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Development of a method for isolation of melanin from archival FFPE tissues of human melanoma for structural studies by pyrolysis-gas chromatography-tandem mass spectrometry

Original Study

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Abstract

Introduction. There is some evidence that pheomelanin produced in skin melanocytes may be involved in the development of cutaneous melanoma, particularly in individuals with a light skin/red hair phenotype. However, nothing is known about possible correlation between the type and/or clinical stage of melanoma and the content of pheomelanin in the tumor tissue. We believe that archival formalin-fixed and paraffin-embedded (FFPE) melanoma tissues could be a good source of melanin pigment for future large-scale research on that issue.

Aim. The aim of this work was to develop a method for isolation and purification of melanin from FFPE samples of human melanoma. To test the suitability of the isolation protocol for planned structural studies, the obtained melanin was analyzed for pheomelanin content by the method based on pyrolysis (Py) coupled with gas chromatography and tandem mass spectrometry (GC/MS/MS).

Material and methods. For melanin isolation, microtome sections of FFPE tissue of primary lesion and lymph node metastases were subjected to a multistep procedure of paraffin removal, tissue rehydration, homogenization, and digestion with the set of proteolytic enzymes. The pigment samples were then pyrolyzed at 500 °C, and the GC-separated thermal degradation products were identified using a triple quadrupole mass spectrometer operating in the multiple reaction monitoring mode.

Results. GC/MS/MS analysis of the pyrolysis products revealed the presence of pheomelanin markers, which allowed quantitation of a pheomelanin component of each of the isolated pigments. Melanin from the FFPE primary melanoma was found to contain 6.6%, and the pigment from FFPE metastatic lymph node 7.5% of pheomelanin.

Conclusions. The developed protocol allows for the isolation of melanin from FFPE melanoma specimens. The pigment can be successfully studied for pheomelanin content by Py-GC/MS/MS method. The results of our study indicate that archival FFPE tumor tissues can be used as a good source of melanin for future structural studies aimed at shedding more light on the role of pheomelanin in the pathomechanism of cutaneous melanoma.

Keywords

FFPE • melanoma • pheomelanin • pyrolysis • GC/MS/MS

Introduction

Epidemiological data indicate that people with light skin exhibit higher risk for developing cutaneous melanoma than dark-skinned individuals [1]. The constitutive color of human skin depends on various genetically determined factors, including the amount and type of melanin, a polymeric pigment synthesized in epidermis by melanocytes. These specialized skin cells produce two structurally and functionally distinct pigments: eumelanin, which consists of indole-type monomers, and pheomelanin, composed of sulfur-containing units of benzothiazine-type and benzothiazole-type [2, 3]. Eumelanin is a sunscreen, antioxidant, and free radical scavenger, and its major physiological role is the protection of skin cells against damage induced by UV radiation [4, 5, 6]. By contrast, pheomelanin – as well as some of the intermediates of its biosynthesis – can act as an endogenous photosensitizer that generates reactive oxygen species and highly mutagenic DNA photoproducts after UV exposure, and that also has UV-independent pro-oxidative activity [5, 7, 8, 9, 10, 11]. A few studies suggest that the elevated proportion of potentially harmful pheomelanin in human skin may be involved in the promotion and progression of cutaneous melanoma. Structural study revealed that dysplastic nevi, one of risk factors for melanoma, contain significantly higher amounts of pheomelanin than common nevi [12]. The proportion of pheomelanin in melanoma cells was also found to be elevated compared to normal melanocytes [13]. However, it is not known whether there is any correlation between the type and/or clinical stage of cutaneous

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melanoma and the content of pheomelanin in the tumor tissue. To address this problem, a large-scale study is needed, and we believe that archival formalin-fixed, paraffin-embedded (FFPE) melanoma tissues could be a good source of melanin for future comparative structural study.

