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Quantitative measurements of the fluorescence intensity — new method for a detection of melanoma cells in histopathological preparations

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Formalin-induced fluorescence (FIF) allows for a detection of human melanoma. Microscopic digital imaging was used to determine quantitatively the FIF intensity. Melanomas were detected with a sensitivity of 82% and a specificity of 66%, *i.e.*, better than reported earlier. Also 70% of melanomas in a vertical and 83% of melanomas in a horizonthal growth phase were classified correctly. Changes in the FIF intensity seem to be correlated with a progress of the disease. Determination of molecules involved in the FIF emission may lead to a discovery of an antigen specific to human melanoma. That might pave a way to elaborating first specific immunohistologic technique for detecting the cells of human melanoma.

1. Introduction

Malignant melanoma is a cause of a worldwide concern. Its incidence has been increasing with a doubling time of 10 years and the disease is potentially lethal. Fortunately the five-year survival rate has doubled since 1950's. It is thought that the decrease in mortality is a result of earlier recognition and treatment [1]. However, the early detection of melanoma is difficult not only for dermatologists and other physicians but often also for histopathologists [2]. Microscopic diagnostics of pigmented skin lesions is based mostly on histomorphological criteria. Immunohistochemical detection of selected antigenes can be used for additional characterization of melanomas and pigmented skin naevi. However, since all the antibodies lack a complete specificity to melanoma even such an approach may not ensure a correct diagnosis. Therefore, there is a clear need of new techniques allowing for a detection of melanoma cells on a microscopic level. We have recently suggested that quantitative measurements of the intensity of the formalin-induced fluorescence (FIF) can be used to differentiate between the cells of melanoma and benign pigmented skin lesions in standard paraffin-embedded skin specimens [3].

Such a fluorescence can be observed only after a fixation of a biologic material with formaldehyde vapour or with a formalin. Similar approach has been successfully applied in the past to a detection and quantitative determination of a concentration of neurotransmitter amines [4].

In this paper we present new results indicating that the quantitative measurements of the FIF intensity are not only useful for a detection of melanoma cells but can also yield additional information important for an assessment of a stage of the disease and for predicting prognosis.

2. Material and methods

2.1. Material

The study material comprised 61 cases of malignant melanoma (superficial spreading melanoma (SSM) – 25, nodular melanoma (NM) – 28, lentigo maligna melanoma (LMM) – 2, lentigo maligna (LM) – 2, acral lentiginous melanoma (ALM) – 1, amelanotic melanoma – 1, intraepithelial melanoma – 2), 3 cases of basal cell carcinoma (BC) and 62 cases of benign pigmented skin lesions (junctional nevus – 12, compound nevus – 18, intradermal nevus – 13, dysplastic nevus (DN) – 7, blue nevus – 1, lentigo simplex – 1, seborrheic keratosis – 10). The formalin-fixed and paraffin-embedded specimens were obtained from files of Oncology Centre, Warszawa, Regional Oncology Centre, Bydgoszcz and of District and City Hospitals, Toruń, Poland.

The sections used for the fluorescence examinations were 4–6 μm thick. Before the measurements the slides were incubated for 30 min at 56 °C, deparaffinized and rehydrated in a standard manner (sequence of xylene, ethyl alcohol and water). A fluorescence intensity standard (in Speck Green 505/515, in Speck Blue 360/430, Microscope Image Calibration Kit, Molecular Probes) was mounted onto the slides before coverslipping them with glycerol solution in phosphate buffered saline (1:9 v/v). After completing the fluorescence examinations the sections were stained with hematoxylin-eosin.

