MEDICAL UNIVERSITY
"Prof. Dr. Paraskev Stoyanov" – Varna

FACULTY OF MEDICINE
Department of General and Clinical Pathology, Forensic Medicine and Deontology

APOPTOSIS AND NECROPTOSIS IN BASAL CELL AND SPINOCELLULAR SKIN CANCER

Dr. Plamen Petrov Vassilev

ABSTRACT

of a dissertation

for the award of the educational and scientific degree of
"Doctor"
Scientific specialty: "Pathological Anatomy and Cytopathology"

Scientific supervisor:
Prof. Maria Angelova Tsaneva, MD, Phd

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The dissertation contains 139 standard pages and is illustrated with 66 tables and 35 figures. The bibliography includes 355 references, of which 6 are in Cyrillic and 349 are in Latin.

The dissertation has been discussed and referred for defence by the Department Council of the Department of General and Clinical Pathology, Forensic Medicine and Deontology at Prof. Dr. Paraskev Stoyanov Medical University – Varna on 17 September 2025.

The public defence of the dissertation will take place on 8 December 2025, Monday, before a scientific jury composed of:

External members:
1. Prof. Julian Ananiev, MD, PhD
2. Assoc. Prof. Dr. Silvia Genova, MD, PhD
3. Assoc. Prof. Ekaterina Softova-Zlatarova, MD, PhD

Internal members:
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Reserve external member:
1. Prof. Dobrinka Radoynova, MD, PhD

Reserve internal member:
1. Assoc. Prof. Kalin Kalchev, MD, PhD

CONTENTS

ABBREVIATIONS USED

In Cyrillic:

BCC – basal cell carcinoma

DNA – deoxyribonucleic acid

CRC – colorectal carcinoma

SCC – squamous cell carcinoma

WHO – World Health Organisation

In Latin:

AIF – Apoptosis-inducing factor

COX-2 – Cyclooxygenase-2

GSDME – Gasdermin-E

IDH - Isocitrate Dehydrogenase gene

MLKL - Mixed lineage kinase domain like pseudokinase

PIN - Prostatic Intraepithelial Neoplasia

PSA - Prostate-Specific Antigen

RIPK - Receptor-interacting serine/threonine-protein kinase

TILs - Tumor-infiltrating lymphocytes

I. INTRODUCTION

Malignant skin diseases are a heterogeneous group of neoplasms that occur worldwide, but with varying frequency. They are mainly categorised into two groups: melanoma and non-melanoma malignant neoplasms. These two groups account for more than 90% of all malignant skin tumours. Skin carcinomas originating in the epidermis are of two main histological types: basal cell carcinoma (BCC) and spinocellular or squamous cell carcinoma (SCC), which together account for 99% of malignant epithelial tumours. BCC is more common, grows slowly and has predominantly local invasive growth, the clinical manifestation of which is destruction of adjacent tissues, especially when located in the face, head and neck. It originates from basal keratinocytes and rarely metastasises (Madan V et al., 2010).

SCC is more common among Caucasians than other races. It is more common in men than in women, and its incidence increases significantly with age. It originates from epidermal keratinocytes (Combalia AA et al., 2020) .

A number of mutations in proto-oncogenes and tumour suppressor genes play a role in the development of BCC and SCC. In recent years, along with the well-known tumour-associated genes, a number of new tumour suppressor genes have been discovered, whose mutations are considered to be of great importance in tumour genesis (Pickering C et al., 2014). Uncontrolled activation of certain signalling pathways can lead to the development of BCC (Saran A et al., 2010).

Therapeutic management of BCC and SCC involves surgical intervention, targeted radiation and chemotherapy (Firnhaber JM, 2012). The latter two therapies induce apoptosis, a natural

process of programmed cell death, through which tumour cells are eliminated. In some cases, the induction of apoptosis is not possible due to the innate or acquired resistance of certain tumours to pro-apoptotic stimuli. In these cases, the therapeutic approach requires the search for alternative ways to destroy tumour cells resistant to apoptosis by initiating other cell death signalling pathways other than apoptosis.

There are various types of cell death: apoptosis, necrosis, autophagy, necroptosis, accidental cell death, anoikis, autosis, entotic cell death, ferroptosis, immunogenic cell death, lysosome-dependent cell death, mitochondrial-induced necrosis, mitotic catastrophe, partanatosis, pyroptosis, and regulated cell death (Galluzzi L et al., 2018). Apoptosis can occur via the so-called "external" and "internal" signalling pathways (You Yu et al., 2017, Xu X et al., 2019). The most obvious sign of both the early and late stages of apoptosis is the activation of cysteine proteases. Detailed examination of caspase-3 expression in cells and tissues by immunohistochemical staining is an important method for elucidating the mechanisms of apoptosis induced by various apoptotic signals (Mazumder S et al., 2008).

Receptors and ligands that activate the extrinsic signalling pathway of apoptosis can also initiate necroptosis (Galluzzi L et al., 2018). The initiation of necroptosis is associated with the expression of receptor serine-threonine kinase 3 (RIPK3) and can occur independently of RIPK1. RIPK3 is a key signalling molecule in the necroptosis pathway. This pathway plays an important role in various physiological and pathological conditions, such as the elimination and destruction of tumour cells (Sun L et al., 2012).

