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Immunohistochemical analysis of melanocyte content in different zones of vitiligo lesions using the Melan-A marker

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Abstract

Introduction: Occurence of vitiligo lesions is caused by the destruction of melanocytes in affected skin and therefore by the reduction of pigment melanin content. Questions remain about the presence of residual melanocytes in the depigmented skin and optimal methods of their identification.
Methods: Skin biopsy samples from 16 patients with non-segmental vitiligo and from 10 healthy volunteers were investigated for Melan-A (A103 clone)+ melanocytes expression by immunohistochemical analysis and for melanin by histochemical studies with section staining by Fontana-Masson method.
Results: For some patients including those with long-standing disease (up to 40 years) Melan-A+ cells and melanin granules were detected in depigmented skin as indication that the residual melanocytes are preserved in vitiligo lesions. More than three-fold decrease of Melan-A+ melanocytes amount was revealed in perilesional normally pigmented skin of vitiligo patients ($P < 0.001$) compared with the skin of healthy volunteers. Clinically intact skin involvement in the pathological process should be taken into consideration if local treatment methods are prescribed.
Conclusion: In some vitiligo patients the residual melanocytes are preserved in depigmented skin. Melan-A marker is useful for identification of melanocytes in vitiligo patients' skin.

Keywords: immunohistochemistry, melanocytes, Melan-A marker, vitiligo

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Introduction

Vitiligo is a chronic disease of unknown etiology characterized by the onset of depigmented spots on various body areas. Currently, the generally accepted hypothesis of the cause of vitiligo is the autoimmune one, according to which the destruction of melanocytes in vitiligo lesions is induced by autoimmune mechanisms (1–3).

In 1991, Cui et al. showed that the depigmented skin of vitiligo patients carries residual melanocytes that can be preserved in hair follicles for a long time (4). These melanocytes are able to act as a reservoir for the repopulation of pigment cells with normal function during disease treatment.

According to some authors, epidermal melanocytes are absent in depigmented skin (5–7). According to others, melanocytes and melanin are found in the area of depigmentation even in long-standing vitiligo lesions (8–10). Such ambiguous results may to some extent be due to differences in markers and research methods.

Immunohistochemical research on various melanocytic antigen expression in the skin provides important information about melanocyte status in vitiligo lesions. Several different markers have been suggested for melanocyte identification, but none of them show absolute specificity and sensitivity (11).

One of the most important melanocytic markers is the Melan-A marker, also known as MART-1 (melanoma antigen recognized by T cells-1). This marker is expressed in melanosomes and the endoplasmic reticulum of melanocytes, and it identifies the melanosomal protein that participates in formation and maturation of melanosomes (12). Forming a complex with Pmel17 protein (also known as gp100 or "silver locus" product), Melan-A plays an essential role in its expression, stability, transport, and processing in melanosomes (13). In turn, Pmel17 protein (identified by HMB-45, HMB-50, and NKI-beteb markers) acts as the main structural protein of the internal matrix fibers of melanosomes.

Melan-A antigen is mostly expressed in melanocytes of the skin and the retina (14). Studying Melan-A expression in vitiligo lesions is of significant interest because this melanosomal protein is one of the immunogenic determinants of melanocytes to which autoreactive circulating CD8+ T-cells were found in patients' blood (15–17). Melan-A–specific cytotoxic T-cells are known to have the phenotype of memory T-cells (CD45RO+) and to express cutaneous lymphocyte antigen (CLA) (15), and so their important role in vitiligo pathogenesis is evident.

The purpose of this study was to investigate the usefulness of the Melan-A marker for identifying and estimating melanocyte content in different zones of non-segmental vitiligo lesions: the zone of depigmented skin, marginal zone (bordering the area), and zone of perilesional normally pigmented skin.

Material and methods

Immunohistochemical and histochemical analyses were performed for 16 patients with non-segmental vitiligo and for 10 healthy volunteers. In vitiligo patients, skin biopsy material was taken from three zones: the zone of depigmented skin, marginal zone (bordering the area), and zone of perilesional normally pigmented skin.