2.2. Fluorescence imaging

Digital images of the fluorescence were recorded at 100× magnification using epifluorescence microscope (BX50, Olympus, Japan) and a cooled CCD camera (DEI-407T, Optronics Engineering, U.S.A.). Computer codes for measurements of the fluorescence intensity were prepared using commercial image analysis software (Image Pro Plus, Media Cybernetics, U.S.A.). Excitation and observation wavelengths were such as described in references [5]–[7]. The fluorescence was excited with a blue light of a high-pressure mercury lamp. The exciting filter transmitted either 366 nm Hg line or 450–480 nm band, while the fluorescence light passed through a suitable dichroic mirror and a barrier filter transmitting respectively above 425 nm or 515 nm. The fluorescence images were typically recorded for central regions of the lesions. Images of two peripheral regions of the lesions (edge), including also cells of a regular

skin, were collected if such regions could have been found on the section. The image integration times were such that none of the pixels showed a saturation effect and varied between 1/60 and 1/2 s.. The images were corrected for bias voltages, thermal noise and for a spatial distribution of sensitivities of the pixels of the CCD matrix. The fluorescence intensity determined for the selected regions of interest was normalized to a unit area and automatically related to the intensity measured for the standard, *i.e.*, expressed as a percentage value of the latter.

3. Results

General characteristics of the observed fluorescence were similar as reported in earlier publications of other authors [5]–[11] and in our previous paper [3]. Briefly, all the lesions investigated emitted the fluorescence. Ultraviolet light (366 nm) excited a yellow-blue fluorescence, while the excitation with a blue light (450–480 nm) induced a yellow-green fluorescence (Fig. 1). The fluorescence was emitted not only by the cells of the pigmented skin lesions – it could be seen also in

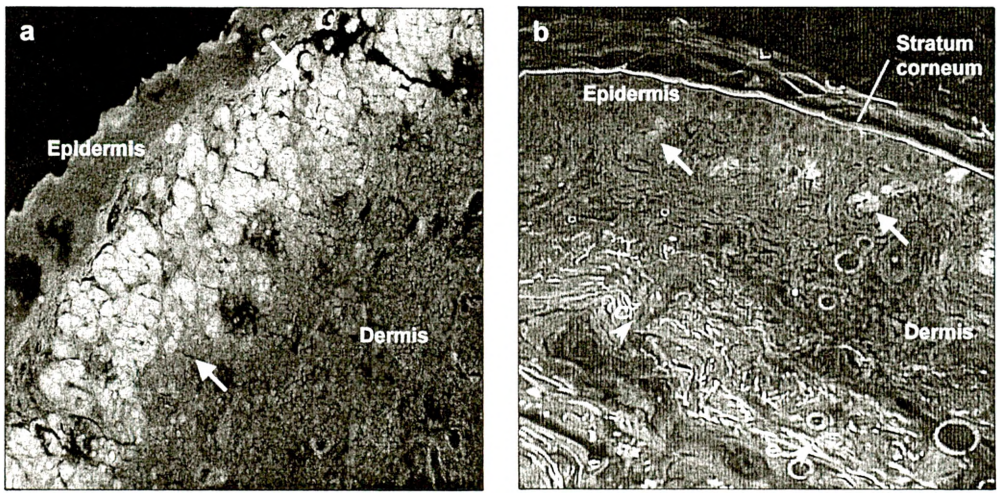

Fig. 1. Formalin-induced fluorescence excited with blue light (450–480 nm). **a** – cells of nodular melanoma (Clark III) localised in dermis (arrows). **b** – fluorescent cells of junctional naevus and elastic (arrows marked "e") and collagen (arrows marked "c") fibres in dermis. Magification ×100.

their vicinity. Additionaly, for a normal skin from excission margins a weak fluorescence was observed in epidermal layer, while in the dermis a stronger fluorescence was emitted by collagen and elastic fibres. Visually, the ultraviolet excited fluorescence was stronger than the fluorescence excited with the blue light. Quantitive comparison of the two intensities was not possible because of different excitation efficiency and different emission band of the fluorescence intensity standard [12]. On the other hand, the ultraviolet excited fluorescence was clearly less specific to the cells of melanomas and to both the type and the stage of the

disease than the fluorescence excited with the blue light. For that reason, from now on we shall concentrate only on the results obtained in studies of the yellow-green fluorescence excited with the blue light (430–450 nm).