Previously, we developed a sensitive method that enables fast and reliable differentiation of pheomelanin and eumelanin, and that allows for detection and quantitation of a pheomelanin component in melanin pigments of various origin [14]. Our method is based on gas chromatography / tandem mass spectrometry (GC/MS/MS) identification of the marker products formed during thermal degradation (pyrolysis, Py) of melanin. The pheomelanin markers are identified in the pigment pyrolysate by simultaneous measuring of a set of their characteristic precursor ion / product ion pairs with a triple quadrupole mass spectrometer operating in a multiple reaction monitoring (MRM) mode. The relative pheomelanin content is then calculated from a standard curve, created with the use of a series of synthetic melanin pigments with known percentages of incorporated pheomelanin. Using the above approach, we have quantified intracellular pheomelanin produced by cultured human melanoma cells [15], and by normal melanocytes with various levels of constitutive pigmentation [16].

The aim of this work was to develop a method for isolation and purification of melanin from archival FFPE specimens of primary melanoma and the tumor lymph node metastases. Melanin isolation in this kind of solid tissue sample is particularly challenging. The pigment itself is insoluble in most solvents, and the extracellular matrix of both tumor tissue and metastatic lymph nodes is abundant in fibrous structural proteins that are resistant to hydrolytic cleavage by common proteases. To assess the suitability of the isolation protocol for the planned structural studies, the pigment obtained from FFPE melanoma samples was analyzed for pheomelanin content by the Py-GC/MS/MS method.

1. MATERIALS AND METHODS

1.1 Material

FFPE tissue specimens of two melanoma patients (one sample from a primary skin lesion and the other from lymph node metastases) were obtained from archives of Department of Tumor Pathology at the Centre of Oncology – Maria Skłodowska-Curie Institute in Gliwice, Poland. The study was approved by the Bioethical Committee for the Institute (decision no. KB/430-50/19).

1.2 Melanin isolation

Microtome sections of each FFPE tissue specimen were deparaffinized by incubation with xylene (70°C, 20 min). The wax removal procedure was repeated using a fresh portion of xylene. After centrifugation (21000g, 10 min), the remaining pellet was washed successively with isopropanol, ethanol (96%, 70%, and 50%), and water to remove traces of xylene and rehydrate the tissue, which was then homogenized in 0.1 M phosphate buffer, pH 6.8, with an ultrasonic disintegrator. Subsequently, the tissue homogenate was centrifuged (21000g, 5 min) and subjected to three steps of digestion with proteolytic enzymes to isolate and purify the insoluble melanin pigment. At the first step, the pellet was freeze-thawed and then suspended in 0.1 M phosphate buffer, pH 6.8, containing 1.5 mg/ml collagenase from *Clostridium histolyticum* (Sigma-Aldrich) and 2mM $CaCl_2$, incubated at 37°C for 72 h and centrifuged for 10 min at 30000g. The dark pellet was washed twice with 0,05M Tris-HCl buffer, pH 7.4, and suspended in the same buffer containing 1.5 mg/ml proteinase K (Sigma-Aldrich) and 3 mM $CaCl_2$. The mixture was incubated at 50°C for 48 h, centrifuged as above, and the pellet was washed twice with 0.1 M phosphate buffer, pH 6.8. Then 2 mg/ml papain (Sigma-Aldrich) in 0.1 M phosphate buffer, pH 6.8, containing 0.5 mM cysteine and 0.5 mM EDTA was added, and the mixture was incubated at 60°C for 48 h. The final melanin pellet, collected by centrifugation (30000g, 10 min), was extensively washed with water, and dried to a constant weight at 37°C.