In healthy volunteers, skin samples taken during cosmetic procedures were used as study material. Skin biopsies were fixated in a buffered 10% formalin solution, processed, and embedded in paraffin. Paraffin blocks were cut into 4- to 5-micron sections on a microtome and placed on glass slides.

Immunohistochemical studies were performed with pre-treat-

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ment demasking in a microwave oven and using a Novostain Universal Detection Kit detection system (Novocastra Laboratories Ltd., UK). Mouse monoclonal Melan-A antibodies, A103 clone (Novocastra Laboratories Ltd., UK) were used for the phenotyping at a dilution of 1:25. Deparaffinization of the sections in xylene was followed by the demasking of antigens by boiling in a citrate buffer (pH 6.0) using a microwave oven for three cycles, 5 minutes each, with a 1-minute break between each cycle. After cooling, the slides were washed in two changes of TRIS-buffer (pH 5.54). In order to prevent endogenic peroxidase activity, a solution of 0.3% hydrogen peroxide with methanol (1:1 ratio) was applied to the sections. Hematoxylin staining of nuclei was performed in all cases.

Melanin granules in the skin were detected through Fontana-Masson histochemical staining of the sections.

The prepared specimens were studied using a Nikon Eclipse E 600 light microscope and photographed with a Nikon D100 digital camera. The amount of Melan-A+ cells was detected in the basal layer of the epidermis, and the amount of melanin-containing cells was detected in the basal and suprabasal layers, the calculation being made per 100 basal keratinocytes. Immunoreactive cells were counted in five fields of vision, and after that the average value was calculated for each specimen.

Statistical analysis was performed using the software package Statistica 6.1 (StatSoft, Inc., USA). Descriptive statistics of quantitative traits was presented as medians and quartiles (Me [Q1; Q3]). The Mann-Whitney U-test was used for comparison of unrelated groups by both quantitative and ordinal traits. Correlation analysis was performed using the Spearman rank correlation coefficient. In testing hypotheses, the differences were considered statistically significant at $P < 0.05$.

Results

The 16 patients examined with non-segmental vitiligo included 11 women and five men. The patients were between 24 and 52 years old (median 38 years), the duration of the disease was between 21 months and 42 years (median 16 years), and the affected area was between 5 and 50% of the body surface (median 20%). All patients had a progressive (unstable) disease stage. The control group consisted of 10 healthy volunteers between 29 and 64 years old (median value 46 years). The group of patients and group of healthy volunteers were not statistically different in age and sex ($P = 0.200$ and $P = 0.130$, respectively).

In the group of healthy volunteers, Melan-A+ melanocytes and melanin granules were found in all skin samples examined. Melan-A+ cells were revealed in the basal layer of the epidermis (Fig. 1), and melanin-containing cells were presented in the cells of the basal and suprabasal layers of the epidermis (Fig. 2).

In some vitiligo patients, expression of Melan-A melanosomal protein and melanin granules was detected in all three zones of vitiligo lesions. In the zone of depigmented skin, Melan-A+ melanocytes and melanin granules were found in six and nine patients, respectively; in the marginal zone, in 13 and 15 patients, respectively; and in the zone of perilesional normally pigmented skin, in all patients examined. For patients with detected Melan-A+ and melanin-containing cells in the zone of depigmented skin, duration of the disease ranged from 21 months to 40 years.

In the patients' skin, Melan-A expression was evident in the cells of basal layer of the epidermis: in the zone of depigmented skin, individual stained cells were detected (Fig. 3a), whereas in the marginal zone and in the zone of perilesional normally pigmented skin the amount varied from one to seven cells per 100 keratinocytes of the basal layer (Figs. 3b and 3c). Regarding melanin granules, in the zone of depigmented skin they were found in individual regions of the basal layer, in the marginal zone they were found in almost the entire basal layer, and in the zone of perilesional normally pigmented skin they were found throughout the basal and suprabasal layers of the epidermis (Figs. 4a–c).