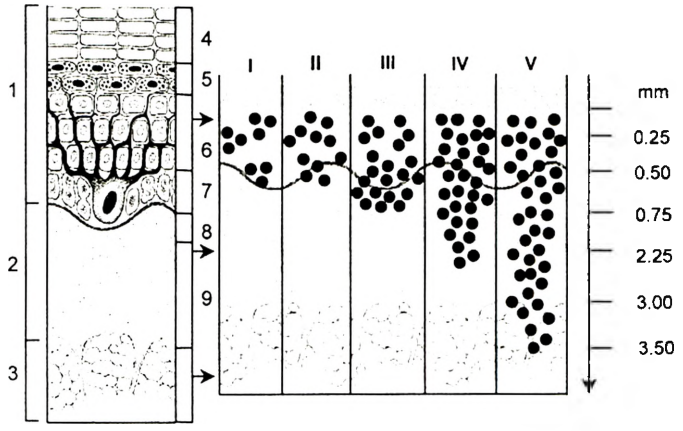

Fig. 2. Schematic representation of a vertical cross-section of human skin. Columns I–V illustrate localisation of the melanoma cells at different stages of the disease classified according to Clark's scale. Arabic numbers designate skin an tissue layers: 1 – epidermis, 2 – dermis, 3 – subcutis, 4 – stratum corneum, 5 – stratum granulosum, 6 – stratum spinosum, 7 – stratum basale, 8 – stratum papillare, 9 – stratum reticulare.

Human skin has a complex structure. From a point of view of this work it is sufficient however to consider only its basic layer structure shown in a vertical section in Fig. 2. It was shown in the earlier studies that the FIF intensity depends on a location of the fluorescent cells. The highest intensity was typically found for the cells located in stratum corneum [3], [6], [7] and in the central regions of the lesions [3]. The emission intensity decreased with a distance from both the centre of the lesion and from the skin surface. At the microscopic magnification ($\times 100$) used in this work the fluorescent cells could be assigned only to: stratum corneum, jointly to stratum granulosum and stratum spinosum, stratum papillare and stratum reticulare. Because of possible future practical applications, the results were also analyzed for more macroscopic structures like epidermis and dermis or jointly for all the layers (Tabs. 1 and 2).

The analysis of average FIF intensities presented in Tables 1 and 2 clearly indicates that for all the lesions investigated in this work the FIF was emitted with a highest intensity by the cells located in the central regions of the lesions. The differences in the intensity levels of the cells from central regions and from margins of the lesions were most significant for the cells located in stratum corneum. Also in that layer there were most significant differences in the fluorescence intensity of the cells of melanomas and naevi or other pigmented skin lesions (Figs. 3–5). Such results confirm the fact that, as suggested in [3], a best differentiation between the

Table 1. Mean fluorescence intensities (% of standard intensity) and respective standard deviations detemined for the cells located in the central regions of the lesions in different skin layers (λ_{ex} = 450–480 nm, λ_{em} = 515 nm).

Skin layer	Melanomas	Naevi	Others
Stratum corneum	5.1±3.4 (n = 61)	2.7±2.4 (n = 45)	1.4±0.5 (n = 12)
Stratum granulosum and stratum spinosum	3.6±2.2 (n = 24)	1.8±1.8 (n = 30)	0.8±0.3 (n = 5)
Stratum papillare	3.1±2.2 (n = 24)	1.7±1.5 (n = 30)	1.2±0.4 (n = 5)
Stratum reticulare	4.2±2.8 (n = 24)	2.4±1.9 (n = 30)	1.6±0.7 (n = 5)
Epidermis	3.9±2.7 (n = 61)	2.0±1.8 (n = 51)	1.1±0.4 (n = 14)
Dermis	3.0±2.2 (n = 61)	2.1±1.5 (n = 51)	1.5±0.6 (n = 13)
All the layers	3.9±2.5 (n = 24)	2.0±1.8 (n = 30)	1.1±0.4 (n = 5)

Table 2. Mean fluorescence intensities (% of standard intensity) and respective standard deviations detemined for the cells located at the edges of the lesions in different skin layers (λ_{ex} = 450–480 nm, λ_{em} > 515 nm).