The mechanisms and signalling pathways of different types

of cell death can be involved in complex interactions. A detailed study of the expression of Caspase-3 and RIPK3 in patients with BCC and SCC would contribute to clarifying the predictive role of proteins, discovering and introducing new therapeutic approaches.

II. OBJECTIVE AND TASKS

2.1. Objective

The aim of this study is to investigate the immunohistochemical expression of the apoptosis marker Caspase-3 and the necroptosis marker Receptor-interacting kinase 3 (RIPK3) in patients with basal cell carcinoma and squamous cell carcinoma of the skin and to determine their predictive value.

In connection with the stated objective, the following tasks were formulated:

2.2. Tasks

1 To study the clinical and morphological characteristics of patients with BCC and SCC.
2 To study the level of immunohistochemical expression of Caspase-3 in BC and SCC tumour tissue.
3 To compare the immunohistochemical expression of Caspase-3 in the tumour tissue of BCC and SCC with adjacent non-tumour tissue.
4 To analyse the expression of Caspase-3 in relation to clinical and morphological indicators: age and gender of patients with BCC and SCC, TILs, tumour necrosis, risk group, degree of differentiation and tumour localisation.
5 To investigate the level of immunohistochemical expression of RIPK3 in the tumour tissue of BCC and SCC.
6 To compare the immunohistochemical expression of RIPK3 in BCC and SCC tumour tissue with adjacent non-tumour tissue.
7 To analyse RIPK3 expression in relation to clinical and morphological indicators: age and gender of patients with BCC and SCC, TILs, tumour necrosis, risk group, degree of differentiation and tumour location.

III. MATERIALS AND METHODS

3.1. Material base for the implementation of the dissertation

Department of General and Clinical Pathology, Forensic Medicine and Deontology at the Medical University of Varna and Diagnostic and Consultative Centre "Sveta Marina" EOOD-Varna.

3.2. Patients and characteristics of the study groups

A total of 91 patients were included in this study, 46 of whom had BCC of the skin and 45 had SCC of the skin for the period from 2015 to 2021. All patients studied underwent radical surgical removal of the skin tumour and the level of immunohistochemical expression of Caspase-3 and RIPK3 was determined.

3.3. Routine examinations

An average of two to three samples were examined from each tumour resection, including tumour parenchyma and adjacent non-tumour tissue. The materials were fixed in 10% neutral buffered formalin and, after appropriate processing, embedded in paraffin with a melting point of 52-54ºC to prepare paraffin blocks. Sections with a thickness of 5 μm were stained with haematoxylin-eosin to assess the histological changes in the tumour and the surrounding non-tumour tissue. The following parameters were determined for the tumours examined: location, histological type of tumour, TILs (tumour infiltrating lymphocytes), degree of tumour differentiation, presence of vascular and perineural invasion, area of necrosis and T stage.

3.4. Criteria for categorising each parameter

Tumour location. Tumours were classified according to their location: head, neck, trunk, limbs, penis/vulva.

Histological type of tumour. The diagnosis is made by applying the WHO classification for non-melanoma skin tumours, respectively the PKC according to the 8th revision of the TNM classification of the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) from 2017 and the BCC classification from 2010.

TNM – stage. The staging of BCC and SCC is based on the WHO classification from 2017 and its revised version from 2023 (Peris K et al. 2023). The TNM classification of head and neck SCC is based on the WHO classification from 2017 (TNM Classification of Malignant Tumours, Eighth Edition, Brierley J, Gospodarowich MK, Wittekind C, 2017).

Based on this classification, the T stage and the degree of tumour differentiation-G were determined. The degree of tumour differentiation is defined for SCC as high, moderate and low differentiation depending on the formation of keratin by tumour cells. In SCC, the risk of progression was determined: low risk: nodular, superficial, pigmented, infundibulocystic, SCC with adnexal differentiation and fibroepithelial; and high risk: basal squamous carcinoma, sclerosing, infiltrating, BCC with sarcomatoid differentiation, micronodular, according to the 2023 European guidelines for the diagnosis and treatment of BCC.

Tumour infiltrating lymphocytes. TILs (tumour infiltrating lymphocytes) were assessed according to the criteria of Klintrup K et al. (2005) Tumours are assessed using a 4-point scale in the areas with the highest TIL infiltration. No TILs – 0;

- Mildly expressed – slight and uneven increase in TILs – 1;
- Moderate – moderately expressed band-like infiltration of TILs with partial destruction of tumour cell islands – 2;
- Pronounced – abundance of TILs located in nests with frequent destruction of tumour cell islands – 3.

Vascular invasion (LVI) and perineural invasion (PNI). Vascular and perineural invasion was assessed in two categories: absent – 0 and present – 1.

Area of necrosis. The degree of necrosis in the tumour tissue was determined semi-quantitatively using a four-point scale:

- Absent - 0
- Less than 10% - 1
- Between 10-30% - 2
- Above 30% - 3

3.5. Specific research methods

3.5.1. Immunohistochemical methods. An indirect immunoperoxidase method was used for immunohistochemical analysis using mini KIT high Ph DAKO K8024.

