RESEARCH ARTICLE

Melan-A expression related to apoptosis of melanocytes in segmental and non-segmental vitiligo [version 1; peer review: awaiting peer review]

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Abstract

Background
Vitiligo is a progressive depigmentation of the skin with unclear etiology. Cell-mediated immunity has been suggested to play an important role in the pathogenesis of vitiligo's progression. Melan-A has a high affinity for specific CD8+ T cells and is one of the critical markers for detecting damage to melanocytes. Our study aims to demonstrate the differences in Melan-A expression associated with apoptosis of melanocytes in patients with segmental vitiligo (SV) and those with non-segmental vitiligo (NSV).

Methods
A cross-sectional study with 64 patients diagnosed with vitiligo, of whom 33 had NSV and 31 had SV. Skin biopsy and direct immunofluorescence were used to examine Melan-A, and the TUNEL staining method was performed to examine melanocyte apoptosis in both groups. Group comparisons were conducted using appropriate statistical methods.

Results
Melan-A expression was significantly higher in the NSV group than in the SV group, and there was a significant difference between the two groups (p=0.001). The median of melanocyte apoptosis in the NSV group was relatively higher than in the SV group, and a significant difference was found between the two groups (p=0.001). The Spearman's rank correlation test between Melan-A expression and melanocyte apoptosis in the NSV group was 0.767 (76.7%) and showed a significant relationship (p<0.05). The same test in the SV group was 0.583 (58.3%) and showed a significant relationship (p<0.05). In both groups, the higher the Melan-A expression, the higher the melanocyte...
apoptosis.

**Conclusion**
Melan-A expression and melanocyte apoptosis are positively correlated. Higher Melan-A expression and melanocyte apoptosis in NSV indicates more severe vitiligo disease compared to SV.

**Keywords**
Melan-A, melanocyte apoptosis, segmental vitiligo, non-segmental vitiligo, disease severity

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How to cite this article: Setyanto B, Kalim H, Poeranto S and Widasmara D. Melan-A expression related to apoptosis of melanocytes in segmental and non-segmental vitiligo [version 1; peer review: awaiting peer review] F1000Research 2022, 11:1211 https://doi.org/10.12688/f1000research.125285.1

First published: 25 Oct 2022, 11:1211 https://doi.org/10.12688/f1000research.125285.1
1. Introduction
Vitiligo is a progressive pigmentation disorder due to the loss of cutaneous melanocytes. To date, the cause of this loss remains unclear. The prevalence of the disease is 0.5–2% of the worldwide population.\(^1\) In South Malang, Indonesia, of the 2,700 patients who visited the Dermatology and Venereology outpatient clinic at Kepanjen Hospital between 2015 and 2019, 187 had vitiligo, amounting to a prevalence of 7%. The peak occurrence of vitiligo is between the first and third decade of life.\(^3\)

Vitiligo does not affect life expectancy, but it affects quality of life because it may cause the patient to feel inferior in their social environment and face stigma from society.\(^7\) The disease’s pathogenesis is not fully understood,\(^5,6\) and no single mechanism has fully been implicated in disease causation.\(^3\) There are 3 main hypotheses for the pathogenesis of vitiligo, namely genetic factors, oxidative stress, and autoimmunity.\(^6–8\)

Clinically, vitiligo is divided into segmental vitiligo (SV) and non-segmental vitiligo (NSV), with the prevalence of SV being less than that of NSV. The course of SV is acute, appearing rapidly within a few weeks and stabilizing within 1–2 years.\(^9\) On the other hand, NSV is characterized by the expansion of lesions on both sides of the body, and the course of the disease is chronic and progressive throughout life.\(^10\) Diagnosis can be made by clinical examination and skin biopsy.\(^11\)

