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PhD THESIS
EXPERIMENTAL AND CLINICAL STUDIES ON MALIGNANT MELANOMA

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SUMMARY

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CONTENT

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PhD THESIS

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Summary

Melanoma is one of the most unpredictable tumours, both in terms of morphology and the evolution of the disease. It can have different clinical forms, which makes it very difficult to diagnose. Despite numerous trials, effective treatment has not been developed, especially for patients in advanced stages, and early detection and removal of a skin lesion is currently the most effective method of treatment.

The paper is structured taking into account the institutional drafting rules. The general part deals with: (a) the detection and diagnosis of skin tumours – the detection and diagnosis of skin cancer and current techniques and methods (photography, dermatoscopy, ultrasound, confocal microscopy, Raman spectroscopy and fluorescence spectroscopy) and (b) risk factors and biomarkers in skin cancers. The special part includes three main chapters, namely: the role of melanoma cell lines in obtaining therapies to combat malignant pathology of the skin organ, melanin and the behaviour of melanoma cell lines in the presence of type B ultraviolet radiation and contributions related to the incidence of skin tumours.

This paper contains three scientific objectives, namely: (i) to study murine and human melanoma cells, especially those containing melanin, to assess their current role in the approach to melanoma, (ii) the behaviour of melanoma cells in the presence of type B ultraviolet radiation and the quantification of melanin and (iii) the analysis of the incidence of skin tumours in a given population in the western part of the country in order to establish the current incidence of this disease.

This paper presents *in vitro* studies on 2D cells related to the quantification of viable cells in certain contexts, cell morphology in the presence of disturbing factors (ultraviolet radiation, melanin) and also analyzes the types of tumour lesions in patients who presented to the clinic, from all age groups, in a certain period.

Detecting and diagnosing skin cancer is a challenge for specialists in the field, who often require teamwork by co-opting specialists in several medical fields and beyond. The most used

principles related to the detection and diagnosis of malignant skin lesions are presented in the paper (Principles and mechanisms involved optically; based on photodynamic; Ultrasound; Bio-electrical impedance; Thermal image).

In the current study, six different melanoma cell lines were selected, both of murine and human origin, namely: mouse melanoma B16-F0 (ATCC® CRL-6322 ™), mouse melanoma B16-F10 (ATCC® CRL- 6475 ™), mouse melanoma B164A5 (ECACC 94042254), human melanoma SK-MEL-5 (ATCC® HTB70 ™), human melanoma SK-MEL-28 (ATCC® HTB72 ™) and human melanoma SH-4 (ATCC® CRL -7724 ™) purchased from the European Collection of Authenticated Cell Culture (ECACC) and the American Type Culture Collection (ATCC) as frozen vials.

The following specific reagents were required for cell culture protocols: specific media – Dulbecco's Modified Eagle's Medium for B16-F0, B16-F10, B164A5 and SH-4, and Eagle's Minimal Essential Medium for SK-MEL-5 and SK-MEL-28, supplements: fetal bovine serum (FCS) at a final concentration of 10 %, a mixture of antibiotic solution (penicillin + streptomycin – final concentration – 1 %), and other reagents such as: trypsin / EDTA solution and phosphate-buffered saline – PBS. Culture media, supplements and reagents were purchased from ATCC, Sigma Aldrich (Germany) and Thermo Fisher Scientific (USA). All procedures were performed under standard conditions, cells were stored at 37° C, 5 % CO_2 and were maintained in culture to monitor macroscopic evolution or microbial contamination.

In vitro models of melanoma are represented by approximately 5, 000 generated cell lines, but only for over 200 cell lines, there is a genetic profile and a biological characterization. In addition to the major role played by cell lines in cancer research, several limitations have also been described, such as: 1) loss of stromal, vascular and cellular immune populations leading to different behaviour in culture compared to the conditions *in vivo*; 2) a possible selection of a subset of clones that respect the conditions for culture growth and 3) the lack of existing microenvironment *in vivo* and the absence of interactions, thus making it difficult or even impossible to recreate these processes that occur *in vivo.*

This study aims to highlight the differences between several murine and human melanoma cell lines, which are frequently used as models for melanoma studies in the literature.

In the experiment that involved the study of cells in the presence of UVB radiation were used: a healthy cell line and two lines of tumour cells, purchased in the form of frozen products

from ATCC. The healthy cell line was represented by primary human epidermal melanocytes (HEMA – ATCC® PCS-200 013 ™), and the tumour cell lines were by human melanomas (SK-MEL-3 – ATCC® HTB-69 ™ and COLO-829 – ATCC® CRL – 1974 ™). Appropriate media for cell culture – Dermal Cell Basal Medium, Adult Melanocyte Growth Kit, McCoy's 5a Medified Modified and RPMI-1640 Medium were purchased from ATCC while all other reagents, such as: fetal bovine serum (FBS), penicillin/streptomycin mixture, phosphate-buffered saline (PBS), trypsin/EDTA combination and Trypan blue were obtained from Sigma Aldrich (Germany). HEMA – primary melanocytes were multiplied in a basal dermal cell medium, in which an adult melanocyte growth kit, 100 U/mL penicillin and 100 g/mL streptomycin mixture were added; SK-MEL-3 were cultured in McCoy 5a modified medium, supplemented with fetal bovine serum at a final concentration of 15 % and COLO-829 were cultured in RPMI 1640 medium, supplemented with fetal bovine serum at a final concentration of 10 %. During cultivation, all cells were kept in optimal conditions for propagation – atmosphere humidified with 5 % CO_2 at 37° C and divided every 48 hours. Cells were counted with an automated cell counting device, Countess II FL, in the presence of Trypan blue reagent.