Skin layer	Melanomas	Naevi	Others
Stratum corneum	3.4±2.5 (n = 61)	2.5±2.3 (n = 45)	1.0±0.4 (n = 9)
Stratum granulosum and stratum spinosum	3.3±2.2 (n = 24)	1.6±1.6 (n = 30)	0.7±0.2 (n = 5)
Stratum papillare	3.0±2.2 (n = 24)	1.6±1.3 (n = 30)	0.9±0.4 (n = 5)
Stratum reticulare	3.7±2.9 (n = 24)	2.3±2.1 (n = 30)	1.2±0.5 (n = 5)
Epidermis	2.9±2.1 (n = 61)	1.8±1.5 (n = 51)	1.1±0.6 (n = 14)
Dermis	2.5±1.8 (n = 61)	2.0±1.6 (n = 51)	1.3±0.5 (n = 13)
All the layers	3.4±2.5 (n = 24)	1.9±1.7 (n = 30)	0.9±0.4 (n = 5)

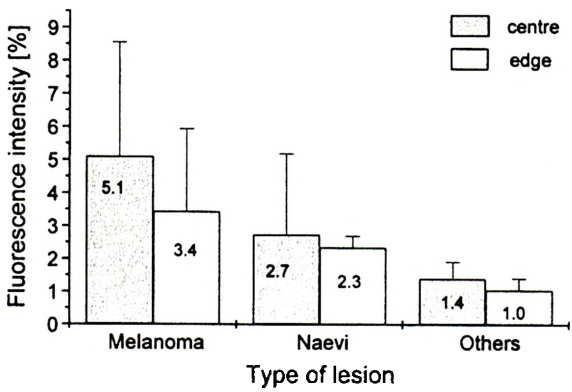

Fig. 3. Mean fluorescence intensities for cells of melanomas, pigmented naevi and other lesions in stratum corneum.

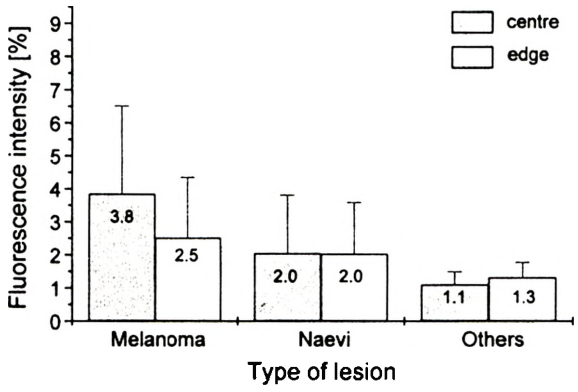

Fig. 4. The same as in Fig. 3, but in the epidermis.

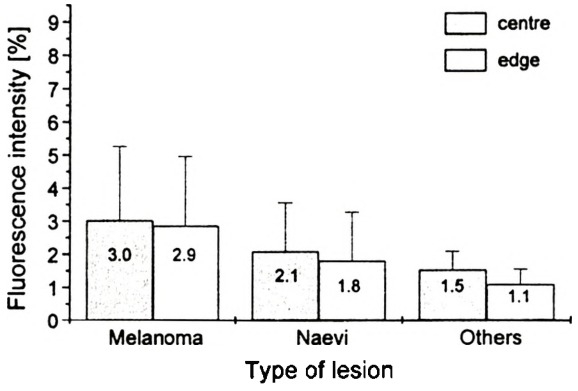

Fig. 5. The same as in Fig. 3, but in the dermis.

cells of melanoma and the cells of the other pigmented lesions can be achieved if the FIF intensity is determined for the cells localised in the central regions of the lesions in stratum corneum.

Diagnostic techniques are typically characterised by their sensitivity and specificity. The sensitivity C is calculated as a percentage of cases for which the melanoma cells found histopathologically have been correctly classified with the fluorescence technique. The specificity S is a value of a similar ratio but calculated for the normal cells. We have shown in our previous work that a simple diagnostic algorithm based on a treshold value of the intensity $I = 2\%$ (referenced to the emission intensity of the standard melanoma if $I \geqslant 2$) yielded $C = 74\%$ and $S = 59\%$. Figure 6 shows the results of the measurements of the FIF intensity (central regions of the lesions, stratum corneum) carried out in this work for a larger than in [3] number of cases. The sensitivity and the specificity calculated for that body of the data are respectively $C = 82\%$ and $S = 66\%$.

