous reports [33–35]. Furthermore, NEAT1 exhibits interaction with miR-101 and suppresses its level in multiple cancer cells [36–40].

Recently, biomarkers have gained importance in the pharmaceutical discovery process. Identifying biomarkers indicating the degree of severity and progression of AA may be utilized to precisely evaluate the disease activity and response to therapeutic interventions. Nonetheless, this is the first research to assess the serum NEAT1 levels and its target miR-101 in AA and determine their diagnostic utility in the disease.

2. Materials and methods

Ethical statement

This work applied the ethical standards of the relevant national and institutional committees on human experimentation with the 2008 revision of the 1975 Helsinki Declaration. The research and scientific ethics committee of the Faculty of Medicine at Fayoum University approved all procedures involving human subjects or patients [**Code: R489**]. Before taking part in this research, all patients supported written informed consent. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations were carried out in creating the ensuing manuscript [41].

2.1. Patient selection and study design

This case-control work involved 72 AA patients. However, the control group comprised 60 middle-aged, healthy people with no previous record of any chronic diseases. They were normal with no complaints; their clinical examination and routine labs (CBC, urine, and stool analyses) were unremarkable. The inclusion and exclusion criteria are described as following.

Inclusion criteria	Exclusion criteria
1 Patients with AA	1 Lactating and pregnant females
2 Patients without receiving any systemic medication for at least four weeks prior to the investigation starting	2 Patients with another autoimmune disorder, including pernicious anemia, SLE, and Hashimoto thyroiditis
3 Patients who had not received any topical treatment for at least two weeks before the research started.	3 Patients with malignancies of the hematological and solid types, such as leukemia and breast cancer.

2.2. Laboratory investigations

The miR-101 and NEAT1 relative expressions were examined. Ten milliliter blood sample was withdrawn using vacutainer equipment. Blood specimens have been drawn into tubes containing separator gels. After 15 min, the tubes were subjected to centrifugation for 10 min at 4000×*g*. Following being separated from whole blood that had coagulated, the serum was stored until it was used to extract RNA at −80 °C [42].

2.3. RNA extraction

RNA extraction was conducted using a miRNeasy (Qiagen, Valencia, CA, USA) extraction kit with 100 μl of serum sample volume. The reaction mixture was first provided with 500 ml of QIAzol lysis reagent before being left at room temperature (RT) for 5 min. Subsequently, chloroform (100 μl) was placed in; however, the mixture was vortexed for 15 s and left at RT for 2–3 min. Subsequently, centrifugation was carried out at 12,000×*g* and 4 °C for 15 min. Then, 100 % ethanol (1.5 times) was inserted in the tube after removing the top aqueous phase. Each 700 μl of this mixture was centrifuged at 8000 g for 15 s at RT in a 2 ml gathering vial on an RNeasy Mini spin column. After transferring the mixture throughout each column, 700 μl of buffer RW1 was incorporated, and the mixture was centrifuged at 8000×*g* for 15 s at RT. The column was then centrifuged at RT and 8000×*g* for 15 s after introducing 500 μl of buffer RPE. The column was transferred into a new 1.5-mL collecting tube and subjected to centrifugation for 2 min at 8000×*g*. The RNA was subsequently eluted by directly pipetting RNase-free water (50 μl) onto the column and centrifuging it at 8000×*g* for 1 min. After the sample was extracted, any residual DNA was removed using DNase, and then the DNase Max Kit was utilized (Qiagen, Valencia, CA, USA) to reverse-transcribe it into cDNA. The RNA was measured using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) spectrophotometer at 260/280 nm.

2.4. Reverse transcription

RNA (1 μg) was reverse transcribed using a high-capacity cDNA reverse transcription (Applied Biosystems, Foster City, CA, USA) kit, as directed by the manufacturer (incubated for 60 min at 37 °C, for 5 min at 95 °C, and then preserved at 4 °C).

2.5. The ncRNA expression by quantitative real-time PCR (qPCR)

Primers and Qiagen's miScript SYBR Green PCR kit were employed for qPCR. The levels of miR-101 gene expression were measured using an internal control, C/D box snoRNA (SNORD 68) [43]. In previous research, the use of GAPDH as an internal control for serum lncRNA has been employed [44]. Predesigned primers for NEAT1 were obtained from Qiagen, Valencia, CA, USA (Accession No: NR_028272.1, Catalogue No: 330,701 LPH15809A). The primer sequences of GAPDH are reverse: 5′-TGGAAGATGGTGATGGGATT-3′ and forward: 5′-CCCTTCATTGACCTCAACTA-3′. The primers for MiR-101 (Catalogue No.YP00204536) and SNORD 68 (Catalogue No. 33712) were obtained from Qiagen, Germany.