Melan-A/MART-1 (melanoma antigen recognized by T cell-1) is a protein with a high affinity for specific CD8+ T cells in perilesional skin and blood that has been detected \textit{in vitro} and is associated with the extent and severity of vitiligo disease.\(^12\) It is one of the critical markers for detecting melanocyte cell damage or melanocyte apoptosis.\(^7\) Melan-A is found mainly in the perilesional zones.\(^13\) Recent studies have shown that circulating T lymphocytes are cytotoxic against Melan-A in most vitiligo patients, as a specific cellular immune response against melanocytes.\(^15\)

Depigmentation levels and vitiligo disease activity can be correlated with an increase in T cells expressing increased antigen-associated receptors.\(^16\) Various studies have shown the role of cytokines in the process of melanocyte cell damage.\(^17\) The notion that CD8+ T cells act as the final procedure in melanocyte destruction is extensive in the pathogenesis of vitiligo.\(^18\) This study aims to compare Melan-A expression and melanocyte apoptosis between SV and NSV and identify correlation between them.

2. Methods
2.1 Subjects
The number of research subjects was calculated using the Lameshow (Cochran) formula, where based on the calculation, the minimum number of samples required was 32 people for each group as research subjects. The subjects consisted of 64 patients diagnosed with vitiligo divided into two groups: the NSV group which contained 33 patients, and the SV group which consisted of 31 patients. All patients attended the Dermatology and Venereology outpatient clinic at Kanjuruhan Kepanjen General Hospital during the period from 21st September 2021 to 22nd March 2022. Each patient’s vitiligo diagnosis was confirmed by anamnesis, clinical examination, and skin biopsy. Anamnesis identifies the presence of milky white patches. In clinical examinations, SV presents within a few weeks and stabilizes within 1–2 years.\(^3\) In contrast, NSV is characterized by the expansion of lesions on both sides of the body, and the disease is chronic and progressive throughout life. The skin biopsy shows if there are melanocytes at the lesion area in the epidermis.\(^14\)

The patients included in this study were aged between 12 and 60 years and had no keloid history. Exclusion criteria included patients with diabetes mellitus, HIV, Cushing syndrome, active infection, trauma, and malignancy, as well as pregnant women, smokers, and those with too much sun exposure (all subjects in this study exposed to the sun less than 2 hours per day). The subjects were enrolled after providing informed consent and detailed demographic and clinical information alongside their family history of vitiligo.

2.2 Methods
Melan-A expression and melanocyte apoptosis were examined by skin biopsy and direct immunofluorescence, followed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method. We used the anti-Melan-A antibody kit from Santa Cruz (catalog number Sc-20032 PE). Double staining immunofluorescence was used to examine the co-distribution of Melan-A and melanocyte apoptosis in the same sample of cells. All skin biopsies were performed at the Dermatology and Venereology outpatient clinic at Kanjuruhan Kepanjen General Hospital, and the samples were given to the clinical pathology department, Faculty of Medicine, Universitas Brawijaya Malang, for further processing.
Skin biopsies were taken in the following way. Perilesional vitiligo was disinfected with 70% alcohol, then 0.25 mL of lidocaine was applied to anesthetize the area to be biopsied. A skin biopsy with a diameter of 3 mm was performed using the punch biopsy method. The skin tissue from the biopsy was placed in an Eppendorf tube. After completion, the wound was cleaned with a 0.9% NaCl solution, a topical fusidic acid applied, and closed using sterile gauze. The tissue was cut to a thickness of 2–3 mm, before the tissue was given a code according to the researcher’s gross code and inserted into a cassette. The tissue was then processed using an Automatic Tissue Tex Processor tool for 90 minutes, according to the standards of the Anatomical Pathology Laboratory of the FKUB, until an alarm signaled completion. The process of blocking and cutting the tissue was performed, which the epidermis was removed from the Tissue Tex Processor machine. Then the tissue block was performed with paraffin according to the tissue code. The tissue was cut with a microtome tool with a thickness of 3–5 microns.