3.5.1.1 Antibodies, staining reagents and working concentrations used are presented in Tables 4 and 5.

The following antibodies were used:

Anti-pro Caspase-3 antibody (E61), cat. No. ab32150, Rabbit Recombinant Monoclonal Caspase-3 antibody, monoclonal rabbit antibody, as a marker for apoptosis (ABCAM's RabMab technology) (Table 4).

Anti-RIPK3 antibody cat. No. ab62344, polyclonal rabbit antibody, marker for necroptosis (ABCAM's RabMab technology) (Table 4).

Negative controls:

For negative controls, instead of the primary antibody, sections from the paraffin blocks used are incubated with normal non-immune serum.

Lung tissue was used for positive controls for Anti-Caspase-3 and liver tissue for Anti-RIPK3.

***Table 4**. Reagents used*

Antibody	Dilution	Positive control	Marker	Manufacturer
Anti-pro Caspase-3 antibody (E61) (ab32150) Rabbit Recombinant Monoclonal Caspase-3 antibody	1:500	Lung	Apoptosis	ABCAM's RabMab technology
Anti-RIPK3 antibody (ab62344) Rabbit Polyclonal RIP3 antibody	1:300	Liver	Necroptosis	ABCAM's RabMab technology

***Table 5.** Staining systems and other reagents*

HRP - DAB system	Original staining system	Dako
Mayer's haematoxylin	Counterstaining	Dako

3.5.1.2. Preparation of biopsy materials for immunohistochemical examination.

- Sections 5 μm thick were prepared from the biopsy materials (fixed in neutral formalin and embedded in paraffin blocks) and placed on silanised slides.
- Deparaffinisation was performed in a descending series of alcohols as follows: 100% ethanol - 3 min, 90% ethanol - 3 min, 80% ethanol - 3 min, 70% ethanol - 3 min, xylene 3 x 10 min. The sections were washed with running water and placed in distilled water.
- Antigen retrieval: antigen retrieval was performed with En Vision FLEX Target Retrieval Solution (working solution)

preheated to 65°C in a PT Link container, and the sections were incubated for 20 min at 97°C and pH=9. After cooling, the samples were washed at room temperature with FLEXWash Buffer (20x) for 1-5 minutes.

3.5.1.3. Immunohistochemical protocol.

The sections were stained according to the FLEX protocol, using a humid chamber at all steps.

Incubation with peroxidase blocking solution (3% H2O2) for 5 minutes at room temperature to block endogenous peroxidase activity. Washing with washing buffer for 5 minutes.

- Incubation with primary antibodies Anti-pro Caspase3 antibody (E61) (ab32150) and Anti-RIPK3 antibody (ab62344), diluted for 20 min at room temperature.
- Wash with washing buffer for 2 x 5 min at room temperature.
- Incubation with labelled HRP polymer for 20 minutes at room temperature.
- Wash with washing buffer for 3 x 5 min at room temperature.
- Incubate the sections with chromogenic DAB peroxidase solution for 2 x 5 min. under continuous microscopy.
- Wash with buffer for 2 min.
- Rinse with distilled water for 2 min.
- Counterstain with Mayer's haematoxylin for 5 minutes.
- Rinse the samples with distilled water for 5 minutes.
- Dehydration in reverse ascending order with 70% ethanol, 80% ethanol, 90% ethanol, 100% ethanol for the same duration as for deparaffinisation.
- Place in mounting medium.

3.5.1.4. Method for assessing the expression of Caspase-3 and RIPK3.

The immunohistochemical expression of Caspase-3/RIPK3 was assessed by examining 10 fields at the highest magnification (x400) for each individual case. The immunohistochemical expression of each antibody was assessed semi-quantitatively using an H-score (histo-score) on tissue sections. For each cell in different fields, the cytoplasmic and nuclear intensity (0, 1+, 2+ or 3+) was determined. The percentage of positive cells for each individual intensity was calculated, and finally the H-score was calculated using the following formula (Ishibasshi H et al., 2003):

- {1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+)}, ranging from 0 to 300.
- The H-score was also used to evaluate the nuclear expression of Caspase-3 and RIPK3. The result was evaluated using the formula: {1x (% nuclei with 1+) + 2x (% nuclei with 2+) + 3x (% nuclei with 3+)}, in a range from 0 to 300.

3.6. Statistical methods

The statistical methods used are consistent with the tasks set, the clinical and morphological indicators studied, and the reporting of the results obtained.

3.6.1. Descriptive statistical analysis

It is used to present the frequency distribution of the analysed indicators by groups, the mean values and standard deviations, and the 95% confidence interval (CI) of change in the mean values for the variables and antibody expression.

3.6.2. Student's t-test for two independent samples

For analysing a statistically significant difference between the mean values of the measured quantitative and qualitative indicators (size in cm, expression intensity, etc.) when comparing two groups.

3.6.3. Analysis of variance (ANOVA)

It is used to assess the presence or absence of the influence of two or more factors on the mean values of the studied indicators. If the level of $p<0.05$, the null hypothesis is rejected, i.e. the factor influences the mean values of the variable under consideration.

