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Fluorescence spectroscopy for early detection and differentiation of cutaneous pigmented lesions

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Many optical techniques are applied recently in clinical practice for obtaining of qualitatively and quantitatively new data from the lesions investigated. Due to their high sensitivity in detection of small changes, spectroscopy techniques are widely used for detection of early changes in biological tissues. One of the most promising approaches is fluorescence detection of normal and abnormal tissues using naturally existing fluorescent molecules (autofluorescence) or added fluorescent markers (exogenous fluorescence). In current study are presented results obtained from autofluorescence detection of a number of benign and malignant cutaneous pigmented lesions. As an excitation source a nitrogen laser at 337 nm is applied and a microspectrometer detects signals from human skin *in vivo*. The main spectral features of benign lesions – base-cell papilloma, compound nevus, heamangioma, dysplastic nevi and malignant lesions – base-cell carcinoma and malignant melanoma are discussed and their possible origins are indicated. Differentiation between benign and malignant forms using detected fluorescent spectra is proposed. Results obtained could be used for development of more complete picture of fluorescent properties of these widely spread skin disorders, as well as to be introduced in clinical algorithms for early detection and differentiation between benign/ dysplastic/ malignant skin lesions.

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1. Introduction

Many up-to-date techniques based on recent progress in optics have been developed for medical applications. Spectroscopy methods have been widely used as probes to acquire fundamental knowledge about physical, chemical, and biological processes in human body [1,2].

Biological tissues contain chromophores that absorb light, as well as fluorophores that absorb and reemit (fluoresce) light. Tissues are heterogeneous by character that promote strong light scattering. The consequent tissue optical properties such as absorption, fluorescence, reflectance and scattering could be used to characterize the tissue and to identify tissues abnormalities using non-invasive and in real time optical techniques. Fluorescence spectroscopy is one of these noninvasive methods that can identify diseases and promote increasing the knowledge in medical diagnosis. [3,4]. Investigators applied this technique for the detection of endogenous fluorophores existed in the tissue under study [5-7] as well as for exogenous fluorescent dyes applied for better differentiation between normal and abnormal skin sites [8, 9]. Recently, sensitizers used for the needs of photodynamic therapy are investigated from the point of view of their fluorescent properties, to combine diagnostic and therapeutic possibilities of these compounds for initial diagnosis [8], as well as for monitoring of biological response to therapy [10].

Autofluorescence (AF) is used for diagnosis and follow-up of dermatological diseases, such as erythrasma and acne [11]. The investigation possibilities of this technique are applied for evaluation of vitiligo [12], erythema [5], and skin photoaging [3]. AF is also applied for skin cancer detection and lesion type determination [1,13,14].

The skin cancer is one of the most spread tumors worldwide and despite of the results achieved in all clinical diagnostic techniques, in the beginning of XXI century the most severe tumor - cutaneous melanoma continues to be an important problem of social health all around the world. For improvement of early diagnosis and for differentiation of risk lesions, an evaluation of autofluorescence characteristics of several common cutaneous benign and malignant lesions is proposed. Pigmented melanoma may simulate benign lesions, including seborreic keratoses, heamangioma, compound and dysplastic nevi, and amelanotic malignant melanoma may mimic a basal cell carcinoma clinically [15].

In the current study, autofluorescence properties of these benign and malignant skin tumors are determined and origins of diagnostically significant spectral features are discussed. Differentiation scheme of benign/dysplastic/malignant pigmented skin lesions is also proposed.