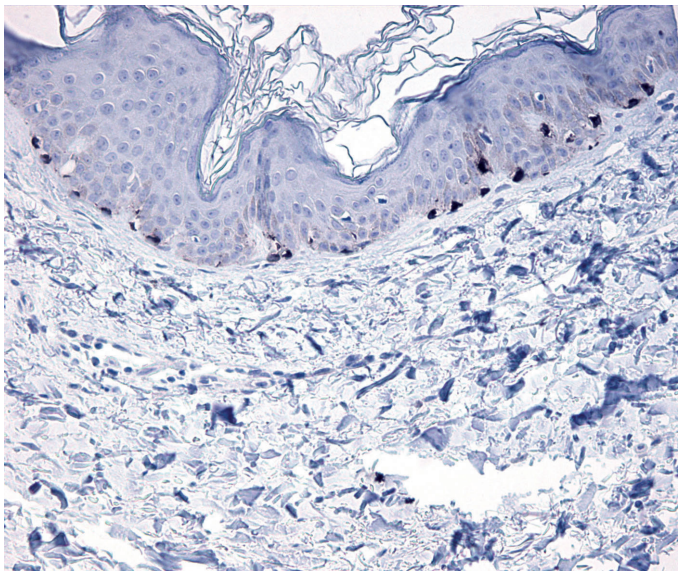

Figure 1 | Expression of Melan-A in the skin of a healthy volunteer. Immunohistochemical reaction with monoclonal antibodies A103, ×200.

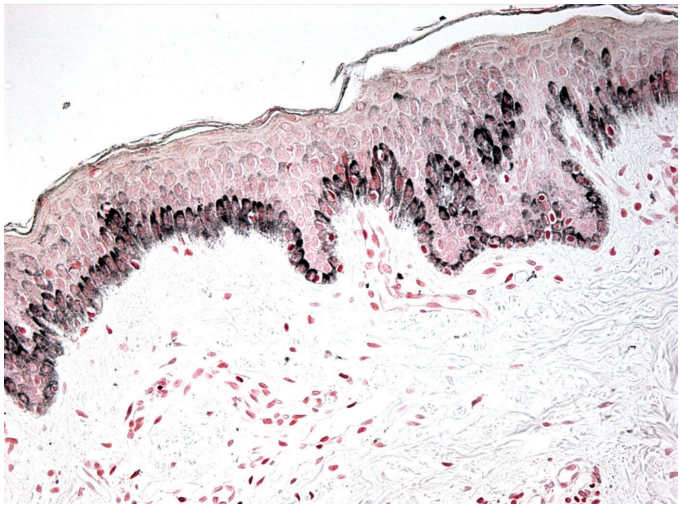

Figure 2 | Melanin granules in the skin of a healthy volunteer. Fontana-Masson staining, ×200.

Statistical analysis showed a decreased amount of Melan-A+ cells in all three areas of vitiligo lesions compared with the skin of healthy volunteers ($P < 0.001$) (Table 1); moreover, its amount in the zone of perilesional normally pigmented skin was over three times lower.

The amount of melanin-containing cells significantly decreased in the zone of depigmented skin ($P < 0.001$) and in the marginal zone ($P = 0.013$), whereas in the zone of perilesional normally pigmented skin it did not differ from the value in the skin of healthy volunteers. In the zone of depigmented skin and in the marginal zone, the amount of Melan-A+ melanocytes correlated with the amount of melanin-containing cells: $r = 0.75$ ($P < 0.001$) and $r = 0.79$ ($P < 0.001$), respectively.