This was followed by hematoxylin and eosin (H&E) staining, which was conducted at the Pathology Anatomy at the Faculty of Medicine, Universitas Brawijaya. There followed the deparaffinization process. The tissue was placed into two tubes of xylol solution for 20 minutes each, after which it was placed into four tubes with ethanol absolute, 90%, 80%, 70%, each 1 mL for 3 minutes (hydration), and then in running water for 15 minutes. It was subsequently stained in Mayers hematoxylin for 1 minute and washed with 4–5 changes of tap water until blue stopped coming off the slides. The tissue was placed in 1% acid alcohol (1% HCl in 70% alcohol) twice for decolorization and rinsed in running tap water before counterstaining in eosin for 3–5 minutes. The slides were then dehydrated in an increasing concentration of ethanol (70%, 80%, 90%, 100%), each 1 mL and were put in two xylene baths for clearing, followed by the immunofluorescence process.

The immunofluorescence process was conducted in the biomedical laboratory at the Faculty of Medicine, Universitas Brawijaya. The slide was heated at 60°C for 60 minutes. Then the slide was immersed in the following solutions in sequence: xylol solution (pure, 1 mL) for 2 × 10 minutes, absolute ethanol (96%, 1 mL) for 2 × 10 minutes, 90% ethanol (1 mL) for 1 × 5 minutes, 80% ethanol (1 mL) for 1 × 5 minutes, 70% ethanol (1 mL) for 1 × 5 minutes, and sterile distilled water for 3 × 5 minutes. The antigen retrieval process was carried out with citrate buffer. The slide was first immersed in a chamber containing citrate buffer at pH 6.0 (Na citrate dehydrate 2.96 g + twin 20 0.5 mL dissolved in distilled water). The slide was then placed in a water bath at 95°C for 20 minutes. After removal from the water bath, we waited for approximately 20 minutes for it to come to room temperature. The slide was then washed with PBS for 3 × 5 minutes. After that, the slide was washed with PBS Triton-X 100 0.2% for 5 × 1 minute. The slide was incubated with 3% bovine serum albumin (BSA) for 30 minutes at room temperature, after which time the BSA solution was discarded. The slide was incubated with Melan-A antibody (Santa Cruz; catalog number Sc-20032 PE) 1:100 in the blocking buffer overnight at 4°C. After overnight incubation, the slide was washed with PBS for 3 × 5 minutes. Then the slide was incubated with DAPI 1:1000 for 5 minutes before washing the slide with PBS for 3 × 5 minutes.

Further, the slide was stained using the TUNEL method conducted at the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang. Tissue pieces were incubated at a temperature of 21–37°C for 15–30 minutes using a proteinase K working solution 15 μg/mL with concentration 100 μg/mL. Then, the slides were immersed in Tris-HCl (0.1 M, pH 7.5) containing 3% BSA and 20% normal bovine serum for 30 minutes at a temperature of 15–25°C. Then the slides were rinsed with PBS at 15–25°C twice and the excess fluid was drained. Then 50 μL of the TUNEL reaction mixture was added to the section before being incubated for 60 minutes at 37°C in a humid and dark atmosphere. The slide was rinsed three times in PBS for 5 minutes each. Mounting medium was used to cover the slide followed by a cover slip. Melan-A expression (red color) and apoptosis of melanocyte expression (green color) were evaluated by an Olympus IX71 fluorescence microscope under 40× magnification. Photos were taken for documentation. The next process was to analyze the Melan-A expression and apoptosis of melanocyte using ImageJ software. The results of the two groups were then compared.

Data were analyzed using descriptive statistics to calculate the percentages, mean values, and standard deviations. Since the data were not normally distributed, we used the non-parametric Mann–Whitney u test. Student’s t-test and Spearman’s rank correlation were used to analyze the variance between the two groups and association among the variables. A p value less than 0.05 was considered statistically significant.

