

USE OF ENDOGENOUS PIGMENT ANALYTES TO ASSESS THE INVASIVE POTENTIAL OF SUPERFICIAL SKIN MELANOMAS

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Abstract

Cutaneous melanoma, which accounts for 72-80% of all diagnosed tumors developing from melanin-producing tissue, is an example of a malignancy whose initiation and development, with rare exceptions, are associated with the realization of endogenous pigmentations in the morphological substrate of the tumor. However, the existing diagnostic paradigm for superficial cutaneous melanomas does not take into account the significance of endogenous pigmentations in assessing the tumor's invasive capacity. This article examines the relationship between changes in the biochemical composition of superficial cutaneous melanomas and their invasive capacity, as well as the possibility of using this relationship in melanoma diagnostics. A comparative pathomorphological, ultrastructural, and spectroscopic study of 128 samples of superficial spreading melanoma removed during radical surgical excision was conducted. A higher frequency and diversity of endogenous pigment analytes were detected during the horizontal phase of tumor growth compared to the vertical phase. Diagnostically significant analytes included carotenoid pigments ($p=0.02257$), porphyrin ($p=0.09080$), and melanin precursors tyrosine ($p=0.01554$) and phenylalanine ($p=0.01753$). The use of a comprehensive pathomorphological and spectroscopic study and multiplex analysis of the obtained results made it possible to simultaneously identify a significant number of statistically confirmed endogenous pigment analytes in superficial melanoma skin samples. These analytes have diagnostic value in assessing the invasive capacity of superficial melanomas.

Key words: superficial melanoma of the skin, endogenous pigment, analyte, melanin, carotenoid, porphyrin, invasive capacity.

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ИСПОЛЬЗОВАНИЕ АНАЛИТОВ ЭНДОГЕННЫХ ПИГМЕНТОВ ДЛЯ ОЦЕНКИ СПОСОБНОСТИ К ИНВАЗИИ ПОВЕРХНОСТНЫХ МЕЛАНОМ КОЖИ

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Резюме

Меланома кожи, на долю которой приходится 70-80% всех диагностируемых опухолей, развивающихся из меланинообразующей ткани, представляет собой пример злокачественной опухоли, инициация и развитие которой за редким исключением сопряжены с реализацией эндогенных пигментаций в морфологическом субстрате опухоли. Вместе с тем, существующая диагностическая парадигма поверхностных меланом кожи не учитывает значение эндогенных пигментаций для оценки способности опухоли к инвазии. Настоящая статья посвящена исследованию связи между изменениями в биохимическом составе поверхностных меланом кожи и их способности к инвазии, а также возможности использования этой связи при проведении диагностики меланомы. Было проведено

сравнительное патоморфологическое, ультраструктурное и спектроскопическое исследование 128 образцов поверхностно распространяющейся меланомы, удаленных во время радикального хирургического иссечения. Выявлена большая частота и разнообразие анализов эндогенных пигментов в горизонтальную фазу роста опухоли по сравнению с вертикальной. К диагностически значимым анализам отнесены пигменты каротиноиды ($p=0,02257$), порфирин ($p=0,09080$), а также предшественники меланина тирозин ($p=0,01554$) и фенилаланин ($p=0,01753$). Использование комплексного патоморфологического и спектроскопического исследования и мультиплексный анализ полученных результатов позволили идентифицировать в образцах поверхностной меланомы кожи одновременно значительное количество подтвержденных статистически анализов эндогенных пигментов, имеющих диагностическое значение в оценке способности поверхностных меланом кожи к инвазии.

Ключевые слова: поверхностная меланома кожи, анализ, эндогенный пигмент, меланин, каротиноид, порфирин, способность к инвазии.

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Introduction

The results of numerous studies on the epidemiology of oncological diseases convincingly demonstrate an increase in morbidity and mortality rates in the vast majority of countries worldwide [1,2]. All this fully applies to cutaneous melanoma, which accounts for over 70% [3] of all diagnosed tumors developing from melanin-producing tissue. The progress observed in recent years in the early and effective diagnosis of melanoma is due to the use of precision immunohistochemical and molecular genetic research methods [4,5]. The diagnostic paradigm for cutaneous melanoma remains relatively conservative and does not take into account the importance of endogenous pigmentations found in the tumor tissue substrate, which play a key role in determining its invasive potential.