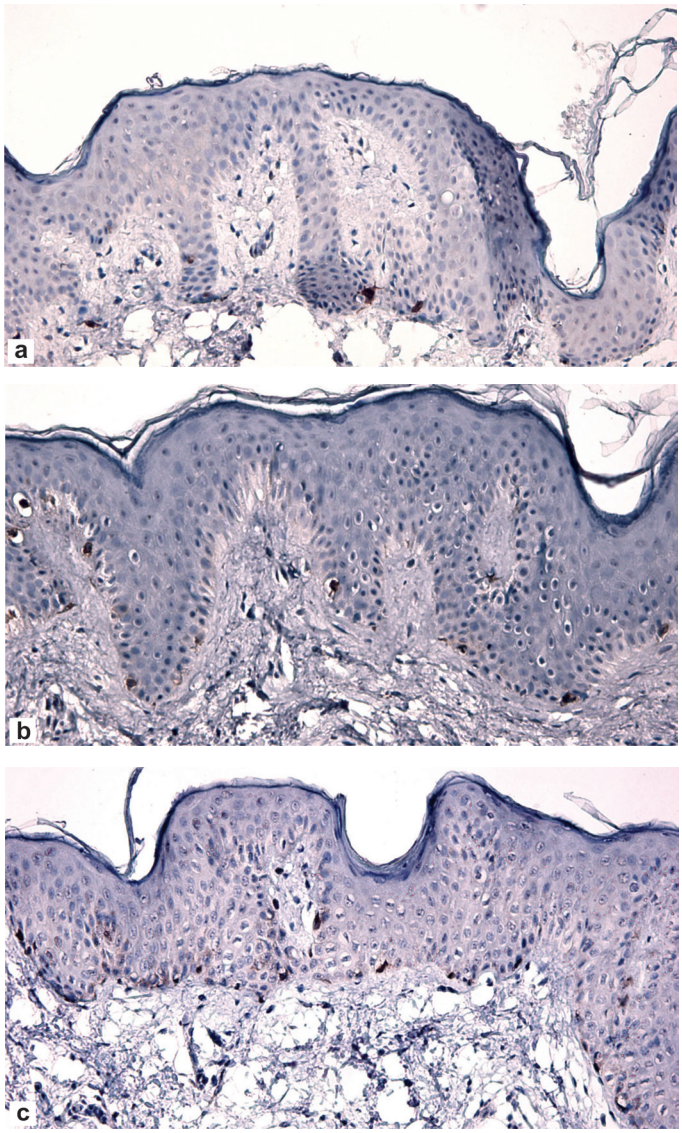

Figure 3 | **a:** Expression of Melan-A in the skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, ×200. Zone of depigmented skin. **b.** Expression of Melan-A in the skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, ×200. Marginal zone. **c.** Expression of Melan-A in the skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, ×200. Zone of perilesional normally pigmented skin.