3. Results
3.1 Subject characteristics
There were 64 patients with vitiligo included in this study, of whom 33 were in the NSV group and 31 in the SV group. The mean age was 22.32 ± 9.20 years in the SV group and 44.79 ± 11.24 years in NSV group. The subjects’ characteristics are presented in Table 1.
3.2 Melan-A expression on perilesional zones

In Figure 1, the results of the Mann Whitney tests showed a p value of 0.001 (<\(\alpha\)=0.05); thus, it can be concluded that there were differences between Melan-A expression in the NSV group and the SV group. The median of Melan-A expression was 16.27±1.86 pixels in the NSV group and 2.70±4.14 pixels in the SV group. Melan-A expression in the NSV group was relatively higher than in the SV group. We then used ImageJ software to calculate Melan-A expression and melanocyte apoptosis from qualitative to quantitative values. The mean result of Melan-A expression in the NSV group was 30.15 pixels and 2.60 pixels in the SV group.

In Figure 2, the results of the Mann Whitney tests showed a p value of 0.001 (<\(\alpha\)=0.05); thus, it can be concluded that there were differences between melanocyte apoptosis in the VNS group and the VS group. The median of melanocyte apoptosis to be 26.45±8.16 pixels in the NSV group and 6.12±5.056 pixels in the SV group. Melanocyte apoptosis was higher in the NSV group than in the VS group. After analysis with ImageJ software, we identified the mean result of melanocyte apoptosis expression in the NSV group to be 51.23 and 5.07 pixels in the SV group.

In Figure 3, the results of the Spearman’s rank correlation test showed that the relationship between Melan-A expression and melanocyte apoptosis in the NSV group was 0.767 (76.7%) and significant (p<0.05). The relationship was positive; thus, the higher the expression of Melan-A, the higher the melanocyte apoptosis. Based on the results of the Spearman’s rank correlation test, the relationship between Melan-A expression and melanocyte apoptosis in the SV group was 0.583 (58.3%) and significant (p<0.05). The relationship was positive; thus, the higher the expression of Melan-A, the higher the melanocyte apoptosis.

Figure 4 indicates that melanocyte apoptosis was more dominant in the NSV group than in the SV group, due to the possibility of redox balance disturbances and more severe melanocyte destruction.

### Table 1. Demographic and clinical characteristics of subjects included in the study.

| Characteristic                        | Non-segmental vitiligo | Segmental vitiligo |
|--------------------------------------|------------------------|--------------------|
| Age (years)                          | 44.79±11.24            | 22.32±9.20         |
| Sex                                  |                        |                    |
| Female                               | 20 (31.25%)            | 15 (23.44%)        |
| Male                                 | 13 (20.31%)            | 16 (25.00%)        |
| Family history of autoimmune disease|                        |                    |
| Yes                                  | 13 (39.4%)             | 1 (3.33%)          |
| No                                   | 20 (60.6%)             | 30 (96.7%)         |
| Duration of the disease (months)     |                        |                    |
| <6                                   | 2 (6.1%)               | 1 (3.2%)           |
| 6–12                                 | 4 (12.1%)              | 5 (16.1%)          |
| >12                                  | 27 (81.8%)             | 25 (80.7%)         |
| Marital status                       |                        |                    |
| Married                              | 29 (87.9%)             | 6 (19.4%)          |
| Not married                          | 4 (12.1%)              | 25 (80.6%)         |
| Social life                          |                        |                    |
| Good                                 | 33 (100%)              | 31 (100%)          |
| Poor                                 | 0 (0%)                 | 0 (0%)             |
| History of therapy                   |                        |                    |
| Corticosteroid topical               | 32 (97%)               | 17 (54.8%)         |
| Unknown                              | 1 (3%)                 | 7 (22.6%)          |
| No therapy                           | 0 (0%)                 | 7 (22.6%)          |
4. Discussion

This study provides important insights about the differences in Melan-A expression associated with apoptosis of melanocytes in SV and NSV patients. Several previous studies have described the role of Melan-A in vitiligo in general, but no studies have investigated the differences in Melan-A expression and melanocyte apoptosis and their correlation in SV and NSV patients. The progressive depigmentation of NSV and its unpredictable disease course highlights its research importance.