2. Experimental

For the needs of autofluorescence detection optimum for excitation wavelength around 340 nm is found as most appropriate [1] for differentiation between normal and neoplastic tissue. Therefore, a compact nitrogen laser emitting at 337 nm, 14 μJ per pulse and 10 Hz pulse repetition rate ("ILGI-503", Russia) has been applied as a most suitable excitation source in our studies. Optical fibers were used to deliver the excitation light and to collect the fluorescence signal. The fluorescence spectra were registered and stored using a fiber-optic microspectrometer (PC2000, Ocean Optics, Dunedin, FL, USA) with a high-sensitivity CCD-array detector (ILX511 linear silicon CCD-array, Sony). The spectra were measured at 5 s integration time, due to low intensity level of the fluorescence signals from pigmented lesions. A personal computer was used to control the system and to store and display the data. The spectra were stored using the specialized microspectrometer software OOI Base ("Ocean Optics", Inc., Dunedin, FL, USA) and were analyzed and graphically displayed by means of another computer program (Microcal Origin 5.0, Microcal Software Inc., Northampton, MA, USA).

Fluorescence measurements of normal skin and lesion' areas were carried out after 10 min of rest for each patient at room temperature ranged from 23 to 25˚C. Several spectra were measured from each pigmented spot and averaged to reduce the influence of inhomogeinity of the lesions. We recorded and averaged five to seven autofluorescence spectra from every suspicious lesion depending on its size, five fluorescence spectra from surrounding normal skin and up to seven spectra from three different anatomical areas of human skin – palm, medial and lateral part of the forearm. These averaged spectra from the health skin area were used like an indicator of the spectral changes in the lesion areas. The resultant spectra are smoothed using Savitzky-Golay algorithm to reduce the instrumental noise of the spectrometric system. A constant distance between the end tip of the optical fibers and the skin surface were applied, using a mechanical stand to avoid any influence of displacement from the normal position on the fluorescence intensity level. All spectra from patients were obtained in the 90^o – angle between optical fibers end tip and skin surface - approximation, that skin surface could be treats as flat surface for the existing conditions of the experiment was made. In the results presented in current paper the averaged by type of lesion fluorescence spectra are presented.

Clinical investigations began when the lesions were classified visually by experienced dermatologist (P.T.) and dermatoscopically (MoleMax II, DERMA Instruments). Each lesion was evaluated using ABCD scoring criteria as follows: Asymmetry (A), Border (B), Colour (C) and Dermoscopic structures (D), according manual [16]. The histological examination was used in the final analysis as a "gold" standard for determination of the lesion type. In this study we had included results from 98 lesions, distributed as follow: 19 - benign nevi, 15 -dysplastic nevi, 12 - pigmented malignant melanoma (nodular and lentigo MM), 16 - keratoses, 17 - basal cell carcinoma, 9 - heamangioma, 6 - amelanotic malignant melanoma and 4 - secondary MM. Other lesions detected during spectral observations in clinic were fibroma, atheroma, and angiokeratoma lesions, but due to low number of the patients they are not included in comparison of the spectral features received.

3. Results

The results, presented in this work, are smoothed and averaged by lesion type, according to their histological diagnosis. Normal skin spectrum applied for comparison with the spectral changes occurred in presence of pathological conditions are smoothed and averaged using all patients normal skin spectra recorded. Every autofluorescence spectrum detected *in vivo* is a superposition of fluorescence spectra of endogenous chromophores existing in the tissue under investigation distorted by re-absorption of tissue pigments, mainly blood and melanin.

The spectral shape of normal skin fluorescence usually has no significant differences from patient to patient. Intensity changes are more pronounced due to different skin phototype and anatomic area, as in both cases different level of melanin pigmentation could be observed. Detected slight differences in spectral shape are only for the case of palm skin fluorescence spectra versus other anatomic sites, where lack of melanin leads to deeper penetration of excitation and respectively for emission light. In this case influence of hemoglobin re-absorption of the fluorescence from deeper dermal layer is well pronounced. This effect was discussed in details in our previous work [17].

