Immunohistochemical analysis of T-cell subsets in the inflammatory infiltrates of alopecia areata and its comparison with androgenetic alopecia

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Background: Androgenic hair loss (AGA) and alopecia areata (AA) are common conditions which sometimes are histologically difficult to differentiate. This study was conducted to detect differentiating features of these two disorders with IHC analysis of T-cell subsets in the inflammatory infiltrates of alopecia areata and androgenetic alopecia.

Methods: This cross-sectional study was conducted at Shohadae-Tajrish Hospital in 2018. Twenty-eight cases of alopecia areata and 32 androgenic hair loss cases were evaluated. H&E and IHC staining for CD3, CD4, and CD8+ T-lymphocytes were performed. Lymphocytic densities were graded in a quintet grading system. The inflammation density in various areas of skin tissue was evaluated. Statistical analysis was performed using SPSS version 21.

Results: Peribulbar lymphocytic infiltration was seen in 88.5% of AA patients and 12.5% of AGA patients (P = 0.000). Presence of melanin, pigmented casts, and lymphocytes in follicular stela were in favor of AA diagnosis. CD3, CD4, and CD8+ T-cells in follicular stela, peri-bulbar, and subcutaneous regions were higher and denser in AA patients, while CD3 and CD4+ T-cells around sebaceous ducts were in favor of AGA diagnosis.

Conclusion: Peribulbar lymphocytic infiltration is among the most important differentiating features of AA and AGA. Infiltration of T-cells in various regions of skin tissue has high diagnostic value. Histologic and immunohistochemical evaluation of skin tissues according to these factors can differentiate these two entities with high precision.

Keywords: hair loss, alopecia areata, androgenetic alopecia

Received: 4 November 2019 Accepted: 18 February 2020

Iran J Dermatol 2020; 23: 21-27

INTRODUCTION

Alopecia is a common dermatologic complaint around the world which is categorized into several conditions. Alopecia areata (AA) is a prevalent disorder with a prevalence of 0.7-3.8% among patients referring to dermatology clinics ^{1,2}. It is estimated that AA is present in 0.1-0.2% of US general population 3, which is associated with a

life-long risk of 1.7% 4. Another form of alopecia is known as androgenetic (AGA) which involves nearly 50% of men and approximately half of women over 40 years of age 5. Due to the fact that both of these entities are so common, definite diagnosis of these disorders is essential.

Differentiation of AA and AGA by histopathology is a challenging issue because both of them are characterized by increases in catagen/telogen ratio and follicular miniaturization ⁶. In cases of hair loss in androgen-dependant regions, this choice between AA and AGA grows more difficult. To make a specific diagnosis, some histologic keys are present. In either disorders, follicular miniaturization or changes in catagen/telogen ratio and empty follicular fibrous tracts which are known as empty stela are found ⁷⁻⁹. In some cases, lymphocytes might be detected in stela. In more complex cases, presence of melanin in stela of AA patients might complicate the diagnosis. Identification of pigmented casts, especially in brunette, leads to more challenging diagnostic puzzles ^{6,10}.

Traditionally, detection of peribulbar lymphocytic infilitration, in addition to eosinophils, approved a diagnosis of AA. But, peribulbar infiltration is routinely not found in subacute AA where an evident follicular miniaturization and increase in catagen/telogn ratio must be present to meet the requirements of AA diagnosis 8. A shift of higher than 50% in catagen/telogen ratio and evident follicular miniaturization with a terminal/vellus ratio of more than 1:7 is essential for making such diagnosis ^{7,11}. The hard problem is where terminal/ vellus ratio is higher than 1:1 but lower than 1:7 in addition to a catagen/telogen shift between 20 to 50 percent; in such settings, histologic discrimination between AA and AGA is very problematical. So, using additional methods to reach an accurate and certain diagnosis is necessary. Several studies have demonstrated that T-cell lymphocyte infiltration in AA is confirmed ⁹, but the details of this infiltration is not extensively reviewed. In addition, status of lymphocytic infiltration in AGA is not clarified well. Thus, we aimed to evaluate and compare the presence and distribution of CD3, C4, and CD8 lymphocytic infiltrations and other pathologic features between AA and AGA samples to determine whether these parameters could contribute to better distinguishing AA from AGA or not.