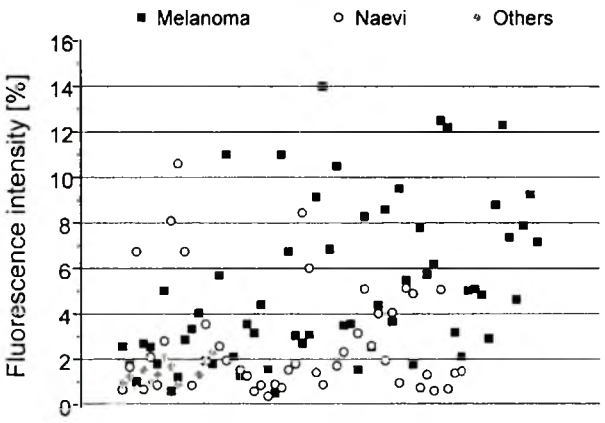

Fig. 6. Results of all the fluorescence intensity measurements carried out for central regions of the lesions in the stratum corneum.

Table 3. Mean fluorescence intensities (% of standard intensity) and respective standard deviations determined for the cells located in the central regions and at the edges of the melanomas in the horizontal and vertical growth phase in different skin layers ($\lambda_{ex} = 450-480$ nm, $\lambda_{em} > 515$ nm).

Skin layer	Horizontal growth phase		Vertical growth phase	
	Centre	Edge	Centre	Edge
Stratum corneum	2.3 ± 2.0 ($n = 27$)	2.1 ± 1.2 ($n = 27$)	6.6 ± 3.4 ($n = 33$)	4.6 ± 2.8 ($n = 33$)
Stratum granulosum and stratum spinosum	1.8 ± 1.1 ($n = 7$)	1.2 ± 0.6 ($n = 7$)	4.3 ± 2.1 ($n = 17$)	4.1 ± 2.1 ($n = 17$)
Stratum papillare	3.3 ± 2.7 ($n = 7$)	1.2 ± 0.6 ($n = 7$)	3.8 ± 2.3 ($n = 17$)	3.7 ± 2.1 ($n = 17$)
Stratum reticulare	1.8 ± 1.2 ($n = 7$)	1.5 ± 0.7 ($n = 7$)	5.1 ± 2.8 ($n = 17$)	4.6 ± 2.9 ($n = 17$)
Epidermis	1.6 ± 0.9 ($n = 27$)	1.7 ± 1.1 ($n = 27$)	4.9 ± 2.6 ($n = 33$)	3.8 ± 2.3 ($n = 33$)
Dermis	1.9 ± 1.2 ($n = 27$)	1.8 ± 1.2 ($n = 27$)	3.6 ± 2.3 ($n = 33$)	3.1 ± 2.1 ($n = 33$)
All the layers	2.6 ± 2.2 ($n = 7$)	1.3 ± 0.6 ($n = 7$)	4.8 ± 2.5 ($n = 17$)	4.3 ± 2.5 ($n = 7$)

An interesting new finding in this study has been a discovery of a distinct difference in the FIF intensity between melanomas in a horizonthal and a vertical growth phase (Tab. 3, Fig. 7). It is thought that melanomas in the horizontal growth phase generally do not have a capacity to metastasize [1]. There are, however, no objective criterion of differentiating between the two phases and the classification is based on microscopic examination of cell distribution in the skin layers. Using a criterion of the threshold intensity $I = 4\%$ of the intensity of the standard, it is possible to classify correctly 70% of melanomas in the vertical growth phase and 83% of melanomas in the horizonthal phase. A choice of the threshold value is always a trade-off between sufficient sensitivity and specificity. Assuming a lower intensity threshold, one can obtain a higher sensitivity of detecting melanomas in the vertical growth phase but at the expense of a less efficient detection of those in the horizonthal growth phase. A correct differentiation between the two phases of the