Materials and Methods

We studied 128 samples of superficial spreading melanoma removed during radical surgical excision and submitted for pathohistological examination in accordance with Order No. 179n of the Russian Ministry of Health dated March 24, 2016, "On the Rules for Conducting Pathological and Anatomical Studies." The first group ($n = 83$) included superficial melanoma samples with a predominantly horizontal growth phase, often with features of Pagetoid melanoma. The second group ($n = 45$) included samples with a predominantly vertical growth phase. The control group ($n = 10$) consisted of facial skin samples (autopsy material) with no clinical or morphological manifestations of skin pathology. Skin samples were fixed for 24–48 hours in 10% neutral formalin in a phosphate buffer (pH 7.4). Skin processing was performed automatically using a Pathos Delta processor (Milestone, Italy), and paraffin embedding was performed using a Leica HistoCore (Leica, Germany). Serial 3–4 μm -thick sections, prepared on a ThermoFisherScientific HM 325 microtome (USA),

were stained with hematoxylin and eosin, picrofuchsin according to van Gieson, and according to Mallory. Melanin was detected by silvering using the Fontana-Masson method. Skin samples directly adjacent to the melanoma and visually unchanged were examined under an electron microscope. Skin fragments, 1 mm^3 in volume, were fixed in a 2.5% glutaraldehyde solution in collidine buffer (pH 7.4) at room temperature for 1.5 h, and post-fixed in a 1% osmium tetroxide solution for 1.0–1.5 h at $+4$ – $+6^\circ\text{C}$. The material was dehydrated in alcohols of increasing concentration (from 50° to 96°) and several portions of ether, and embedded in Araldite. Semi-thin sections were stained with methylene blue, and ultra-thin sections were contrasted with uranyl acetate and lead citrate [6].

Tissue structure was visualized in ultrathin sections using a Zeiss EVO LS10 scanning electron microscope (Zeiss, Germany) with a STEM detector.

Spectroscopic examination of the biological samples was also performed using an InSpektr M spectrometer, version M-532 (RU dated June 18, 2021, No. RZN 2015/2419, manufacturer: RamMix LLC, Russia), consisting of an Olympus CX41 optical microscope and a spectrometer unit with an excitation wavelength of 532 nm. The laser spot diameter at the focal point was 10 μm , and the laser power was 10 mW. Instrument control, spectral recording, and spectral acquisition were performed using the specialized InSpektr software, also developed by RamMix LLC (Chernogolovka). The program identified chemical substances and recorded changes in their quantitative and qualitative composition in the biological sample under study using spectra combining Raman and fluorescence signals. The spectra of biological molecules identified during the study were assigned to the corresponding substance class and displayed as separate columns or fields in the database. Their detection or absence was reflected as a binary indicator (yes/no). Raman spectroscopy was used to

identify both certain pigments (porphyrin, carotenoids) and analyte components related to melanin pigment precursors (tyrosine, phenylalanine) (Table 1).

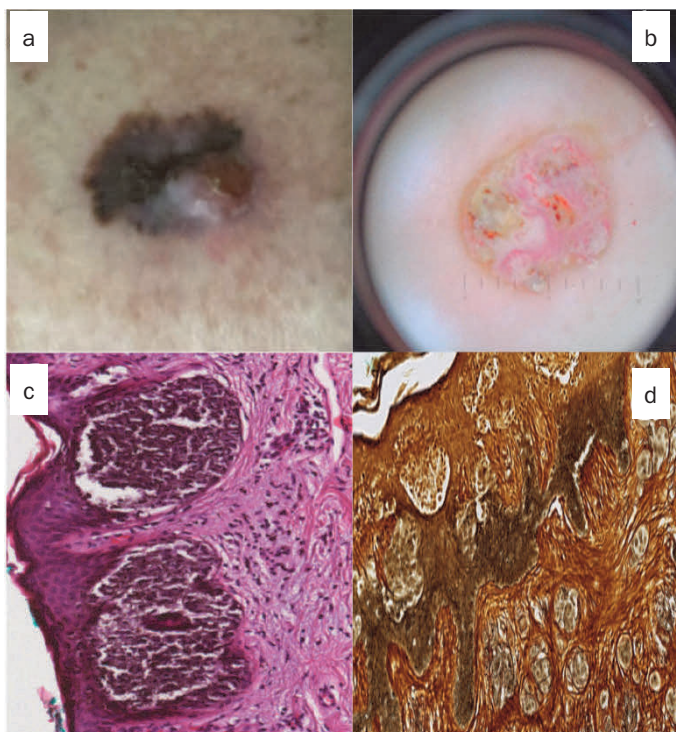
To compare the identified changes, the results of studies of control skin tissue samples obtained from individuals without skin pathology were used. Database creation and subsequent statistical processing were performed using specialized software: R-4.3.0 (The R Foundation, Austria) and RStudio Desktop (Version 2023.06.1, RStudio Inc, USA). Additional R packages within this software were used for descriptive statistics, building regression models, and data visualization.