MATERIALS AND METHODS

This cross-sectional study was conducted on biopsies performed from 2011 to 2017 at Shohada-e-Tajrish Hospital in Tehran; 28 cases of AA and 32 cases of AGA were reviewed. The final diagnosis of all cases was confirmed by two independent expert histopathologists. In all cases, a 4-mm punch biopsy was performed. An expert laboratory technician

performed the process of IHC staining for CD3, CD4, and CD8 markers. H&E staining had been performed on 5-µm sections. H&E findings included peribulbar lymphocytic infiltration, eosinophils, and lymphocytes in fibrous tracts, melanin, and pigmented casts in fibrous tracts, hyperkeratosis, and dilatation of follicles' opening and follicular miniaturization. Lymphocytic densities were graded in a quintet grading system (0: no infiltration, trace: scant lymphocytic infiltration, 1+: mild lymphocytic infiltration, 2+: moderate lymphocytic infiltration and 3+: severe lymphocytic infiltration). These lymphocytic infiltrations were evaluated in follicular epithelium, papillary dermis, reticular dermis, subcutis, peri-bulbar dermis, peri-sebaceous ducts, and empty follicular fibrous tracts (stela). Patients' age and gender were also recorded.

All acquired data were analyzed in SPSS software (version 20, SPSS Inc., Chicago, IL, USA). Descriptive analysis of data was performed in form of overall and in-group frequency and percentage of parameters. For comparison of parameters between two groups, t-test, chi-square test, or U-Mann Whitney test were used as needed. Parameters' cut-off for discrimination between two diagnoses were determined with ROC curve analysis, and their associated AUC, sensitivity, and specificity were calculated. P-value < 0.05 was considered as statistically significant.

RESULTS

We reviewed 32 AGA and 28 AA patients. Mean age of the patients was 39.3 ± 14.77 and 31.17 ± 14.00 years for AGA and AA groups, respectively (P = 0.040). Gender distribution of patients in each group was as follows: 26 (81.3%) females and 6 (18.8%) males in the AGA group (female-to-male ratio = 4.3:1) and 23 (82.1%) females and 5 (17.9%) males in the AA group (female-to-male ratio = 4.6:1) (P = 0.929).

Overall inflammation density in AGA patients was trace in 12 (37.5%) patients, 1+ in 15 (46.9%) patients, and 2+ in 5 (15.6%) patients, while in AA patients, 4 (14.3%) cases of trace, 17 (60.7%) cases of 1+, and 7 (25%) cases of 2+ inflammation density was seen (P = 0.122). The overall inflammation severity was not statistically significant between two groups. Peribulbar lymphocytic infiltration density showed significant difference between

two groups as in the AGA group; there was no infiltration in 28 (87.5%) cases and trace infiltration in 4 (12.5%) cases, but in AA patients, 3 cases (11.5%) showed no evidence of infiltration; 10 (38.5%) cases had trace infiltration; 7 patients (26.9%) had 1+; and 6 patients (23.1%) had 2+ infiltration densities (P = 0.000). Figure 1 depicts lymphocytic infiltration around sebaceous ducts in H&E and CD4 IHC staining in a case of AGA.

Further findings from H&E examination and comparison between two groups of AGA and AA

in different parameters are shown in Table 1.

Analysis of inflammation density with CD3, CD4, and CD8+ T-lymphocytes reveals that there is no difference between AA and AGA in reticular dermis and follicular epithellium. While inflammation density in stella, peribulbar, and subcutaneous regions is significantly in favor of AA diagnosis (all CD3, CD4, and CD8+ T-lymphocytes), inflammation in peri-sebaceous duct regions (CD3 and CD4+ lymphocytes) is highly suggestive of AGA. Figure 2 shows infiltration of CD3+ lymphocytes in stela and