The PCR cycling conditions protocol was followed: 10 min at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. The procedure used the Rotor-Gene Q System on a 20-μl reaction mixture (Qiagen). Using the cycle threshold (Ct) method, endogenous control of the target genes was assessed. The ΔCt of microRNAs was determined by deducting the Ct values of SNORD 68 from miR-101. By deducting the GAPDH Ct values from the lnc-NEAT1 Ct values, the ΔCt of lncRNAs was determined.

The miR-101 expression levels were determined *via* equation $(2)^{-\Delta\Delta Ct}$ [45]. Fold changes for the control were assigned to 1. A fold change of less than one indicated down-regulation, whereas a fold change greater than one indicated up-regulation [46].

2.6. Statistical analysis

The process of data collection and coding was conducted in order to facilitate the handling and evaluation of the data. The data was entered twice into Microsoft Access. Data analysis was carried out employing the Statistical Package for Social Science (SPSS) software version 22 on Windows 7 (SPSS Inc., Chicago, IL, USA). Simple descriptive analyses use the arithmetic mean as a measurement of central tendency, percentages, and numbers for qualitative data, and standard deviations as a measurement of dispersion for quantitative parametric data. The inquiry involved quantitative data analysis, which was assessed for normality using the one-sample Kolmogorov-Smirnov test. Subsequently, suitable inferential statistical tests were chosen. For quantitative, non-parametric data, to compare two independent groups, the Mann-Whitney test was used. For quantitative parametric data, a *t*-test is employed for comparing quantitative measures between two independent groups. Sensitivity and specificity tests were used for evaluating novel test with ROC (Receiver Operating Characteristics) curve. Bivariate Pearson correlation test to test the correlation among variables. A p-value below 0.05 was deemed significant.

3. Result

3.1. Demographic and disease character in different study groups

Our results showed no significant difference in sex and age between groups with a p-value over 0.05. Among alopecia cases, 61.1 % show gradual onset of disease; however, in 80.6 % of cases, the onset time was late, with the mean number of attacks (1 ± 0.8) ranged between (1 and 5) attacks, 63.9 % of them treated with steroids. All cases showed a progressive course, complaint stress, and showed no associated disease or manifestations.

For clinical findings, among alopecia cases, 69.4 % showed one patch, and 16.7 % of cases had an affected beard. The mean extent percentage is 6.64 ± 18.6. All cases lack afflicted eyebrows, eyelashes, fingernails, or body hair. Moreover, all cases lack white hair (Tables 1 and 2).

Table 1
Comparisons of demographic characters.

Variables	Cases (N = 72)		Control (N = 60)		p-value
Age (years)	29.1	10.6	31.9	8.6	0.09
Sex					
Female	52	72.2 %	37	61.7 %	0.3
Male	20	27.8 %	23	38.3 %	

Table 2
Frequency of various clinical results between study participants.

Variables (n = 72)	Clinical data	
	Number	%
Scalp lesion		
One patch	50	69.4 %
Two patches	6	8.3 %
Three patches	6	8.3 %
Four patches	6	8.3 %
Frontal, vertex, bitemporal, occipital, marginal	2	2.8 %
Totalis	2	2.8%
Beard		
None	60	83.3 %
Affected	12	16.7 %
Other non-affected areas		
Eyebrow (non-affected)	72	100 %
Eyelashes (non-affected)	72	100 %
Nail (non-affected)	72	100 %
White hairs (non-affected)	72	100 %
Body (non-affected)	72	100 %
	Mean ± SD	**Median/Range**
Extent of lesion (%)	6.64 ± 18.6	20 (1–100)

3.2. Comparisons of NEAT1 and miR-101 markers fold change between cases and controls

Table 3 demonstrates that the mean levels of fold change of miR-101 are significantly low, with a p-value below 0.05 among cases with negligible disparities in NEAT1 level between groups (Fig. 1).

3.3. Expression levels of NEAT1 and miR-101 in different disease characteristics among cases

Female cases showed a lower mean NEAT1 level in comparison to male patients (p-value = 0.003). In addition, patchy variants had a lower level of NEAT1 than the totalis type, but without significant difference. Further, there was no marked distinction in the NEAT1 level regarding other disease characteristics. The miR-101 marker showed no significant difference as regards different disease characteristics among cases (Table 4).

3.4. Correlation between NEAT1 and miR-101 fold change and study variables

The NEAT1 level positively correlates with the number of attacks and the level of miR-101 among cases. Furthermore, miR-101 shows no marked correlation with the patient's age, number of attacks, or lesion extent, p-value >0.05 (Table 5).

Table 3
Comparisons of NEAT1 and miR-101 markers fold change.