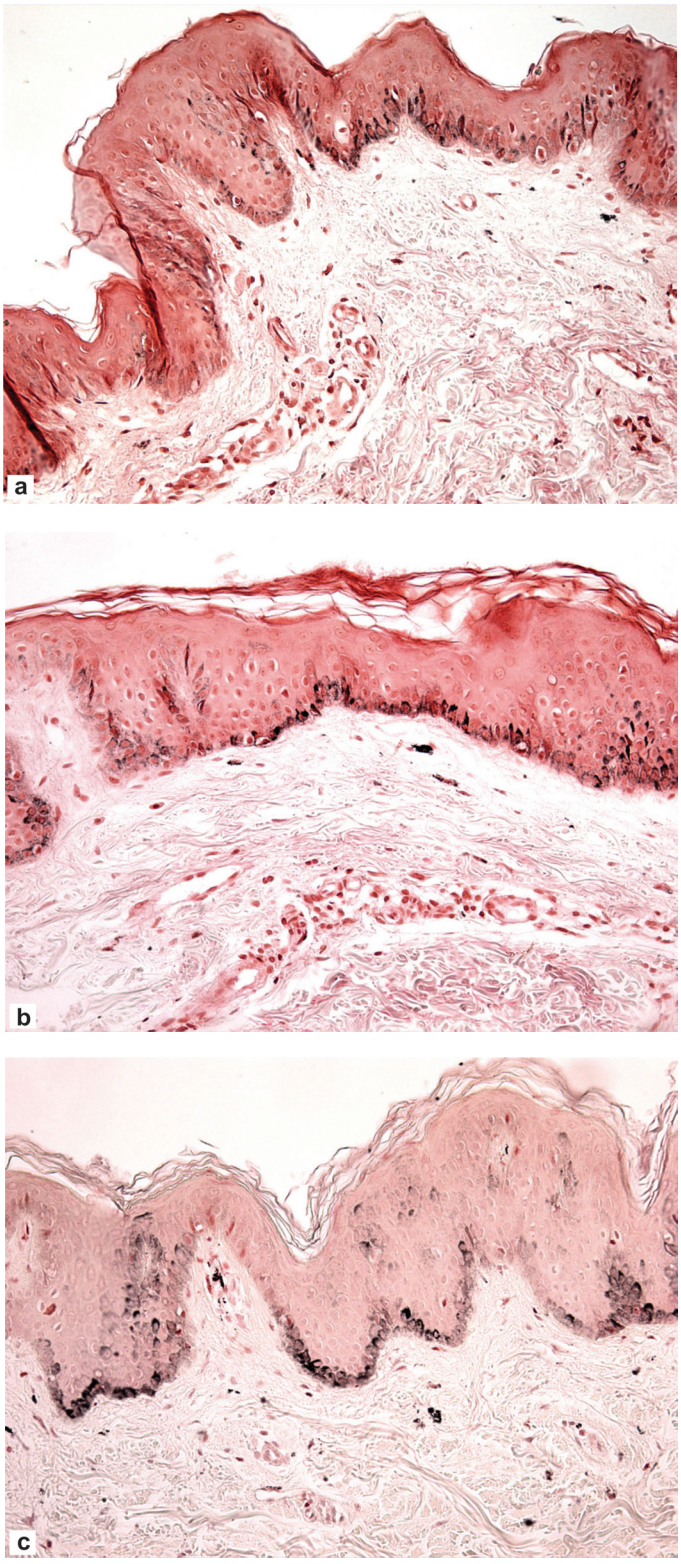

Figure 4 | **a:** Melanin granules in the skin of a vitiligo patient. Zone of depigmented skin. Fontana-Masson staining, ×200. **b.** Melanin granules in the skin of a vitiligo patient. Marginal zone. Fontana-Masson staining, ×200. **c.** Melanin granules in the skin of a vitiligo patient. Zone of perilesional normally pigmented skin. Fontana-Masson staining, ×200.

Discussion

In recent years, the Melan-A marker has been increasingly used in diagnosing various pigment neoplasms and diseases of the skin (18). It is well expressed in melanocytes of healthy skin (12, 19, 20) and transformed melanocytes of benign and malignant neoplasms of melanocytic genesis (21–25).

In a number of studies, the Melan-A marker was shown to be more specific and sensitive than the S-100 and HMB-45 melanocytic markers (14, 20, 22–24). The S-100 marker, which identifies the protein S-100, is not specific enough because it is expressed not only

Table 1 | Content of Melan-A+ melanocytes and FM+ cells in the epidermis of patients with vitiligo and healthy volunteers (amount of cells per 100 basal keratinocytes, median values and quartiles)

Populations of cells	Healthy volunteers (n = 10)	Patients with vitiligo (n = 16)			P_1	P_2	P_3
		Lesional skin	Marginal zone	Area of perilesional normally pigmented skin			
Melan-A+ melanocytes	10.9 [9.6; 12.6]	0 [0; 1.0]	1.0 [0.2; 2.6]	3.4 [2.5; 5.2]	‹ 0.001	‹ 0.001	‹ 0.001
FM+ cells	52.0 [38.4; 100.2]	1.0 [0; 7.8]	18.1 [5.6; 55.9]	54.6 [40.0; 65.1]	‹ 0.001	0.013	0.598

Legend: Melan-A+ melanocytes: cells expressing Melan-A antigen (determination with A103 monoclonal antigens); FM+ cells: cells containing melanin granules (staining with the Fontana-Masson method); P1, P2, and P3: levels of statistical significance during comparison of parameters in healthy volunteers and patients, respectively, in lesional skin, the marginal zone, and the area of perilesional normally pigmented skin

in melanocytes but also in adipocytes, Schwann cells, and myoepithelial cells (24, 26–30). The HMB-45 marker, which identifies gp100 (glycoprotein of premelanosomes), is considered more specific than the S-100 marker but it also does not show absolute specificity because it can be expressed in the cells of sweat glands and non-melanocytic tumors (24, 31, 32). It also has less sensitivity than Melan-A (20, 23, 33).

The Melan-A marker also does not have absolute specificity for melanocytes and may show immunoreactivity for cells of non-melanocytic origin: steroid-producing tumors, angiolipomes, and some others (18, 34–39). Moreover, Melan-A can be expressed by macrophages, but its immunohistochemical staining is weak and granulous, in contrast to the strong expression of melanocytic cells (40).