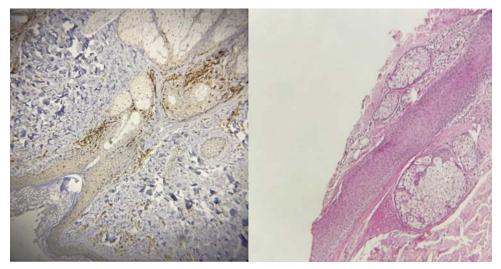


Figure 1. Lymphocytic infiltration around sebaceous ducts in a case of AGA, CD4 IHC staining and H&E staining (×100)

Table 1. Comparision of H&E study findings in AA and AGA groups

Parameters	Infiltration grading	AGA	AA	P-value	
Peribulbar lymphocytic infiltration	0	28 (87.5%)	3 (11.5%)		
	Trace	4 (12.5%)	10 (38.5%)	— — 0.000	
	1+	-	7 (26.9%)	— 0.000	
	2+	-	6 (23.1%)	_	
Presence of eosinophils in stela	Present	2 (6.3%)	5 (17.9%)	- 0.162	
	Absent	30 (93.8%)	23 (82.1%)		
Melanin in stela	Present	6 (18.8%)	14 (50%)	- 0.010	
	Absent	26 (81.3%)	14 (50%)		
Lymphocytes in stela	0	14 (43.8%)	5 (17.9%)		
	Trace	18 (56.3%)	12 (42.9%)	- 0.004	
	1+	-	10 (35.7%)	- 0.001	
	2+	-	1 (3.6%)	<u> </u>	
Pigmented casts in stela	Present	-	4 (14.3%)	- 0.027	
	Absent	32 (100%)	24 (85.7%)		
Follicular miniaturization	Present	28 (87.5%)	17 (60.7%)	0.047	
	Absent	4 (12.5%)	11 (39.3%)	- 0.017	
Hyperkeratosis	Present	24 (75%)	20 (71.4%)	- 0.755	
	Absent	8 (25%)	8 (28.6%)		
Dilatation of follicles' opening	Present	22 (68.8%)	24 (88.9%)	0.063	
	Absent	10 (31.3%)	3 (11.1%)		

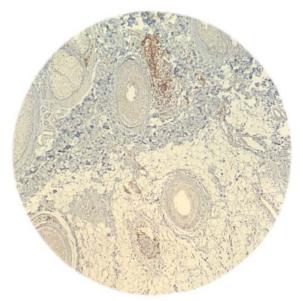


Figure 2. Infiltration of CD3+ lymphocytes in stela and peribulbar region in a patient with AA diagnosis (×100)

peribulbar region in a patient with AA diagnosis. The practical details are listed in Table 2.

To differentiate between two diagnoses, it is essential to establish a cut-off value for parameters which were significantly different between AA and AGA. We evaluated sensitivity, specificity, and AUC of these parameters for distinguishing between AA and AGA based on these cut-off values. The best parameter for this purpose was pribulbar lymphocytic infiltration with AUC of 0.911, sensitivity of 88.5%, and specificity of 87.5%. The details are thoroughly reviewed in Table 3.

DISCUSSION

Since the time Headington ¹² suggested punch biopsy sectioning in 1984, this procedure has got growing popularity among pathologists because it allows visualization of all follicles present in biopsy, so it makes morphologic and quantitative assessment feasible ¹³. But still, using this procedure