1.3 Py-GC/MS/MS analysis

The total amount of melanin isolated from given FFPE tissue was placed into a pyrolysis device (Pyrojector II, SGE Analytical Science) and thermally degraded at 500°C. Pyrolysis products were transferred with a stream of helium directly to the split/splitless injector of the Agilent Technologies 7890A gas chromatograph. The GC separations were performed on Agilent HP-5ms capillary column (5% diphenyl, 95% dimethyl polysiloxane, 60 m x 0.32 mm i.d. x 0.5 μm film thickness) with helium as the carrier gas. The GC oven temperature was programmed from 35°C (constant for 5 min) to 100°C at a rate 5°C/min, then to 260°C at a rate 10°C/min. The final temperature was constant for 16 min. The GC column outlet was connected directly to the EI ion source of the Agilent Technologies 7000 GC/MS Triple Quad mass spectrometer. The temperatures of GC/MS interface, the ion source and the quadrupoles were 240°C, 230°C, and 150°C, respectively. An ionization energy of 70 eV was applied. The tandem mass spectrometer operated in MRM mode, with nitrogen as the collision gas and helium as the quench gas. The MRM settings used are listed in Table 1.

MassHunter GC/MS Acquisition B.07.01 and MassHunter Workstation Qualitative Analysis B.07.00 (Agilent Technologies) software were used for data collection and mass spectra processing. A series of synthetic eumelanin/pheomelanin copolymers (melanin standards with known percentages of incorporated pheomelanin), as well as a standard curve for pheomelanin quantitation in the pigment isolated from FFPE samples, were prepared as described previously [16].

Table 1. Multiple reaction monitoring (MRM) settings for the analysis of melanin pyrolysate by GC/MS/MS

Time segment	Retention time [min]	Compound (Type*)	MRM transition		Collision energy [V]
			Precursor Ion [m/z]	Product Ion [m/z]	
1	9.7	Thiazole (P1)	85	58	15
			58	57	12
	11.1	Toluene (NS1)	92	91	15
			91	65	16
2	16.2	Styrene (NS2)	104	77	27
			104	78	15
			103	77	14
3	19.2	Phenol (NS3)	94	65	26
			94	66	16
	22.1	Methylphenol (NS4)	108	107	16
			107	77	16
4	24.9	1,2-Benzenediol (NS5)	110	63	30
			110	64	20
			110	92	15
	25.8	Benzothiazole (P2)	135	108	18
			108	82	12
5	26.9	Indole (NS6)	117	89	30
			117	90	16
			90	63	27
6	28.2	4-Hydroxybenzothiazole (P3)	151	96	23
			151	123	12
			123	96	10
	28.5	Methylindole (NS7)	130	77	27
			130	103	14
			103	77	10
7	29.3 – 29.9	2,3-Dihydro-5H-1,4-benzothiazin-5-one (P4)	165	110	18
		and its isomers (P4' and P4'')	165	136	22
			136	109	16
8	30.7 – 30.9	Methyl-	179	110	15
		-2,3-dihydro-5H-1,4-benzothiazin -5-one (P5, P5')	179	150	20
			178	109	20
	31.2	4-Hydroxy-6-ethylbenzothiazole (P6)	179	164	15
			164	109	20
			164	136	15

** P – pheomelanin marker, NS – non-sulfur-containing pyrolysis product*

2. RESULTS AND DISCUSSION

Fig. 1 displays reconstructed total ion current MRM chromatograms of the marker pyrolysis products (MRM pyrograms) of melanin isolated from the FFPE specimens. As shown in Table 1 (Material and Method section), we designed MRM settings to monitor the two distinct groups of products in each pyrolysate: heterocyclic compounds with 1,3-thiazole or 1,4 thiazine rings, which are characteristic of thermally degraded pheomelanin (the pheomelanin markers, P) and the products devoid of sulfur, such as toluene, phenol, methylphenol, indole and methylindole. The latter group of compounds are always formed in high yields during the pyrolysis of melanin, irrespective of its structural type and origin, and therefore we use them for normaliza-

tion of the pyrolytic data, which is essential for pheomelanin quantitation [16].