The Melan-A/MART-1 protein, which is located in the melanosome and plays a role in both protein formation and maturation, is one of the activation stimulants for CD8+ T cells, along with GP100 and tyrosinase. Melan-A has an important role in the expression, stabilization, transport, and processing of the melanosome in melanocytes. Melan-A is

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Figure 1. (a) The median of Melan-A in the NSV group is relatively higher than in the SV group. Under the Olympus IX71 fluorescence microscope under 40× magnification; (b) expression of Melan-A in NSV; (c) expression of Melan-A in SV.
usually found in individuals who have HLAA2, although it can also be found in others, but the amount is only 1 in 1000
CD8+ T cells and in the naïve state.20,21 Several studies have shown better sensitivity and specificity of Melan-A as a
marker of melanocytic differentiation, compared to markers S100 and HMB-45. The HMB45 marker is not specific for
melanocytes because it can also be expressed by sweat gland cells and nonmelanocytic tumors. However, HMB45, which
is a monoclonal antibody, reacts with a melanosomal protein (GP100) which can not only express activated melanocytes,
but can also express fetal melanocytes, thereby confirming melanocyte activity. The S100 marker can also detect
melanocyte activity but has the disadvantage of being able to react positively not only to melanocytes, but also to
Langerhans cells, neurons, tissue, muscle cells, and apocrine glands. Circulating cytotoxic T lymphocytes are found in
most vitiligo patients, where T cells express an increase in lymphocyte-associated antigen receptors, which in turn
correlates with the degree of depigmentation and vitiligo disease activity.20,21 Histologically, Melan-A appears to be most
dominant when located in perilesional zones.22-24 CD8+ T cells infiltrate the perilesional skin, where vitiligo is most
active, and preferentially localize to the epidermis and dermis, adjacent to melanocyte.25

**Figure 2.** (a) The median of melanocyte cell apoptosis in the NSV group is higher than in the SV group. TUNEL
method using the Olympus IX71 fluorescence microscope under 40× magnification; (b) melanocyte cell
apoptosis (yellow arrow) in the NSV group; (c) melanocyte cell apoptosis (yellow arrow) in the SV group.
The increased amount of Melan-A can be caused by several factors, such as stress, ultraviolet exposure, pollution, and chemical materials that are immunogenic and have both autoreactivity and a high affinity for CD8+ T cells that destroy melanocytes, leading the process of apoptosis to occur.\(^{20,26-28}\) The presence of neoantigens resulting from damage to melanocytes activates pattern recognition receptors, in turn activating dendritic cells through damage-associated molecular patterns (DAMPs) followed by reactive CD8+ T cells that continuously destroy the melanocytes.\(^{18,29,30}\)

Melanocyte apoptosis is a programmed and schematic mechanism that occurs in vitiligo. DNA in melanocytes is cut into fragments with enzymes from endonuclease, which catalyze DNA. In electrophoresis, the melanocytes are seen to shrink, which indicates preparation for cell death. A typical feature of apoptosis is a “step ladder pattern”, whereby the cells appear wavy. In the end, the cells become fragmented and release apoptotic bodies.\(^{31-33}\) However, in addition to apoptosis, several authors have recently suggested that neoantigens can also be caused by another form of melanocyte cell death such as necroptosis, pyroptosis, ferroptosis, oxeiptosis, and parthanatos, which are characterized by an inflammatory process. Apoptosis that is too long can also cause necrosis and vice versa. In other words, it can be said that both prolonged apoptosis and necrosis can produce neoantigens and trigger autoimmunity.\(^{34}\)

The autoimmune mechanism leading to apoptosis can be through either the extrinsic or intrinsic pathway. The extrinsic pathway occurs through FAS ligand and FAS receptors, as well as involving FAS-associated death domain (FAAD).
The subsequent FAAD-like interleukin F-beta converting enzyme (FLICE) activates procaspase 8, converting it into caspase 8. Caspase 8 activates procaspase 3 to caspase 3, which in turn activates apoptosis.35,36 Furthermore, the extrinsic pathway also involves the movement of perforin and granzyme into cells. Perforin makes holes or pores in the surface of the target cell membrane, which, besides causing direct damage, also facilitates the entry of granzyme into the cell. Granzyme will stimulate the target cells to undergo apoptosis. The intrinsic pathway occurs in cells and involves pro-apoptotic factors such as Bax and Bak, as well as anti-apoptotic factors such as Bcl-2 and Bcl-xl, which affect the cytochrome. Then cell death can occur through caspases.37–39