Statistical significance of differences was assessed at a significance level of $p < 0.05$.

The study protocol was reviewed and approved by the Ethics Committees of the State Budgetary Healthcare Institution "Moscow Multidisciplinary Clinical Center "Kommunarka" of the Moscow Department of Health (extract from protocol No. 6 dated August 8, 2023) and the Federal State Budgetary Educational Institution "Tyumen State Medical University" of the Ministry of Health of the Russian Federation (extract from protocol No. 121 dated May 22, 2024).

Results and Discussion

Macroscopically, all melanomas examined exhibited at least three of the five ABCDE features [7] (Fig. 1a) and were confirmed dermoscopically (Fig. 1b). In the first group of samples ($n = 83$), the tumor was localized in the dermoepidermal junction, epidermis, and partially in the papillary dermis (Fig. 1cd).



Atypical melanocytes could be located singly (Fig. 2a) or formed tumor clusters, which was expectedly accompanied by the development of intercellular

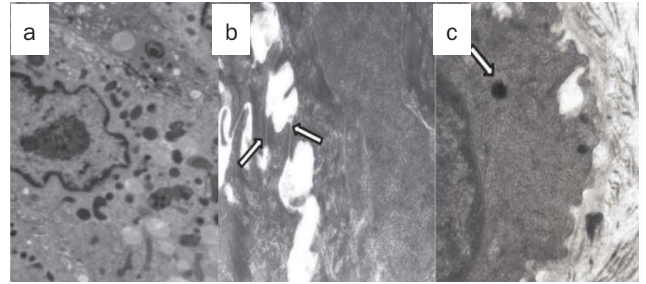


Рис. 2. Ультраструктурные особенности клеточной составляющей поверхностной меланомы кожи:

а – атипизм ультраструктур меланоцита опухоли, нарушение ядерно-цитоплазматического отношения, гетерогенность меланосом. Трансмиссионная электронная микроскопия $\times 9000$; б – включения меланина и межклеточный отёк (спонгиоз) между базальным и шиповатым слоями эпидермиса. Десмосома – белая стрелка. Трансмиссионная электронная микроскопия $\times 11000$;

с – липофусцин в цитоплазме кератиноцита базального слоя эпидермиса - белая стрелка. Трансмиссионная электронная микроскопия $\times 12000$.

Fig. 2. Ultrastructural features of the cellular component of superficial skin melanoma:

а – atypia of tumor melanocyte ultrastructures, violation of the nuclear-cytoplasmic ratio, heterogeneity of melanosomes. Transmission electron microscopy $\times 9000$;

б – inclusions of melanin and intercellular edema (spongiosis) between the basal and spinous layers of the epidermis. Desmosome - white arrow. Transmission electron microscopy $\times 11000$;

с – lipofuscin in the cytoplasm of the keratinocyte of the basal layer of the epidermis - white arrow. Transmission electron microscopy $\times 12000$.

Рис. 1. Поверхностно-распространяющаяся меланома кожи туловища:

а – неравномерности границ новообразования и распределения пигмента, цветовое разнообразие в отсутствие выраженной асимметрии и возвышения; б – фотофиксация изображения новообразования, полученного при помощи дерматоскопа;

с – педжетоидная поверхностная меланома, атипичные меланоциты и формируемые ими комплексы в области дерматоэпидермального абриса, эпидермисе и частично сосочковом слое дермы. Окраска гематоксилином и эозином $\times 150$;

д – скопления меланина в эпидермисе и дерме. Серебрение по Фонтана-Массону $\times 70$.