MRM pyrograms of melanin isolated from FFPE tissue of both primary melanoma (Fig. 1, panels A and D) and lymph node metastases (panels B and E) were quite similar, and were dominated by the non-sulfur-containing products. However, all the monitored compounds characteristic of pheomelanin structural units were present in the pyrolysates. The most abundant pheomelanin markers were thiazole (P1, 5-10% of relative intensity) and hydroxybenzothiazole (P3, 5-6%). The abundance of other pheomelanin markers (P4-P6) were substantially lower. The MRM pyrolytic profiles of the pigments isolated from FFPE specimens were similar to the profile of synthetic melanin standard with 10% of incorporated pheomelanin (Fig. 1, panels C and F). However, interestingly enough, the ratio of the relative content of P4' isomer of 2,3-dihydro-5H-1,4-benzothiazin-5-one to the P4" isomer in both pyrolysates of melanoma melanin was different, as compared to the pyrolysate of the synthetic melanin. The results of our recent Py-GC/MS/MS studies of synthetic eumelanin/pheomelanin copolymers show that eluted later from the GC column P4" isomer of 2,3-dihydro-5H-1,4-benzothiazin-5-one is the pyrolytic marker of the pheomelanin monomer units formed via oxidative polymerization of 5-S-cysteinyl-3,4-dihydroxyphenylalanine (5-S-cysteinylDOPA), while the P4' isomer arises from the units derived oxidatively from 2-S-cysteinylDOPA. 5-S-cysteinylDOPA is regarded as the major precursor of human epidermal pheomelanin, and indeed, our previous study shows that the pyrolysate of such a pigment, produced by normal melanocytes, contains mainly the P4" isomer [16]. The results presented here strongly suggest that malignant melanocytes may preferentially use 2-S-cysteinyl conjugate of DOPA for pheomelanin synthesis. We intend to address this issue in our next research.

To obtain a standard curve for pheomelanin quantitation in the pigment isolated from FFPE specimens, the ratio of the total peak area of all pheomelanin markers and the total peak area of all non-sulfur-containing pyrolysis products, taken from the MRM programs of a series of synthetic eumelanin/pheomelanin copolymers, was plotted against the percentage of the pheomelanin component in the melanin standard (0.5-20 %), and a high degree of linearity was achieved ($R^2 > 0.995$). Using the linear regression equation we calculated that the pigments from the FFPE primary melanoma and FFPE melanoma lymph node metastases contain 6.6 and 7.5 % of pheomelanin, respectively. For obvious reasons (only two samples tested, each one from different donor), it is difficult to assess the significance of the obtained results of the quantitative analysis, and that was not our goal in this work. Moreover, the only pheomelanin percentage data available in the literature are for normal skin. For example, according to Del Bino et al. [17], human epidermis comprises about 26% of pheomelanin, regardless of the constitutive skin color. Similar results (26-36%, depending on the skin photo-