Table 2. Details of immunohistochemical staining for CD3 and CD4+ T-lymphocytes

IHC staining/region	Alopecia type	0	Trace	1+	2+	3+	<i>P</i> -value
CD3/stela	AGA	8 (25%)	24 (75%)	-	-	-	- 0.000
	AA	1 (3.6%)	10 (35.7%)	13 (46.4%)	2 (7.1%)	2 (7.1%)	- 0.000
CD3/peribulbar	AGA	27 (84.4%)	5 (15.6%)	-	-	-	- 0.000
	AA	4 (15.4%)	9 (34.6%)	7 (26.9%)	5 (19.2%)	1 (3.8%)	0.000
CD3/reticular dermis	AGA	-	24 (75%)	7 (21.9%)	1 (3.1%)	-	0.468
	AA	-	17 (60.7%)	9 (32.1%)	2 (7.1%)	-	0.400
CD3/subcutaneous	AGA	30 (93.8%)	2 (6.3%)	-	-	-	- 0.001
	AA	14 (51.9%)	10 (37%)	3 (11.1%)	-	-	0.001
CD3/follicular epithellium	AGA	2 (6.3%)	27 (84.4%)	2 (6.3%)	1 (3.1%)	-	- 0.501
	AA	3 (10.7%)	21 (75%)	4 (14.3%)	-	-	0.501
CD3/peri-sebaceous duct	AGA	3 (9.4%)	13 (40.6%)	11 (34.4%)	5 (15.6%)	-	- 0.013
	AA	6 (21.4%)	19 (67.9%)	2 (7.1%)	1 (3.6%)	-	0.013
CD4/peri-sebaceous duct	AGA	-	8 (25%)	14 (43.8%)	10 (31.3%)	-	0.006
	AA	1 (3.6%)	18 (64.3%)	7 (25%)	2 (7.1%)	-	0.006

Table 3. Sensitivity, specificity and AUC of cut-off values for distinguishing AA from AGA

Parameter	Cut-off	Sensitivity (%)	Specificity (%)	AUC	95% CI
Peribulbar lymphocytic infiltration	Trace	88.5	87.5	0.911	0.828-0.994
Melanin in stela	Presence of melanin	50	81.2	0.656	0.515-0.797
Lymphocytes in stela	Presence of lymphocytes	82.1	43.7	0.740	0.612-0.868
Follicular miniaturization	Presence of miniaturization	60.7	12.5	0.366	0.233-0.510
Pigment casts	Presence of casts	14.3	100	0.571	0.424-0.719
CD3 in stela	Trace	60.7	100	0.835	0.729-0.941
Peribulbar CD3	Trace	84.6	84.4	0.884	0.789-0.979
Subcutaneous CD3	Trace	48.1	93.7	0.713	0.576-0.850
Peri-sebaceous duct CD3	Trace	78.6	9.4	0.292	0.160-0.424
Peri-sebaceous duct CD4	1+	32.1	25	0.258	0.131-0.384

AUC: Area under the curve

needs high levels of expertise and precision, and in some cases, differentiation of several types of alopecias becomes so challenging. As mentioned above, in this study we aimed to assess the challenging diagnosis of AGA and AA.

Overall, histologically distinguishing of two alopecias, especially in the absence of peribulbar inflammation, is very tough because both AGA and AA show hair follicles' miniaturization 14. In our study, miniaturization in AGA is significantly higher than AA (87.5% vs. 60.7%). In Whiting et al. 15 and Dy et al.'s ¹⁰ studies, a higher rate of miniaturization in AA was seen, but this rate was not statistically significant. On the other hand, Horenstein et al. 16 and Miteva et al. 17 reported, similarly, a higher rate of miniaturization in AGA in comparison to AA. Conflicts between various studies could be attributed to the region where biopsy was taken. As hair follicles' miniaturization is a slow process which is mainly found in the center of lesions, miniaturization in margin of lesions might occur later in the process.

One of the important morphologic parameters which was clearly higher in areata alopecia was peribulbar inflammation. This can also be seen in syphilitic alopecia and lupus, but it is very rare in AGA ¹⁸. Peribulbar inflammation was seen in 88.5% of AA patients, while only 12.5% of AGA patients showed peribulbar inflammation. This feature is usually reported in early stages of AA, so there are broadly variable reports of 12 to 100% regarding the occurrence of this feature ^{16,18,19-24}.

Another differential feature in our study was the presence of pigmented casts and melanin depositions. Pigmented cast was not seen in any case of AGA, but 14.3% of AA patients showed these casts (P = 0.027). Melanin in stela was also detected in 50% of AA patients and 18.8% of AGA patients, which is an approximately three-fold ratio (P = 0.010). It is reported that the type and location of pigmented casts and melanin depositions vary between two disorders. In AA, pigmented casts are usually larger and higher in number and are located in peribulbar region, while in AGA, they are found in telogen/catagen follicles ²⁵. Peckham et al. ⁸ reported that in 84% of AA patients, melanin was found in stela and it is a key diagnostic feature of AA. In an Indian study by Singh et al., in 83% of horizontal biopsies and 80% of vertical biopsies in AA, melanin was found ¹⁹.