Fig. 1. Superficial spreading melanoma of the skin of the trunk:

а – uneven boundaries of the neoplasm and distribution of pigment, color diversity in the absence of pronounced asymmetry and elevation;

б – photographic recording of an image of a neoplasm obtained using a dermatoscope;

с – pagetoid superficial melanoma: atypical melanocytes and the complexes they form in the area of the dermoepidermal outline, epidermis and partially the papillary layer of the dermis. Hematoxylin and eosin staining $\times 150$;

д – accumulations of melanin in the epidermis and dermis. Silvering according to Fontana-Masson $\times 70$.

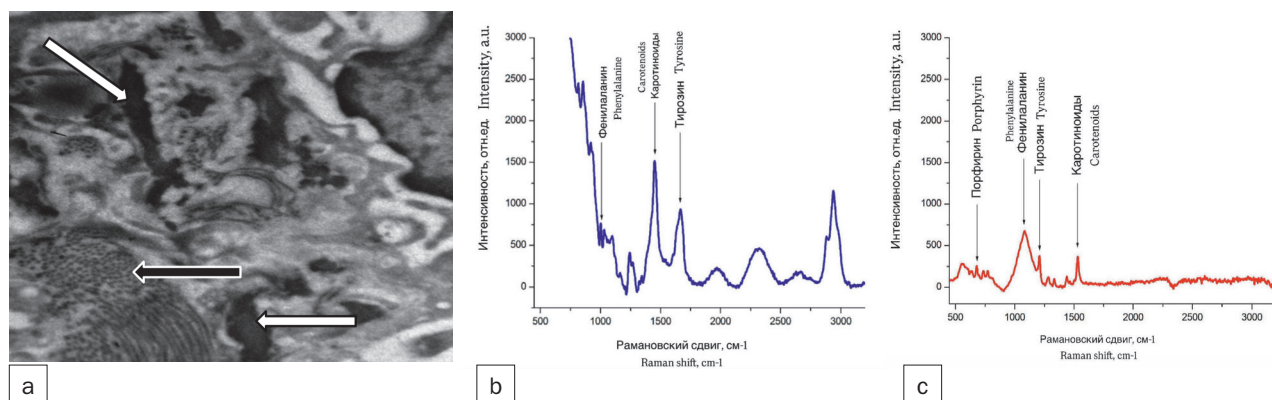


Рис. 3. Поверхностно-распространяющаяся меланома кожи со спектральными образцами горизонтальной и вертикальной фаз роста:

a – игольчатые отложения порфирина (белые стрелки) и коллаген (черная стрелка) в субэпидермальном пространстве дермы. Трансмиссионная электронная микроскопия x 12000;

b – спектральная характеристика меланомы в фазу горизонтального роста (пояснения в тексте);

c – спектральная характеристика меланомы в фазу вертикального роста (пояснения в тексте).

Fig. 3. Superficial spreading melanoma of the skin with spectral patterns of horizontal and vertical growth phases:

a – needle-like deposits of porphyrin (white arrows) and collagen (black arrow) in the subepidermal space of the dermis. Transmission electron microscope x 12000;

b – spectral characteristics of melanoma in the horizontal growth phase (explanations in the text);

c – spectral characteristics of melanoma in the vertical growth phase (explanations in the text).

edema in the spinous layer of the epidermis (Fig. 2b). Keratinocytes in the basal layer of the epidermis often contained small lipofuscin inclusions (Fig. 2c), which differed from melanin grains by their ultrastructural heterogeneity and lower electron density.