3.7. Statistical data processing

The statistical analysis of the data was performed using the STATISTICA software package (StatSoft Inc, USA, STATISTICA Manual (Data analysis software system), Version 13.0, 2022). The graphs were constructed in Microsoft Excel for Windows.

IV. RESULTS AND DISCUSSION

4.1. Clinical and morphological characteristics of patients with BCC and SCC

Table 6 presents the clinical and morphological characteristics of the studied patients with BCC and SCC.

Table 6. *Clinical and morphological characteristics of the studied patients with BCC* and *SCC*

Characteristics		Number (n) of patients	%
Total number of patients examined		91	10
1. Gender	Men	45	49.5
	Women	46	50
2. Histological type	Basal cell carcinoma (BCC)	46	10
	Nodular BCC	24	52.17
	Superficial BCC	10	21.74
	Fibroepithelial BCC	7	15.22
	Micronodular BCC	5	10.87
	Squamous cell carcinoma (SCC)	45	10
	Conventional SCC	33	73
	Verucose SCC	5	11
	Keratoacanthoma/highly differentiated SCC	7	15.5
3. Differentiation	BCC	4	10
	Low risk	34	73.91
	High risk	12	26.09
	SCC	45	10
	G1	30	66.67
	G2	15	33
4. Area of necrosis	BCK	4	10
	Absent /Group I/	41	89.13
	Below 10% /Group II/	5	10.87
	SCC	45	10
	Missing /Group I/	34	75.55
	Below 10% /Group II/	8	17.78
	Between 10-30% /Group III/	3	6.67

5. Tumor-infiltrating lympho-cytes (TILs)	BCC	46	10
	Absent	18	39.13
	Slightly pronounced	16	34.7
	Moderately pronounced	12	26.09
	SCC	45	10
	Missing	15	3
	Slightly pronounced	22	48
	Moderately pronounced	8	17.78
6. Vascular invasion (LVI)	BCC	4	10
	Absent	46	100
	SCC	45	100
	Missing	45	10
7. T stage	BCC	46	100
	T1	46	100
	SCC	45	100
	T1	43	95.56
	T2	2	4.44
8. Localisation	BCC	46	10
	Chapter	37	80.43
	Trunkus	4	8.7
	Limbs	2	4.35
	Genitals (penis, vulva)	3	6.52
	SCC	45	10
	Chapter	31	68.89
	Neck	2	4.44
	Trunkus	3	6.67
	Limbs	8	17.78
	Genitals (penis, vulva)	1	2.22

Of the 91 patients analysed, 46 had BCC and 45 had SCC. The average age of the patients was 58.3±14.47 years. The average age of patients with BCC was 56.5±13.50 years, and that of patients with SCC was 60.2±15.32 years. By gender, the patients were distributed as follows: 45 (49.5%) men and 46 (50.5%) women. There were 25 (55.6%) male patients with SCC and 20 (44.5%) with BCC. Among women, 20 (43.5%) patients had SCC, and 26 (56.5%) had BCC.

Of the 46 patients with BCC studied, 24 (52.17%) had the nodular subtype (Figure 12), 10 (21.74%) had the superficial subtype (Figure 13), 7 (15.22%) had a fibroepithelial subtype

(Figure 14), and 5 (10.87%) had a micronodular subtype (Figure 15). Of the 45 patients with SCC studied, 33 (73.33%) are of the conventional subtype (Figure 16), 5 (11.11%) were of the verrucous subtype (Figure 17) and 7 (15.56%) were of the keratoacanthoma/highly differentiated SCC subtype (Figure 18). We found that BCC is more common in women, with a ratio of 1.3:1, while SCC is more common in men, with a ratio of 1.25:1.

In terms of the degree of differentiation, 30 (66.67%) patients with SCC fall into the G1 group (Figure 19) and 15 (33.33%) cases fall into the G2 group. Of the BCCs, 34 (73.91%) patients are at low risk and 12 (26.09%) are at high risk.

The analysis of necrosis revealed that 8 (17.78%) of the SCC and 5 (10.87%) of the BCC had necrosis covering up to 10% of the tumour area (Figures 20 and 21). Necrosis between 10% and 30% of the tumour area was found in 3 (6.67%) of the SCC.

In each individual case, tumour-infiltrating lymphocytes (TILs) were determined in the tumour tissue. TILs were absent in 15 (33.33%) of SCCs and in 18 (39.13%) of BCCs, were weakly expressed in 22 (48.89%) of SCCs and in 16 (34.78%) of BCCs (Figure 22) and are moderately expressed in 8 (17.78%) of squamous cell carcinomas (Figures 23 and 24) and in 12 (26.09%) of basal cell carcinomas. No lymphovascular invasion was found in the skin tumours examined.

According to the T stage of the tumour, we found that SCC in 43 (95.56%) cases was in stage T1 and only in 2 (4.44%) cases in stage T2. All 46 (100%) patients with BCC were in stage T1. According to the location of the tumour, 2 (4.44%) of the SCCs were in the neck area. 31 (68.89%) of the SCCs and 37 (80.43%) of the BCCs were located in the head. 3 (6.67%) of the SCCs and 4 (8.7%) of the BCCs were located in the trunk. 8 (17.78%) of the SCCs and 2 (4.35%) of the BCCs are located on the extremities, and 1 (2.22%) of the SCCs and 3 (6.52%) of the BCCs are located in the genital area.