In our study, it was found that there were differences between Melan-A expression in the NSV group and the SV group. The result of Melan-A expression in the NSV group was higher than in the SV group. Besides, we identified that melanocyte apoptosis in the NSV group was higher than in the SV group. In both groups, there was a positive correlation between Melan-A expression and melanocyte apoptosis; the higher Melan-A expression, the higher the melanocyte apoptosis. Our study shows that in NSV, Melan-A is more immunogenic, which causes more autoreactivity in CD8+ T cells. These specific CD8+ T cells secrete TNFα, IL6, IL8, perforin, and granzyme and induce endoplasmic reticulum-associated degradation (ERAD), which eventually causes melanocyte damage and cell death. It can increase the acceleration of the autoimmune process and the progression of vitiligo disease.12 It is said that Th1 and Th2 imbalances and Melan-A play a major role in the progression of SV to NSV.9 A descriptive study conducted by Vaniary et al., which evaluated the expression of Melan-A in vitiligo patients, found that the Melan-A expression on depigmented skins of vitiligo patients was below the average value, although this study did not compare Melan-A in vitiligo and in healthy skin.40 Our result is consistent with the study by Arora et al., which showed that an increase in Melan-A causes autoreactivity to CD8+ T cells and increasingly causes more severe damage to melanocytes.12 From the study conducted by Chen et al., the role of the role of CD8+ T cells as executors is explained by the high frequency of serum melanocyte-specific CD8+ T cells in vitiligo patients compared with healthy people. In addition, serum CD8+ T cell levels are also associated with disease severity.18 Altogether, these findings provide strong evidence that Melan-A expression correlates with melanocyte apoptosis in both NSV and SV. In addition, in NSV patients, there was an acceleration of the autoimmune process and progression of vitiligo disease, which was indicated by higher expression of Melan-A and melanocyte apoptosis than in SV.

5. Conclusion
In conclusion, the expression of Melan-A in NSV is higher than in SV, and the apoptosis of melanocytes in NSV is higher than in SV. There is a positive correlation between Melan-A expression and melanocyte apoptosis. The higher Melan-A expression, the more melanocytes are undergoing apoptosis in NSV and SV. Future multicenter studies with larger sample sizes and correlations with other vitiligo biomarkers are necessary to fully understand the pathogenesis of NSV and SV.

Ethics
This research has been declared to be ethically feasible by Health Research Ethic Committee Faculty of Medicine Brawijaya University July 28th 2021 with number 218/EC/KEPK-S3/07/2021.

Informed consent
Written informed consent for publication of the patients’ details was obtained from the participants or from the parents/guardians of participants under the age of 18.

Data availability
Underlying data
Zenodo: Melan-A Expression Related to Apoptosis of Melanocytes in Segmental and Nonsegmental Vitiligo, https://doi.org/10.5281/zenodo.6997417.

The project contains the following underlying data:

- Analysis Result.docx (analysis result data).
- DAPI and Apoptosis SV (Immunofluorescence).
- DAPI and Melan-A NSV (Immunofluorescence).
- DAPI and Melan-A SV (Immunofluorescence).
- DAPI and Apoptosis NSV (Immunofluorescence).
- DAPI and Apoptosis SV (Immunofluorescence).
- Data and Demographic.docx (demographic data of subjects)
- Melan-A and Apoptosis SV (Immunofluorescence)
- Melan-A and Apoptosis NSV (Immunofluorescence)
- Result-2.docx (result data)

Reporting guidelines
Figshare: STROBE checklist for Melan-A expression related to apoptosis of melanocytes in segmental and non-segmental vitiligo, https://doi.org/10.6084/m9.figshare.21163072.v1.41

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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