Porphyrin accumulations in the form of needle-like formations were localized predominantly in the dermoepidermal junction and dermis (Fig. 3a). In samples from the second group ($n = 45$), tumor complexes and individual atypical melanocytes were localized in the papillary and reticular layers of the dermis. Tumors with a vertical growth phase were characterized by higher rates of invasion, tumor substrate thickness, mitosis, and the frequency of both pigments and their precursors compared to those in the first group (Table 1). The spectra of the control group skin samples were characterized by high initial fluorescence values and an abundance of both non-specific and specific identifiable analytes, including melanin precursors (phenylalanine and tyrosine) with a distribution of molecular vibration intensities of phenylalanine at 1003.8 cm^{-1} and 1045.1 cm^{-1} , and tyrosine at 1172.4 cm^{-1} . In comparison, the spectra of skin samples with superficially spreading melanoma in the horizontal growth phase showed higher total initial fluorescence (from 3000 arbitrary units) and a diverse composition of specific, so-called “pigment” analytes, with molecular vibration intensities at 1002 cm^{-1} (phenylalanine), 1614.4 cm^{-1} (tyrosine), 1122 cm^{-1} (porphyrin), and 1480 cm^{-1} (carotenoids) (Fig. 3b).

The initiation and development of the vertical phase of melanoma growth in the spectra were accompanied by low values of the initial fluorescence of biological

objects, minimal differences between the initial, maximum, and residual fluorescence values, and the presence of Raman peaks of analytes of precursors of proteinogenic chromoproteins (Fig. 3c).

To statistically evaluate the relationship between each of the two tumor growth phases and the analytes detected in it by Raman spectroscopy, binary logistic regression was used as a statistical analysis method. In this model, the dependent or studied variable was the tumor growth phase, and the independent variables were the analytes detected during spectroscopy [8, 9]. Two working hypotheses were formulated. The first, designated as H_0 , assumed the absence of differences in the frequencies of determination of tyrosine, phenylalanine, porphyrin and carotenoids between the horizontal and vertical phases of tumor growth. The alternative hypothesis H_1 assumed the presence of statistically significant differences between the tumor growth phases and the identified analytes. The obtained results demonstrated the statistical significance of carotenoids ($p = 0.02257$), tyrosine ($p = 0.01554$) and phenylalanine ($p = 0.01753$) (Table 2).

It should be noted that each coefficient in the resulting model represents the logarithmic odds ratio of transition to the vertical growth phase for each unit increase in the corresponding analyte value, holding all other variables constant. Carotenoids, tyrosine, and phenylalanine have a negative effect on the probability of transition to the vertical growth phase of superficial spreading melanoma, as their estimated values are less than zero. This means that detection of these components by Raman spectroscopy increases the probability of a tumor being in the horizontal growth phase.

Таблица 1

Выявленные при рамановской спектроскопии пигменты и их предшественники в различные фазы роста поверхностно-распространяющихся меланом кожи

Table 1

Pigments and their precursors identified by Raman spectroscopy in different growth phases of superficial spreading melanomas of the skin

№	Признак Characteristic	Количество, % Count, %	
	Фазы роста опухоли / The phase of tumor growth	128 (100%)	
1	Радиальная / Horizontal	83 (64,8%)	
	Вертикальная / Vertical	45 (35,2%)	
	Компоненты, выявленные при помощи рамановской спектроскопии/ Raman Spectroscopy-detected biomarkers		
2	Тирозин / Tyrosine	86/128 (67,2%)	
	Фенилаланин / Phenylalanine	69/128 (53,9%)	
	Порфирин / Porphyrin	75/128 (58,6%)	
	Каротиноиды / Carotenoids	62/128 (48,4%)	
	Частоты выявления компонентов посредством рамановской спектроскопии в различные фазы роста меланомы / Raman Spectroscopy-based detection rates of biomarkers during melanoma growth phases		
	А) Фаза радиального роста / A) Horizontal growth phase		
3	Тирозин / Tyrosine	63/83 (75,9%)	
	Фенилаланин / Phenylalanine	54/83 (65,1%)	
	Порфирин / Porphyrin	44/83 (53,0%)	
	Каротиноиды / Carotenoids	47/83 (56,6%)	
		Б) Фаза вертикального роста / B) Vertical growth phase	
	Тирозин / Tyrosine	23/45 (51,1%)	
	Фенилаланин / Phenylalanine	15/45 (33,3%)	
	Порфирин / Porphyrin	31/45 (68,9%)	
	Каротиноиды / Carotenoids	15/45 (33,3%)	

Таблица 2

Результаты расчета коэффициентов, включенных в модель бинарной логистической регрессии

Table 2

Results of calculating the coefficients included in the binary logistic regression model