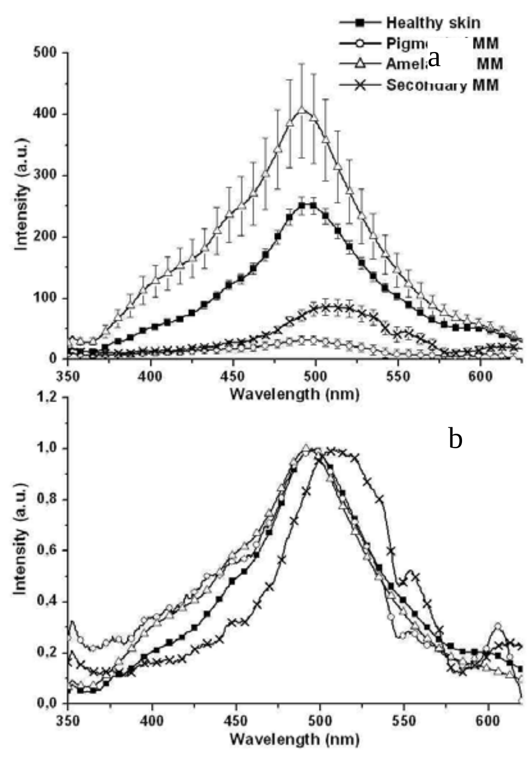

Fig. 1. Comparison of the fluorescence signals from normal skin, pigmented, amelanotic and secondary melanoma lesions with their standard deviation for all cases averaged (a) and normalized with respect to maximum (b) to reveal spectral shape differences.

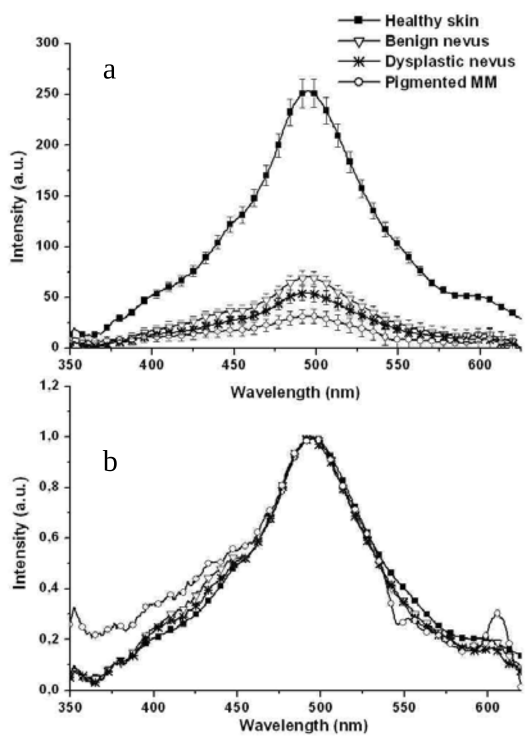

Fig. 2. Comparison of the fluorescence signals from normal skin and pigmented benign and dysplastic nevi, and malignant melanoma with their standard deviation for all cases averaged (a) and normalized with respect to maximum (b) to reveal spectral shape differences.

On Fig. 1a are presented autofluorescence spectra of healthy human skin and different MM lesions – pigmented, amelanotic and secondary melanoma with their standard deviation, obtained from averaging of all spectra detected from given type of lesion. On the Fig. 1b are presented spectra from the same normal and abnormal skin sites but normalized with respect to maxima of the fluorescence spectra to reveal the spectral shape changes occurred. Amelanotic melanoma lesions are characterized with fluorescence signal higher than surrounding normal skin due to lack of melanin in the tumor area. Pigmented MM has lowest fluorescence intensity related to huge level of melanin content in these lesions, which leads to high absorption of excitation and emission signals. Secondary MM in the four cases observed during our clinical measurements visually has lower melanin pigmentation compared with pigmented primary melanoma lesions but higher level of hemoglobin content related to inflammatory processes and neovascularisation. The hemoglobin absorption is well pronounced in all cases – as minima in the fluorescence signal at the region around 400-440 nm and narrower minima at 540 and 575 nm, see Fig. 1b.