This study used the Melan-A marker (A103 clone) to identify melanocytes and evaluate their amount in various zones of non-segmental vitiligo lesions. Our results indicate that the Melan-A marker is expressed both in the skin of healthy volunteers and in the skin of vitiligo patients. In vitiligo patients, Melan-A+ melanocytes and melanin granules (the sections were stained using the Fontana-Masson method) have been found in all the zones of vitiligo lesions.

The determined presence of Melan-A+ cells and melanin granules in depigmented skin of patients, including those with long-standing disease (up to 40 years), indicates that the residual melanocytes are preserved in vitiligo lesions. The data obtained appear to confirm the results of other authors. Tobin et al. also detected residual melanocytes in the epidermis of depigmented patients' skin even in cases of long-standing disease (up to 25 years) (8). Expression of tyrosinase was revealed in some of these cells by dopa reaction. Moreover, during ultrastructural analysis of depigmented skin, mature melanin granules were found in the basal and suprabasal layers of the epidermis in 10 of 12 patients examined. In the authors' opinion, these melanin granules were synthesized by residual melanocytes. Based on the results, the authors concluded that active (partly functioning) residual melanocytes are preserved in the depigmented skin of vitiligo patients.

Kim et al. investigated the lesional skin of 100 vitiligo patients and found the presence of melanin granules in 16% of the cases (staining with the Fontana-Masson method) and the expression of NKI-beteb+ melanocytes in 12% of the cases (9). De Francesco et al. found tyrosinase+ and HMB-45+ cells in lesional skin in three of 14 untreated vitiligo patients (21.4%). These three patients displayed > 75% repigmentation after 9 months of narrow-band UVB phototherapy (41). Seleit et al. demonstrated the presence of HMB-45+ cells (indicating differentiated and active melanocytes) in 44% of cases in interfollicular epidermis and in 46.7% of cases in follicular epidermis in lesional vitiligo skin (10). In their latest work, they detected HMB-45+ melanocytes and TRP2+ (Tyrosinase Related Protein 2) melanocytes in 25% and 75% of vitiliginous white hair, respectively (42).

Our findings show that in patients with non-segmental vitiligo the pathological process may involve not only depigmented skin but normally pigmented, clinically intact skin as well. Our results are consistent with the results of studies by Le Poole et al., who found a more than two-fold decrease of NKI-beteb melanocytic marker expression (43), and by Tobin et al., who demonstrated the failure of maturation and transfer of melanosomes from melanocytes into keratinocytes of the basal and suprabasal layers of the epidermis in this area (8). Wańkowicz-Kalińska et al. revealed the association of microscopic signs of melanocyte destruction in clinically intact skin of patients with generalized vitiligo with skin infiltration with T-cells (44).

Recently, new data became available on defects in normal-appearing skin of vitiligo lesions. Wagner et al. demonstrated altered adhesion between melanocytes and keratinocytes in the epidermis of vitiligo patients that, in turn, was linked to the absence of and discontinuous distributions of E-cadherin in clinically normal skin (45). Anbar et al. used electron microscopy to detect degenerative changes in perilesional melanocytes of non-segmental vitiligo patients in the form of vacuolization of the melanocytic cytoplasm, pyknosis of nuclei, and peripheral margination of chromatin (46). Ding et al. found that melanocytes from perilesional vitiligo skin contain few mitochondria (47). The structures of those mitochondria are irregular in various shapes and sizes, most of which are swollen with obscure cristae and vacuolization, especially in active vitiligo.

Conclusion

This study indicates the usefulness of the Melan-A marker for identifying melanocytes in non-segmental vitiligo patients' skin and for estimating their content in different zones of the vitiligo lesions. Expression of melanosomal protein Melan-A and melanin granules was found in all three zones of vitiligo lesions. For some patients, including those with long-standing disease (up to 40 years), Melan-A+ cells and melanin granules were detected in depigmented skin as an indication that the residual melanocytes are preserved in vitiligo lesions, and these results are consistent with the data of other authors. A greater than three-fold decrease in Melan-A+ melanocyte amount was revealed in perilesional normally pigmented skin of vitiligo patients, as compared to the skin of healthy volunteers. The possibility of clinically intact skin involvement in the pathological process should be taken into consideration if local treatment methods are prescribed.

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