Коэффициенты Coefficients	Оценка Value	Стандартная ошибка Standart mistake	Уровень значимости p Significance level of p
Intercept (свободный член) / Intercept (free member)	1,3934	0,5923	0,01865*
Каротиноиды / Carotenoids	-1,0158	0,4454	0,02257*
Порфирин / Porphyrin	0,7829	0,4629	0,09080
Тирозин / Tyrosine	-1,1103	0,4589	0,01554*
Фенилаланин / Phenylalanine	-1,0596	0,4461	0,01753*

*статистически значимые показатели анализов фазы роста меланомы

*statistically significant indicators of melanoma growth phase analyte values

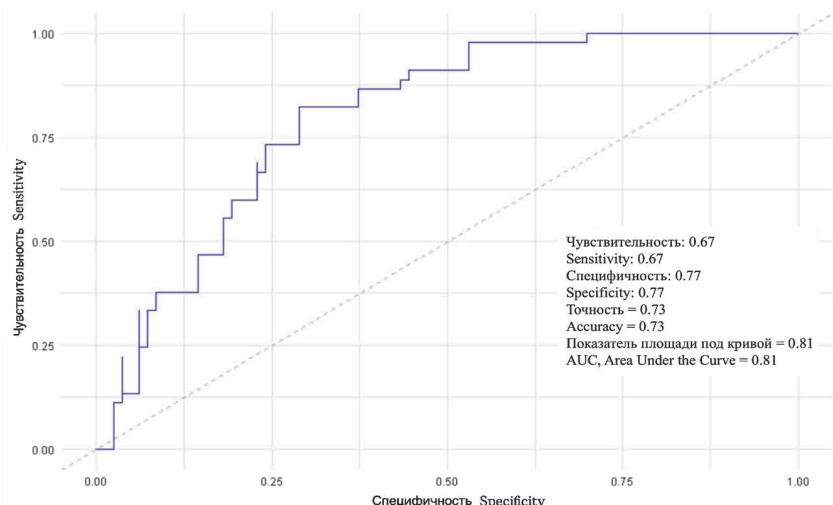


Рис. 4. ROC-анализ оценки модели перехода меланомы кожи в вертикальную фазу роста.

Fig. 4. ROC-analysis for assessing the model of cutaneous melanoma transition to vertical growth phase.

The quality of the binary classification was assessed using a Receiver Operating Characteristic (ROC) curve (Fig. 4). The area under the curve (AUC) was 0.81, indicating the good ability of the model to differentiate tumor growth phases. The specificity of model was 0.77, sensitivity was 0.67, and the proportion of correctly classified cases among all examples (accuracy) reached 0.73.

Based on the obtained results, a model for predicting the vertical growth phase of superficial spreading melanomas based on Raman spectroscopy data is presented as follows:

$$p = \frac{1}{1 + e^{-(1.3934 - 1.0158 \times \text{Carotenoids} + 0.7829 \times \text{Porphyrin} - 1.1103 \times \text{Tyrosine} - 1.0596 \times \text{Phenylalanine})}}$$

where:

- p is the probability that the melanoma is in the vertical growth phase
 - e is the base of the natural logarithm ≈ 2.718
- If $p > 0.5$, the model predicts a vertical growth phase; if $p < 0.5$, it indicates a horizontal growth phase.

It should be noted that, despite the obtained significance level for porphyrin being $p = 0.09080$, which exceeds the established one for this study, we considered it necessary to include it in the prognostic

model. This decision is based on data from reputable scientific sources indicating a high concentration of porphyrins in malignant tumors [10-12]. The lower number of melanoma cases in the vertical growth phase observed in our study compared to the horizontal phase may explain the lack of statistical significance of porphyrin and does not allow to confidently exclude it from the model.

Conclusion

Despite significant advances in the diagnosis and therapy of melanomas, treatment of these tumors remains a challenge, as the stability of positive results is not always achieved. The need to update existing diagnostic criteria became evident after the publication of R. Virchow's seminal work on oncology [13]. Over the years, this approach has been repeatedly revised due to the introduction of highly accurate diagnostic methods. Today, medical science seeks to understand the nature of melanoma precisely through the lens of its metabolic features, suggesting that it is the metabolic products, including pigments, that may serve as potential diagnostic markers [14-15]. The present study aims to assess the importance of endogenous pigmentation in identifying the invasiveness of cutaneous melanoma.

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