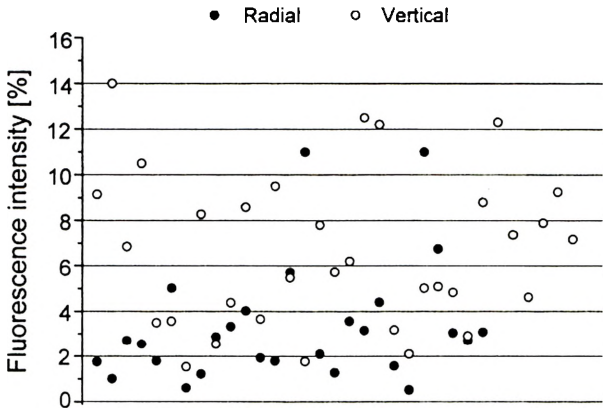

Fig. 7. Results of all the fluorescence intensity measurements carried out for the melanomas in radial and vertical phase of growth (central regions, stratum corneum).

Table 4. Mean fluorescence intensities (% of standard intensity) and respective standard deviations determined for the cells located in the central regions of the melanomas for different stages of the disease classified according to Clark's scale ($\lambda_{ex} = 450-480$ nm, $\lambda_{em} > 515$ nm).

Skin layer \ Stage	Clark I	Clark II	Clark III	Clark IV	Clark V
Stratum corneum	2.1 ± 1.4 ($n = 8$)	3.9 ± 2.9 ($n = 19$)	6.6 ± 3.6 ($n = 19$)	6.2 ± 3.5 ($n = 11$)	8.1 ± 1.1 ($n = 3$)
Stratum granulosum and stratum spinosum	1.0 ± 0.5 ($n = 2$)	2.2 ± 1.2 ($n = 5$)	4.3 ± 1.9 ($n = 9$)	3.9 ± 2.6 ($n = 6$)	6.0 ± 0.7 ($n = 2$)
Stratum papillare	0.9 ± 0.4 ($n = 2$)	1.9 ± 1.0 ($n = 5$)	4.5 ± 2.5 ($n = 9$)	2.2 ± 1.5 ($n = 6$)	5.2 ± 0.9 ($n = 2$)
Stratum reticulare	0.8 ± 0.1 ($n = 2$)	2.2 ± 1.2 ($n = 5$)	5.2 ± 2.7 ($n = 9$)	3.9 ± 2.5 ($n = 6$)	8.6 ± 0.7 ($n = 2$)
Epidermis	1.8 ± 1.6 ($n = 8$)	2.9 ± 2.3 ($n = 19$)	4.8 ± 2.6 ($n = 19$)	4.7 ± 2.9 ($n = 11$)	6.2 ± 1.8 ($n = 2$)
Dermis	1.8 ± 1.8 ($n = 8$)	2.5 ± 2.1 ($n = 19$)	3.8 ± 2.4 ($n = 19$)	2.8 ± 1.5 ($n = 11$)	5.6 ± 3.1 ($n = 3$)
All the layers	0.9 ± 0.4 ($n = 2$)	2.2 ± 1.1 ($n = 5$)	4.9 ± 2.4 ($n = 9$)	3.8 ± 2.5 ($n = 6$)	7.3 ± 1.0 ($n = 2$)

disease is very important in predicting prognosis. Thus, if this result is confirmed in future studies involving a larger number of cases, the measurements of the FIF intensity may serve not only as a tool for detecting the melanoma cells but can also offer an important prognostic technique. The work on gathering new data and optimising the algorithm is now in progress in our laboratory.

It should also be noticed that the average FIF intensities were in all the skin layers higher for nodular melanomas (NM) than for the superficial spreading melanomas (SSM). There is also a trend in the data indicating that for all the skin

layers a progress of the disease determined in the Clark's scale is associated with the increase in the FIF intensity (Tab. 4). At the same time, however, a scatter of the intensities obtained for individual cases of each type of melanoma is too large to allow for a differentiation between NM and SSM or for a determination of the Clark's stage of the disease on a basis of the emission intensity.