Variables	Cases (n = 72)		Control (n = 60)		p-value
	Median of fold change	IQR	Median of fold change	IQR	
NEAT1	0.884	2.79	0.96	0.06	0.70
miR-101	**0.754**	0.962	0.915	0.11	**0.001***

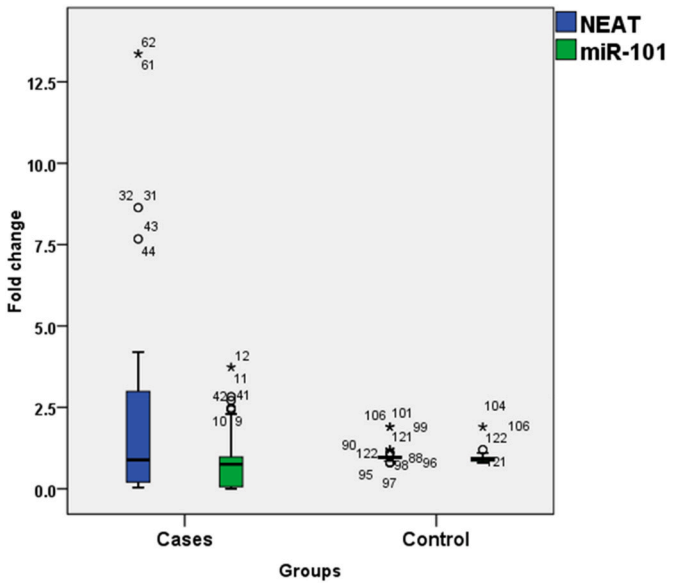

Fig. 1. Box plot for NEAT1 and miR-101 fold change in AA patients compared to controls.

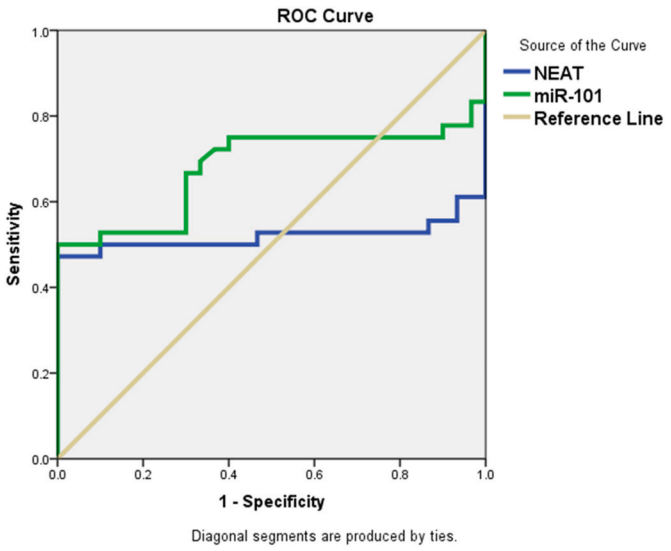

Fig. 2. ROC curve for NEAT1 and miR-101 markers in alopecia cases diagnosis.

Table 4
Comparisons of NEAT1 and miR-101 fold change in different disease characteristics among cases.

Variables	NEAT1			miR-101		
	Median	IQR	p-value	Median	IQR	p-value
Sex						
Male	1.1	2.6	**0.003***	0.78	0.84	0.3
Female	0.75	7.3		0.61	0.92	
Variant						
Patchy	0.59	2.6	0.18	0.42	0.88	0.24
Totalis	1.96	2.01		0.87	0.01	

Table 5
Correlation between NEAT1 and miR-101 markers and study variables.

Variables	NEAT1		miR-101	
	r	p-value	r	p-value
Age (years)	*0.023	0.8	0.12	0.17
Number of attacks	**0.30**	**0.01***	0.16	0.2
Extent of lesion (%)	0.15	0.2	0.19	0.1
miR-101	**0.52**	**0.001***	–	–

3.5. Predictive power of miR-101 and NEAT1 in AA patients

The sensitivity and specificity test for the miR-101 marker shows a significant specificity of 60 % and sensitivity of 75 % at a cut-off of 0.897 and p-value of 0.001. On the other hand, NEAT1 levels show no significant sensitivity to differentiate between cases and controls, with a p-value of 0.07 (Fig. 2).

4. Discussion

AA is a widespread issue that impacts nearly all age groups. It is thought to be a source of concern, particularly in women, for psychological or cosmetic purposes. AA is a prevalent, progressive condition linked to hair follicle cycle abnormalities. Various mechanisms precisely regulate the regression (catagen), rest (telogen), and cyclic phases of hair growth (anagen) in hair follicles [47]. Since the precise processes driving AA and successful targets for therapy are not known, patients with hair loss currently have limited treatment options [48]. The majority of current medical approaches are palliative, necessitating improvements in AA diagnosis and clinical therapy. Nonetheless, the precise mechanism related to hair follicle cycling involving microRNAs and noncoding RNAs remains obscure. Non-coding RNAs, or ncRNAs, are found all over the human genome. Non-coding RNAs (ncRNAs) are important modulators of numerous biological activities, including cell division, the cell cycle, death, and epigenetic modification [49]. The current work aimed to examine the serum NEAT1 and miR-101 levels in AA patients and determine their correlation with the degree of severity. To the best of our knowledge, this was the first study to investigate the levels of NEAT1 in patients with alopecia areata.