On the Fig. 2a are presented averaged spectra of normal skin, compared with melanin-pigmented benign, dysplastic and malignant cutaneous lesions. Significant fluorescence intensity decrease, which correlated with the type of pigment lesion, was observed for all lesions. The fluorescence intensities of the maximum of the two kinds of nevi investigated are very close one to another (see Fig. 2a). The malignant melanoma fluorescence intensity is much lower than that of normal skin and nevi and could be separate from pigmented nevi spots. However, no indicative discriminations of the fluorescence spectral shape between normal skin and nevi were observed (see Fig. 2b). There are not indications about any significant accumulation of fluorescent chromophores in these types of lesions. Spectral shape differences in malignant melanoma lesions are related to metabolic changes (NADH fluorescence increase in the region around 430-460 nm) and blood content increases (deeper minima at 420, 540, 575 nm). These spectral shape changes are clearly observed in comparison of normalized with respect to maximum between normal skin, benign and dysplastic nevi and malignant melanoma, Fig. 2b.

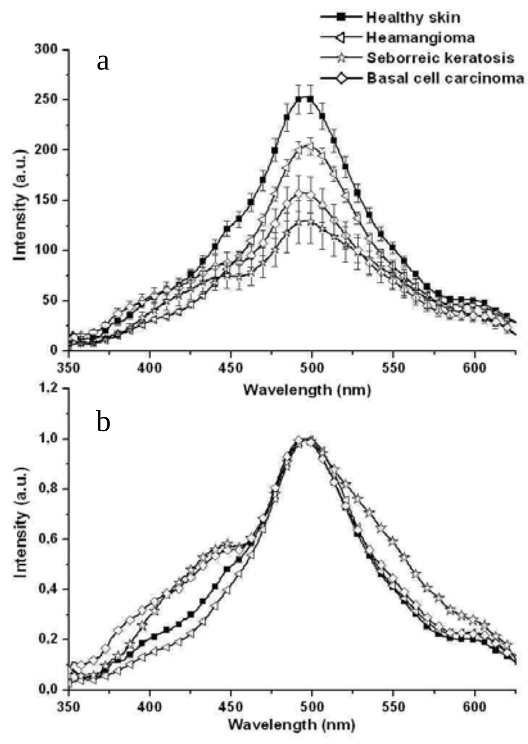

Fig. 3. Comparison of the fluorescence signals from normal skin, heamangioma, keratoses and basal cell carcinoma lesions with their standard deviation for all cases averaged (a) and normalized with respect to maximum (b) to reveal spectral shape differences.

On the next Fig. 3 fluorescence spectra of non-melanin pigmented skin lesions are presented in comparison with the spectrum of averaged normal skin. On Fig. 3a are presented autofluorescence spectra of healthy human skin and benign heamangioma and keratoses and malignant basal cell carcinoma with their standard deviation, obtained from averaging of all spectra detected from given type of lesion. On the Fig. 3b are presented spectra from the same normal and abnormal skin sites but normalized with respect to maxima of the fluorescence spectra to reveal the spectral shape changes occurred.