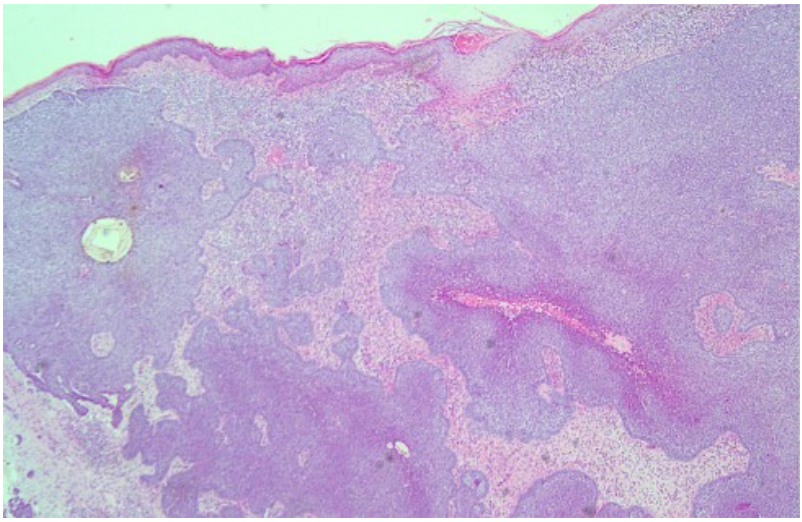


Figure 12. *Nodular type basal cell carcinoma. Large lobules of basal cells with slight pleomorphism, with a palisade peripheral arrangement among fibromyxoid stroma. Coloured. HE x200*

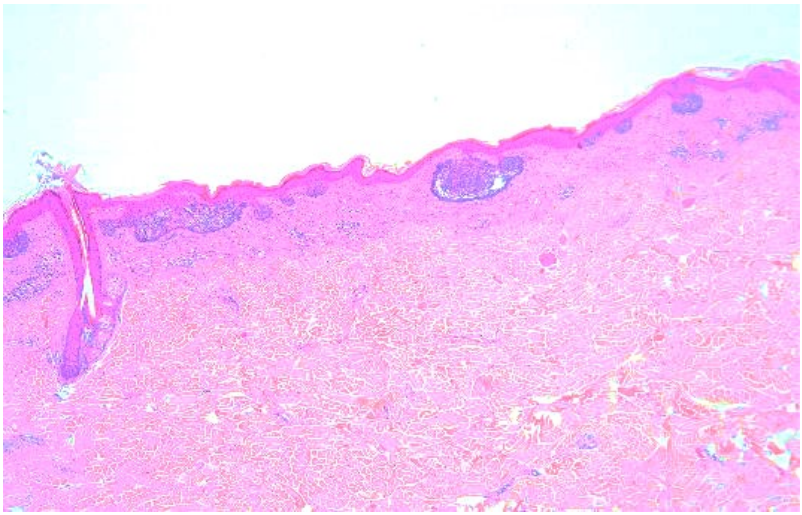


Figure 13. *Superficial basal cell carcinoma. Isolated lobules of basaloid cells associated with the basal layer of the epidermis. Stained with HE x200*

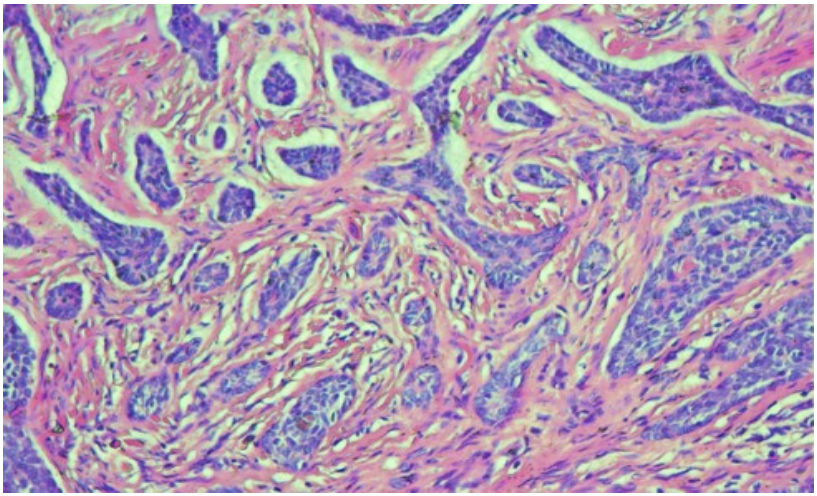


Figure 14. *Pincus fibroepithelioma, a type of basal cell carcinoma of the skin. Anastomosing epithelial strands among significant dermal fibrosis. Stained with HE x200*