Serum levels of NEAT1 were lower in patients with AA, but there was a substantial disparity between the patient group and healthy controls. There was no marked disparity in NEAT1 levels regarding other disease characteristics. The NEAT1 level shows a positive association with the miR-101 level among cases. In contrast, NEAT1 levels demonstrate no significant sensitivity to distinguish between cases and controls. In agreement with our results, Ding and coworkers reported a reduction in NEAT1 expression; however, there was no substantial disparity in androgenetic alopecia. Moreover, they hypothesized that lncRNA NEAT1 absorbs 10 miRNAs, indicating a possible role for lncRNA NEAT1 in epidermal cell proliferation. They identified six ceRNA networks involved in the hair follicle cycle: Neat1/Tug1-miR-22-3p-Cdkn1a, Neat1-miR-27a-3p-Plk2, Neat1-miR-27b-3p-Gspt1, Neat1-miR-30a/e−5p/miR-146a-5p-Notch1, Neat1-miR-126a-3p-Slc7a5, and Neat1/Tug1-miR-148a-3p-Gadd45a/Mafb/Mitf [47]. Additionally, NEAT1 is linked to cutaneous tumorigenesis [50].

Our findings revealed a significantly low mean miR-101 concentration among cases, but the miR-101 marker shows no significant difference regarding different disease characteristics. Specificity and sensitivity tests for the miR-101 marker show a significant specificity of 60 % and a sensitivity of 75 % at a cut-off value of 0.897 p-value of 0.001.

In accordance with our findings, numerous studies have linked miR-101 to autoimmune diseases. Particularly, Yang et al. discovered that miR-101 influences inflammatory marker release like IFN, IL-10, TNF-α, and IL-1 in SLE through the control of the MAPK pathways [34], as inhibiting p38 MAPK activation lowers IL-6 and IFN-γ in SLE [35].

In addition, Wang and colleagues discovered that the IL-1b level in non-small-cell lung cancer (NSCLC) is significantly elevated. IL-1b inhibited miR-101 expression and induced a rise in its target gene levels, Lin28B, a suppressor of the tumor-suppressive let-7 family of microRNAs. NSCLC cell proliferation and migration were stimulated by

the IL-1b/miR-101/Lin28B pathway, which is dependent on cyclooxygenase-2 (COX-2) activity. This signaling connects inflammation signaling to NSCLC cancer cell migration and proliferation and thus could partially clarify inflammation-induced tumorigenesis. Additional investigations are required to figure out the precise pathway through which miR-101 could influence the pathogenesis of AA [51]. In agreement with our results, Wang and coworkers demonstrated down-regulation of the mouse homologs of three miRNAs (mmu-miR-1, mmu-miR-101a and mmu-miR-705) predicted to target *Icos* and *Cxcl11*. The miR-101 family has been reported to facilitate Roquin-mediated degradation of *ICOS* mRNA [52]. Further studies with a large sample size are needed to clarify the role of miR-101 and NEAT1 in AA and their relation to different variants and the prognosis of the disease.

5. Conclusion

In summary, this work found that miR-101 possesses the opportunity to act as a diagnostic biomarker for AA. The small number of subjects that participated in the current investigation presents a limitation. To validate the current findings and demonstrate the potential for employing them as diagnostic targets in AA, additional investigation is needed.

Funding

None.

Institutional review board statement

The study was authorized by the research and ethics committee of Fayoum University's Faculty of Medicine [Code: R489 (Aug_2023)], following the ethical considerations of the 1964 Helsinki Declaration and its later amendments.

Informed consent statement

Informed consent was obtained from all participants involved in the study.

CRediT authorship contribution statement

Randa Erfan: Methodology, Data curation. **Olfat G. Shaker:** Investigation, Formal analysis. **Mahmoud A.F. Khalil:** Writing – review & editing, Writing – original draft. **Amel Raouf Hassan:** Investigation, Formal analysis. **Abeer K. Abu-El-Azayem:** Methodology, Investigation. **Amira Samy:** Methodology, Investigation, Conceptualization. **Haitham Abdelhamid:** Methodology, Investigation. **Aeshah A. Awaji:** Writing – review & editing, Writing – original draft, Conceptualization. **Hassan Salem El sayed:** Methodology, Investigation. **Asmaa Mohammed:** Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

None.

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