4. Discussion

The phenomenon of the higher FIF intensity of melanoma cells compared to the emission of the cells of other pigmented lesions has been known for more than 30 years [3], [5]–[11]. Practically all the authors suggested that the FIF intensity could be a useful indicator for differentiating between melanomas and benign pigmented skin lesions. On the other hand, because of a subjective and qualititative character of the visual microscopic examinations of the FIF intensity, the suggested approach has not found its way to clinical practice. Only in 2001 CHWIROT *et al.* [3] carried out first quantitative measurements of the fluorescence intensity of the formalin-fixed paraffin-embedded melanomas and other pigmented lesions using photometric digital recording and an analysis of microscopic images. The authors found that all the pigmented lesions emitted FIF. Thus, the earlier observations of a lack of the FIF of some of melanomas and of many benign pigmented lesions were most probably an effect of a lower sensitivity of techniques used in 1970's and 1980's.

The most important outcome of the study of CHWIROT *et al.* [3] was, however, a suggestion that a simple algorithm based on the intensity threshold allowed for a correct classification of melanomas with a sensitivity of $C = 74\%$ and a specificity of $S = 59\%$. The same algorithm used in this work for a larger than in [3] number of cases yielded $C = 82\%$ and $S = 66\%$. Such values of the diagnostic parameters seem to sufficiently justify a conclusion that the quantitative determination of the intensity of the formalin-induced fluorescence of the paraffin-embedded pigmented skin lesions can become a useful auxiliary technique for histopathological detection of melanoma. The method has also a virtue of being relatively easy to implement. Preparation of the material is the same as for standard histopathological examinations with only one additional step, *i.e.*, the application of a commercially available fluorescence intensity standard on the microscopic slides.

Molecular mechanisms associated with a transformation of normal melanocytes to malignant melanoma cells are still unknown. The set-backs in a detection and a therapy of the disease can be to a large extent attributed to that lack of knowledge. The fact that the cells of malignant melanoma emit the FIF with the significantly higher intensity than the cells of other pigmented skin lesions indicates that the malignant transformation of the melanocytes is associated with the increased level of the molecules emitting the observed fluorescence. Those can be either newly synthesised molecules or molecules present in normal melanocytes but synthesised/accumulated at an increased level in the transformed cells. The nature of

the molecules emitting the FIF is not known. Majority of the authors points to dihydroxyphenylalanine (DOPA) or its derivatives. However, the microspectrofluorimetric data suggest that DOPA is not the main emitter since the spectra of DOPA models differ from the spectra recorded for the melanocytes [3], [5]. Our microspectrofluorimetric studies [3] indicate that the same substance is responsible for the emission of the FIF in the normal melanocytes and the melanoma cells. The substance would be then present in the transformed cells at a higher level. Such a hypothesis is additionally supported by a finding that melanomas in the vertical growth phase emit the FIF with higher intensity than those in the horizonthal phase. A rationale for the idea of the two phases of the growth is the assumption that there are two distinct stages of the progress of malignant melanoma:

– uncontrolled proliferation of tumour cells mostly in superficial dermis and without capacity to metastasize (horizonthal phase),

– continued proliferation, deeper in the dermis and with a presence of the cells able to metastasize (vertical phase).

The concept of the two growth phases, very important in predicting prognosis for a patient, is based on indirect evidence and one line of such evidence points to different immunologic responses of the host in each of the two phases to the presence of tumour cells [1]. Our observation that the intensity of the FIF changes significantly with a transition from the horizonthal growth phase to the vertical phase indicates that a division of the melanoma growth into two phases may reflect also changes at metabolic level. Such a conclusion is further supported by the evidence of an increase in the average FIF intensity of the melanomas with the progress of the disease determined using the Clark's scale.

Better understanding of the mechanisms leading to the increase in the FIF intensity with the malignant transformation of the melanocytes and with the progress of the disease may pave a new way to a discovery of antigenes specific to melanoma. Such a development might have important implications for a diagnostics and perhaps also for a therapy of malignant melanoma.

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