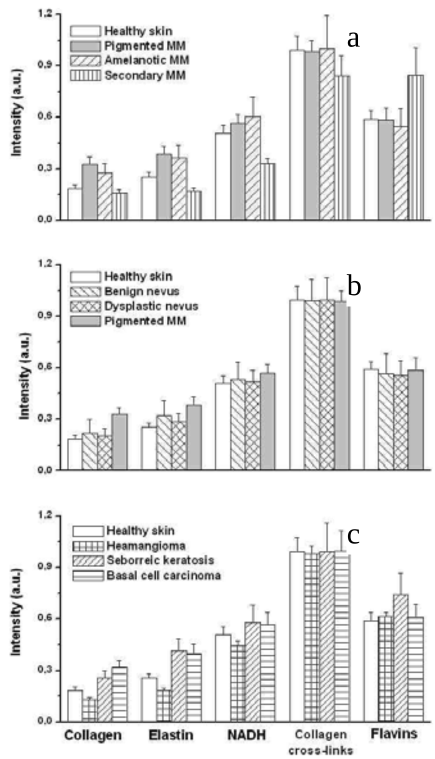

Fig. 4. Relative distributions of fluorescence signals for five main fluorescent compounds in the skin – collagen, elastin, NADH, collagen cross-links and flavins - (a) fluorescence maxima distribution for normal skin vs. different MM lesions; (b) fluorescence maxima distribution for normal skin vs. different melanin-pigmented benign and malignant lesions; (c) fluorescence maxima distribution for normal skin vs. heamangioma, keratoses and basal cell carcinoma.

In general, a decrease of fluorescence intensity of lesions is observed in all cases investigated, see Fig. 3a. However, for these pathologies intensity decrease is not highly expressed – about 1.5 to 3 times lower than the fluorescence intensity of normal skin. Spectral shape changes detected are very informative and important for assessment of diagnostic and differentiation criteria for evaluation of benign lesions and normal skin vs. carcinoma lesions. For keratoses is typical the relative increase of the fluorescence intensity at the 440 nm region and at 520-560 nm region. First is the area of NADH fluorescence, and the maximum observed could be related to increased metabolism activity in the lesion. Second appearance of fluorescence maximum is related to increasing content of keratin and flavins [7].

Fluorescence spectra of carcinoma cases have some spectral changes, related to re-absorbance of fluorescent signal in the regions at 420 nm, 540-575 nm by the hemoglobin. This effect is related to hyper-vascularization of malignant neoplasia. A relative increase of the fluorescence peak at 440 nm was registered also in the case of base-cell carcinoma.

In the case of hemoglobin-pigmented heamangioma lesions stronger re-absorption of the fluorescence signal in the regions 420, 540-575 nm is observed. No other significant fluorescence spectral changes are observed which could give us link for appearance of new chromophores in these cutaneous lesions.

4. Discussion

In this *in vivo* study, it was observed significant differences in the intensity of the fluorescence signal of the normal, benign and malignant cutaneous tissues. Skin tissue consists of many kinds of chromophores with different absorption spectra, different quantum efficiencies, which have not been completely studied. However, major of them are melanin, haemoglobin and water. The water absorption spectrum covers ultraviolet and near- and far-infrared [18] and it will not be considered in this study. Melanin absorption has influence over the whole visible spectral region and fluorescence intensity of the skin decrease significantly with the melanin content increasing [19]. This effect is clearly observed when compare the fluorescence spectra of normal human skin vs. melanin-pigmented lesions. Other significant absorber is hemoglobin in the blood vessels of skin. In visible spectral range it is characterized with strong absorption bands in the region of 420 nm, as well as at 540-575 nm region [7, 19]. Hemoglobin in its two forms – oxy-hemoglobin (HbO_2) and deoxy-hemoglobin (Hb) re-absorb the fluorescence of skin proteins and coenzymes, and this could be observed in the cases of neo-and hyper-vascularization in malignant lesions – MM and basal cell carcinoma and in heamangioma. Neovascularisation process and related to it hemoglobin re-absorption of the fluorescence signal could be slightly observed even in case of pigmented MM.

This could be one of the diagnostic criteria for evaluation of malignancy stage. When the fluorescence spectra of the malignant lesions are examined the significant decrease of intensity in the region >520 nm is observed. Basal cell carcinoma is characterized with low concentration of melanin and the structure of the hemoglobin absorption could be easily observed, with the two minima at 540 and 575 nm in the spectra of fluorescence obtained. Even for pigmented malignant melanoma, nevertheless of strong melanin absorption, which not allows deep penetration of the light in the lesion, minima at 540 and 575 nm are detectable. However, in the blue spectral region where absorption of melanin is higher, minimum at 420 nm is not well pronounced, due to limited possibility for deeper fluorescence detection from the lesion. In this short wavelength spectral region main contribution to the signal observed has autofluorescence of proteins and NADH.