Presence of eosinophils in perifollicular region is considered an important diagnostic feature in the literature. In our study, 17.9% of AA and 6.3% of AGA patients showed eosinophils in perifollicular region (P = 0.162). Elston *et al.* ²⁶ found eosinophils in 53% of AA patients, while this rate in Peckham *et al.*'s study ⁸ was 44%. As mentioned earlier, this rate was much lower in our study. Chaitra *et al.* ²² and Singh *et al.* ¹⁹ reported no evidence of eosinophil in their evaluations.

Lymphocytes in stela were also suggested as an indicator of AA. In the current study, 82.1% of AA patients had such sympyoms, while in AGA, only 56.3% had lymphocytes in stela (P = 0.001). In various studies of AA, 41 to 94 % of patients had lymphocytes in stela 8,17,19 .

Dilatation of follicles' opening was seen in 68.8% of AGA patients and 88.9% of AA patients. This difference was not statistically significant (P = 0.063). Miteva $et\ al.\ ^{17}$ found this feature in 91% of patients with incognita alopecia. Muller $et\ al.\ ^{24}$ also found this condition in 58% of AA patients. There are no reports on this feature in AGA studies. The role of this feature in discrimination of these two disorders needs further investigations.

Immunohistochemical staining for CD3, CD4, and CD8+ T-lymphocytes revealed no difference between AA and AGA in reticular dermis and follicular epithellium. While inflammation density in stella, peribulbar, and subcutaneous regions is significant in favor of AA diagnosis (all CD3, CD4, and CD8+ T-lymphocytes), inflammation in peri-sebaceous duct regions (CD3 and CD4+ lymphocytes) is highly suggestive of AGA. Previous studies have reported that perifollicular inflammation was present in 14.3-100% of AA patients 17,20,22,23, while in AGA patients, this rate was 0-85% 11,17,27. The majority report sets forth the following finding of fact: inflammation in AA is more severe than AGA. What we found in our study was that severity of inflammation depends on the region of study in the tissue; in some parts, inflammation of AA is more severe, and in some other parts, AGA is more inflamed. It is speculated that in AA, the underlying cause of inflammation is the nature of the disease but in AGA, coincidence of seborrehic capitis; inflammation due to sun light or application of some topical agents on the skin leads to inflammation. ROC curve analysis showed that these parameters contribute to distinguishing AA from AGA, to some various degrees. The most helpful parameters in our study were peribulbar lymphocytic infiltration in H&E stained slides, CD3+ lymphocytes in peribulbar region, and stela in IHC stained slides (AUCs of 0.911, 0.884, and 0.835, respectively). Other studies have also emphasized the importance of these parameters for more accurate diagnosis. Kolivras et al. 5 showed that CD3+ lymphocytes are reliable histopathologic keys in favor of AA diagnosis (sensitivity and specificity of CD3 staining in that study was higher than 80% for various regions; when two regions were combined, this rate reached almost 100%). In a study by Kamyab et al. 28, it was revealed that inflammation density of AA was higher than AGA regarding CD3 (specificity of 86.7% and sensitivity of 96.7%) and CD8 (specificity of 50% and sensitivity of 86.6%) staining. It was also mentioned that intrafollicular CD3 and CD8 stained T-cell infiltrations were significantly higher in AA samples in comparison to AGA samples (P-values of 0.017 and < 0.000, respectively).

The main limitation of the study is its relatively small sample size which did not allow comparison of alopecias with different degrees of clinical severity. Recruitment of total duration of disease from the onset in further analyses can lead to more comprehensive results.

CONCLUSIONS

Peribulbar lymphocytic infiltration is the most reliable parameter in distinguishing AA from AGA. Staining for CD3 (T-lymphocytes' cell marker) has significant diagnostic value in establishing accurate diagnosis.

Conflict of Interest: None declared.

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