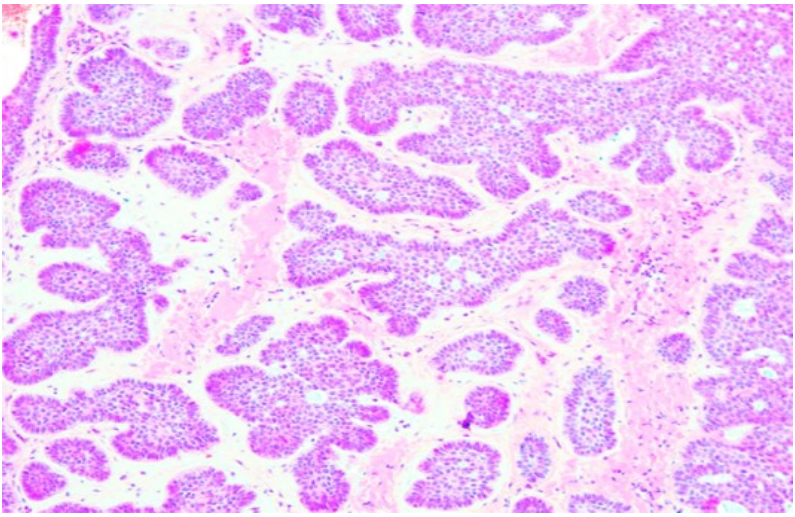


Figure 15. *Micronodular type of basal cell carcinoma of the skin. Small nests of basal cells with peripheral palisade arrangement. Stained with HE x200*

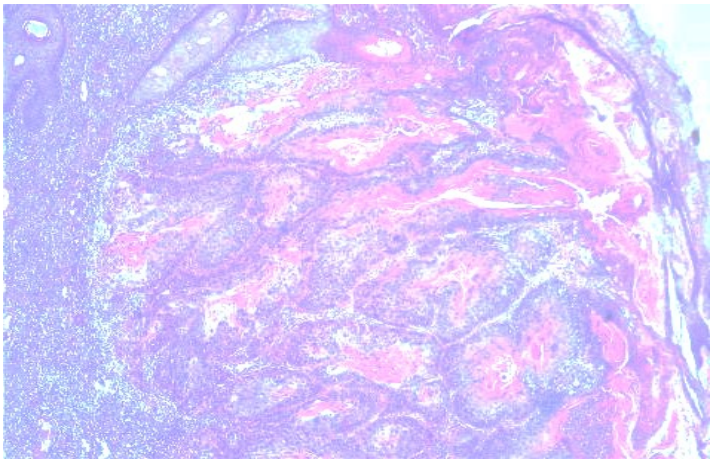


Figure 16. *Conventional squamous cell carcinoma represented by atypical cells with polygonal shape, intracellular formation and extracellular deposition of keratin in the form of cancer pearls. Coloured. HE x200*

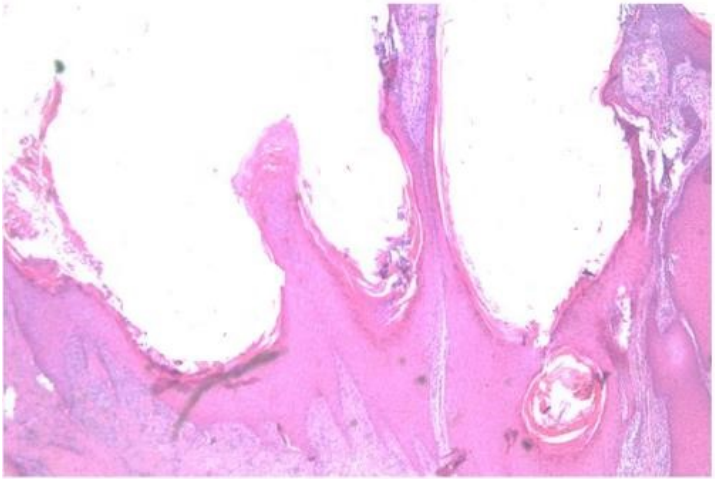


Figure 17. *Verrucous squamous cell carcinoma of the skin with characteristic tumour growth pushing the stroma (pushing borders). Coloured HE x200*

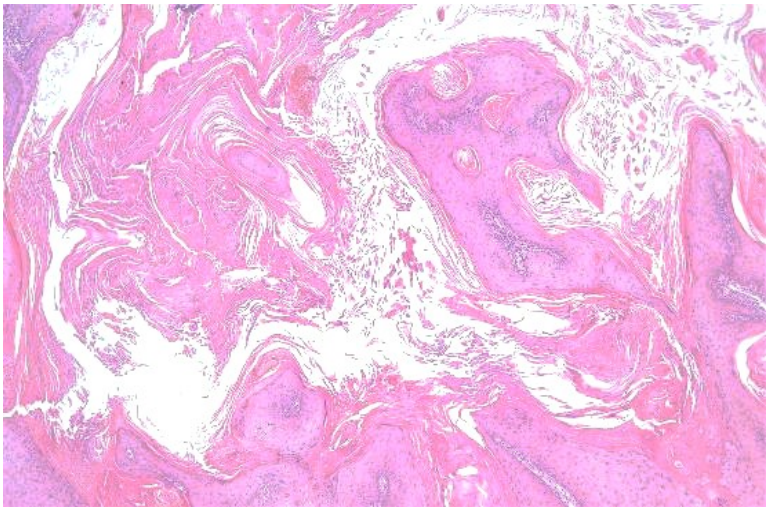


Figure 18. *Keratoacanthoma/well-differentiated squamous cell carcinoma with characteristic crater-like pattern. Coloured. HE x200*