To determine the medium melanin content of the tested samples, it was necessary to obtain standard melanin curves in different culture media specific to each cell line.

The UVB irradiation protocol involved the use of cells at a confluence of min. 80 – 85 % and the main steps were: removal of the environment before UVB exposure, washing with PBS, exposure to 312 nm, at two different doses – 30 mJ/cm^2 and 60 mJ/cm^2 with a Biospectra system (Vilber Lourmat, France).

Cell viability was assessed by the Alamar blue test: 10, 000 cells/200 µL medium/well were seeded in a 96 – well plate, incubated for 24 hours after UVB exposure and analyzed spectrophotometrically at 570 nm wavelengths and 600 nm with an xMark ™ Microplate spectrophotometer (BioRad) as described above.

Related to the incidence of skin tumours an analytical, observational, retrospective study of cases of tumours in the county of Timiș, located in western Romania, was performed. The patients presented between August 2015 and September 2019 at the County Emergency Clinical Hospital "Pius Brînzeu" Timișoara, Clinical Department of Reconstructive Plastic Surgery and Burns – Austria House. A total of 2026 cases included patients of all ages. The diagnosis followed the clinical, dermoscopic, histopathological criteria and, when necessary, the

immunohistochemical ones. The processed data included the characteristics of patients by sex, age and tumour location.

The present thesis has fully achieved its proposed objectives, namely: (i) the study of murine and human melanoma cells, especially those containing melanin, to assess their current role in addressing melanoma, (ii) the behaviour of melanoma cells in the presence of type B ultraviolet radiation and the quantification of melanin; and (iii) the analysis of the incidence of skin tumours in a given population in the western part of the country in order to establish the current incidence of this disease. Following the experimental and literature studies carried out, correlated with the objectives and methods approached, the following main conclusions can be drawn:

2D cells may develop different behaviour, depending on *in vitro* conditions, origin, treatments, and other factors. To get a proper result and interpretation, scientists need to know the specifics about these changes. Murine metastatic cells (B16-F10 and B16-F0) are comparable to human metastases such as SK-MEL-5 and SK-MEL-28. The significant and rapid progress of *in vitro* technology is extremely useful in elucidating the key mechanisms that occur in the evolution of melanoma. The data provided by the behaviour of different melanoma cell lines is a key point in finding new candidates to slow the progression of melanoma, reduce the side effects of current therapies and increase the life expectancy of patients diagnosed with this terrible disease.

The 2D cells studied to determine the melanin content are as follows: A375, SK-MEL-28 and SK-MEL-5 do not produce melanin, SK-MEL-1 – is a human melanoma cell line that produces melanin, COLO 829 – in small passages produces melanin, SH-4 – are cells that produce melanin, SK-MEL-3 – produce melanin. Of the 3 lines of mouse melanoma checked, the one that produces melanin in a higher concentration is B16F10, followed by B16F0 and B164A5. As can be seen in the case of line B16F0, the longer the time in culture is prolonged and the number of passages increases, the lower the concentration of melanin is. As for healthy cell lines of human origin, melanin was detected in human melanocytes, in while in the case of keratinocytes and fibroblasts no melanin content was recorded.

Melanocytes and SK-MEL-3 cells have a similar melanin content, while COLO-829 cells have a higher melanin content. In future studies, the effect of type A and type B radiation on the cells in this study will be evaluated. Treatment with UV type B radiation had the most harmful effect on COLO-829 cells. Immediately after exposure, the cells appeared significantly affected

after both doses (30 and 60 mJ/cm^2), but after 24 h the cells treated with the dose of 30 mJ/cm^2 began to regain their specific morphological appearance. The same cannot be said for cells exposed to a dose of 60 mJ/cm^2, in which case, over 90 % of the cells have an apoptotic appearance, the cells not being attached to the culture plate.

Shortly after UVB exposure, at two different doses of radiation, melanocytes are affected, but after 24 hours they are fully recovered. In the case of human melanoma cells, UVB radiation is extremely toxic to melanin-producing cells, namely COLO-829, in a much larger way than those that do not produce melanin in culture – SK-MEL-3 – but have the ability to produce pigmented tumours in animal models.

The study on the incidence of tumours in a given population revealed the following: people aged 60 – 69 years were the most numerous (~ 22 %), followed by those in the age category 70 – 79 years (~ 18 %), while those under 10 years of age accounted for 0. 5 %; of the total number of patients included in the database, 2. 6 % had a diagnosis of melanoma, 6. 5 % had a diagnosis of basal cell carcinoma, while a significantly lower percentage, 0. 25 % were diagnosed with squamous cell carcinoma. The diagnosis of malignant melanoma in the early stages is essential for increasing life expectancy and skin self-examinations, especially in older men (taking into account that men are more prone to developing the disease), may prove to be of particular value.

The opinion of specialists is that melanoma can be treated and correct and early diagnosis is critical. Awareness-raising is needed and medical staff should advocate for programs dedicated to the population at risk.