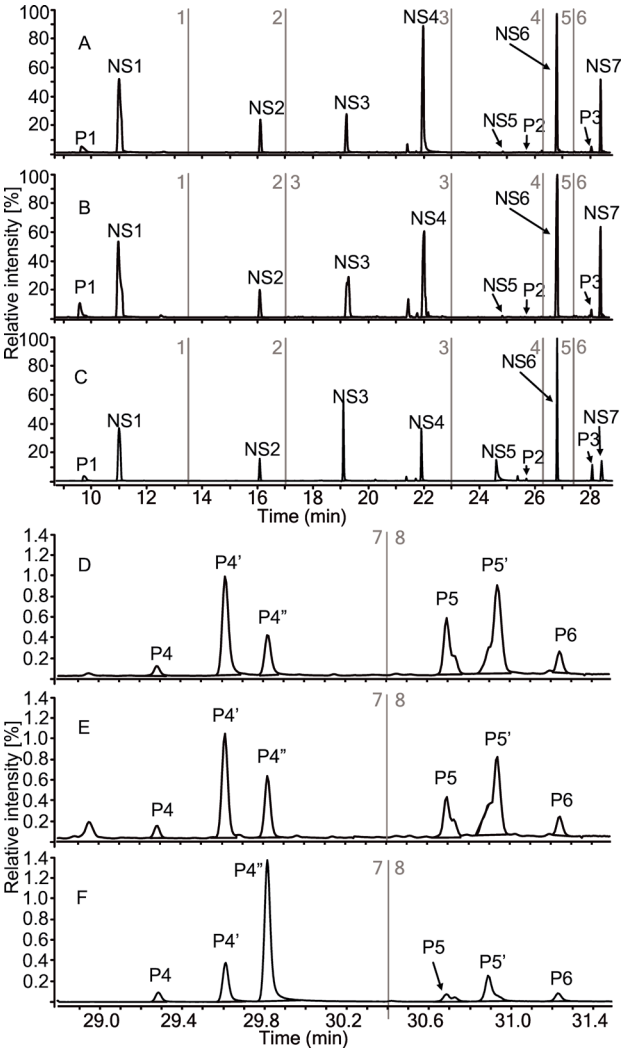


Fig. 1. Reconstructed total ion current MRM chromatogram of the pyrolysate of melanin isolated from FFPE primary melanoma (panels A and D) and FFPE melanoma lymph node metastases (panels B and E). Panels C and F show the pyrograms of synthetic eumelanin from L-3,4-dihydroxyphenylalanine (L-DOPA) with 10% incorporation of pheomelanin units. For peak designation see Table 1.

type) were reported recently by Lerche et al. [18]. Importantly, the above findings are based on an analytical method different from that used in our study: on chemical degradation of unfixed skin sections or punch biopsy specimens taken from healthy individuals, without previous isolation of melanin pigment. Reported here, the pheomelanin content of melanin from melanoma, less than one would expect in light of the published results related to normal skin, could also be a characteristic feature of the particular kind of tumors whose FFPE tissues were examined. There is some evidence that neoplastic transformation of melanocytes is accompanied by abnormal melanogenesis. It seems that these alterations include not only the total melanin content (primary melanoma lesions are usually hyperpigmented, rarely

amelanotic), but also its quality in terms of the proportion of pheomelanin-type structural units. Furthermore, our previous Py-GC/MS/MS study revealed that cultured human melanoma G-361 cells, which were found to be completely eumelanotic, start to produce pheomelanin in a dose-dependent manner when exposed to valproic acid and 5,7-dimethoxycoumarin, the potential chemopreventive agents that are capable of inducing the differentiation process of tumor cells, thus converting them to normal melanocytes [15]. This suggests that at least some kinds of cutaneous melanoma tumors, as undifferentiated or poorly differentiated cells, may contain much less pheomelanin than normal skin.

3. CONCLUSIONS

The developed multistep procedure of paraffin removal, tissue rehydration, homogenization and enzymatic digestion allows for the isolation of melanin from FFPE melanoma specimens. The pigment can be successfully studied for pheomelanin content by the Py-GC/MS/MS method. The results of our study show that archival FFPE tissues could be used as a source of melanin in future research aimed at shedding more light on the role of pheomelanin in the induction and progression of cutaneous melanoma.

Authors' Contribution

S.K.: research concept and design, carrying out the experiments, acquisition of data, data analysis and interpretation, writing – original draft preparation, visualization, literature review, final proofreading and approval of the version for publication; **A.D.L.**: research concept and design, supervising the project, carrying out the experiments, data analysis and interpretation, writing – original draft preparation, writing - review and editing, literature review, final proofreading and approval of the version for publication, funding acquisition; **A.S.W.**: research concept and design, carrying out the experiments, histopathological evaluation and selection of biological material, writing – review and editing, final proofreading, and approval of the version for publication; **D.L.**: research concept and design, histopathological evaluation, and selection of biological material, writing – review and editing, final proofreading, and approval of the version for publication.

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Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

The study was approved by the Bioethical Committee for the Institute (decision no. KB/430-50/19).

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