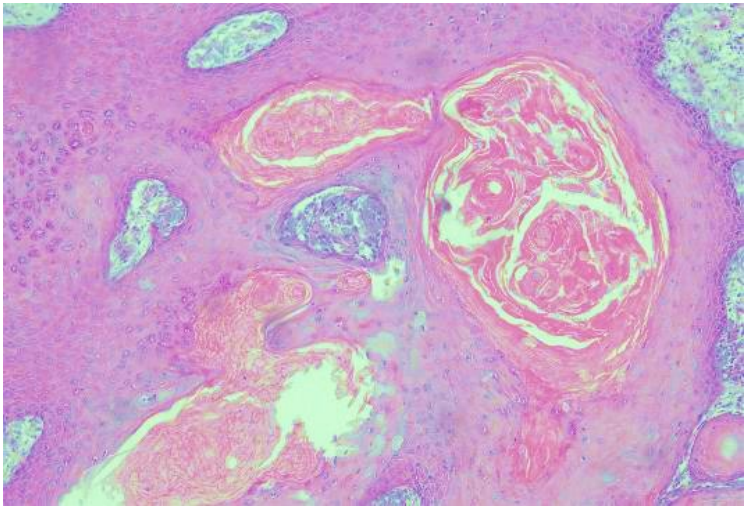


Figure 19. *Well-differentiated squamous cell carcinoma with intra-cellular formation and extra-cellular deposition of keratin in the form of cancer pearls. Stained with HE x200*

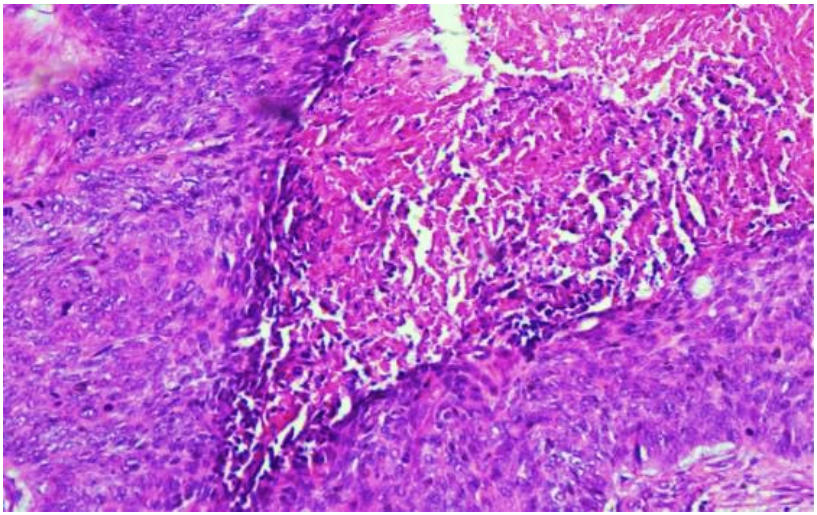


Figure 20. *Tumour necrosis in squamous cell carcinoma covering up to 10% of the tumour area. Stained with HE x200*

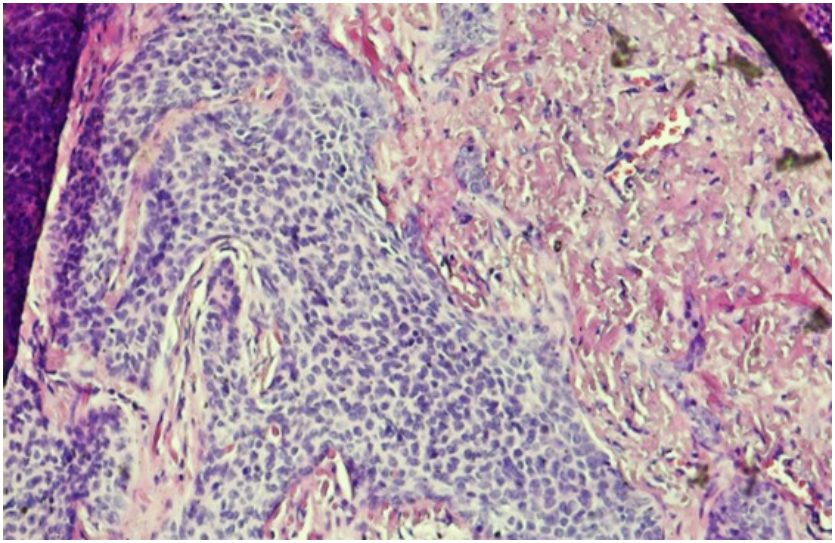


Figure 21*. Tumour necrosis in basal cell carcinoma up to 10% of the tumour area. Stained with HE x200*