Main fluorophores in the human skin are co-ferments, amino-acids, different proteins [1, 3, 5, 7]. The emission spectrum of the normal skin consists of one main maximum at 480-490 nm region, which is commonly related to structural proteins, and mainly with collagen cross-links fluorescence and weaker signals at 440 and 400 nm, related to the NADH and proteins [3, 4, 7]. According existing references we choose several wavelengths to reveal the relations between fluorophore content in different skin samples.

In Fig. 4 a, b, c normalized fluorescence intensity, of five main fluorophores in the skin samples investigated – collagen, elastin, NADH, collage cross-links and flavins are presented, at 395, 410, 450, 490 and 530 nm respectively. All spectra shown in Fig. 4 were drawn normalized to their local maxima, in order to compare the skin lesions measured. There are big deviations between signals obtained from specific type of fluorophore depending on the skin pathological condition.

In the case of different MM lesions slight increase of NADH fluorescence is observed for pigmented and amelanotic MM *vs*. normal skin, which is related to higher metabolitic activity of the lesions. For the secondary MM the spectra are highly distorted by hemoglobin absorption and the real fluorophores distribution could not be evaluated by such comparison, see Fig. 4a.

In the case of benign and dysplastic nevi there were not observed significant differences *vs.* normal skin. Only intensity changes could be used for valuable differentiation algorithms. However, fluorescence intensity increase in the short wavelength region is observed for pigmented melanoma, related to higher metabolism activity (NADH) and growth of lesion (collagen, elastin), see Fig. 4b.

Most significant changes are observed in non-melanin pigmented lesions as NADH fluorescence is significantly higher for keratoses and basal cell carcinoma. Collagen and elastin spectral compounds also show higher signal for these two lesions. Flavins fluorescence combined with keratin fluorescence give raise of about 30 % for the keratoses lesions, which are characterized with keratinocytes overgrowth [16], see Fig. 4c.

As we observed in Fig. 4, there is significant distinction between spectra for all five fluorophores detected in relation to the skin pathological condition. However, there are some chromophores that could be included additionally in such comparison of the fluorescence properties of different skin samples, as well as some more precise addressing of the fluorophore type, for example – collagen, which has more than one chemical configuration existing in the skin tissue [5].

According to the results obtained in this study we could evaluate the general origins of the fluorescence spectral features for several benign, dysplastic and malignant skin lesions. Significant differences between pigmented melanoma and seborreic keratoses, heamangioma, compound and dysplastic nevi are observed. For the case of differentiation of amelanotic malignant melanoma vs. basal cell carcinoma also good results are obtained. In such way autofluorescence spectroscopy could be used for better differentiation of mimicking benign and malignant lesions [15], which will improve diagnostic accuracy significantly.

Melanoma incidence and mortality rates are increasing in many countries. Although the “best” method for detection and differentiation of malignant nevi is still a matter of debate, there is no doubt that early detection of malignant melanoma is the most effective tool to improve the survival rates and prognosis for patients. To overcome the subjectivity related to the experience of individual dermatologists [20], optical imaging techniques have been proposed to provide qualitative measurements in an objective and reproducible way [21].

5. Conclusions

The most important for every diagnostic procedure developed is its possibility to differentiate malignant from non-malignant lesions. Where it is absolutely accurate, than 100 % of the lesion types would be predicted. But every diagnostic test is imperfect in its own way – one procedure will miss many cases and make a few false diagnoses, another will miss a few cases, but the number of false diagnoses will be much higher. Using autofluorescence detection of skin benign and malignant pigmented lesions we obtain very good diagnostic performance for distinguishing of malignant melanoma lesions *in vivo* from other simulating benign and malignant pathologies. Our next step will be to cover other skin lesions diagnosis using autofluorescence detection technique and development of algorithms and equipment for early non-invasive skin cancer detection.

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