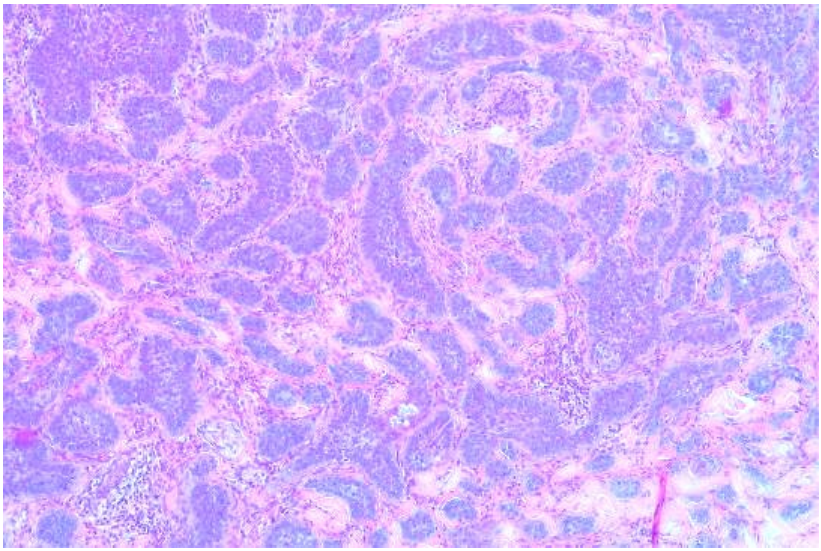


Figure 22. *Mild infiltration of TILs in the tumour tissue of basal cell carcinoma. Stained with HE x200*

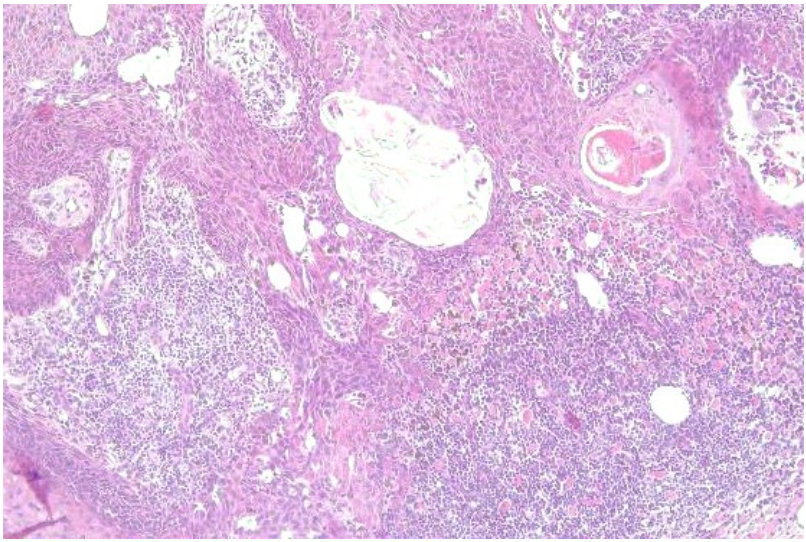


Figure 23*. Moderate infiltration of TILs in the tumour tissue of squamous cell carcinoma. Stain: HE x200*

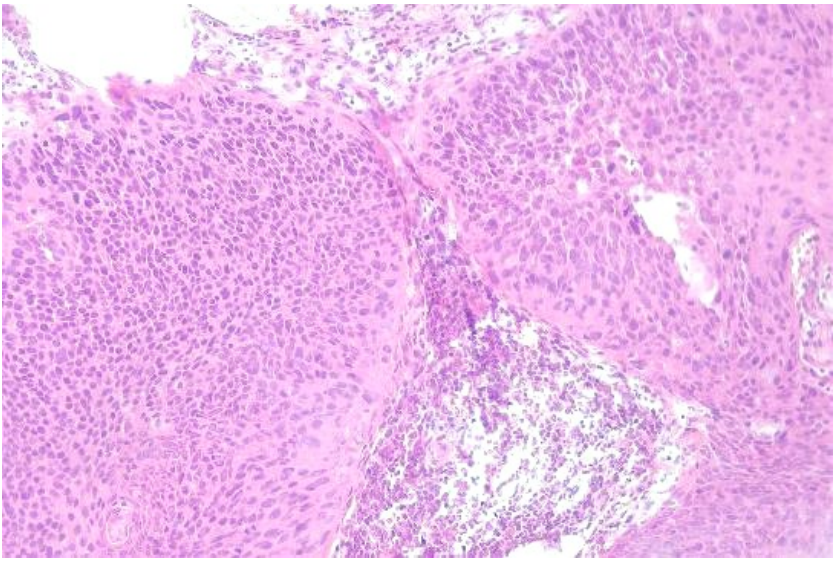


Figure 24. *Moderate infiltration of TILs with a distinct nests arrangement. Stain: HE x200*

4.2.1 Cytoplasmic and nuclear expression of Caspase-3 in tumour tissue in BCC and in adjacent non-tumour tissue

The mean value of cytoplasmic expression in BCC tumour tissue determined by H-score is 20.20±60.44, with a minimum value of 0 and a maximum value of 300 (Figure 25). The mean value of nuclear expression in BCC tumour tissue of Caspase-3 is 17.87±56.74, with a minimum value of 0 and a maximum value of 293 (Figure 26).

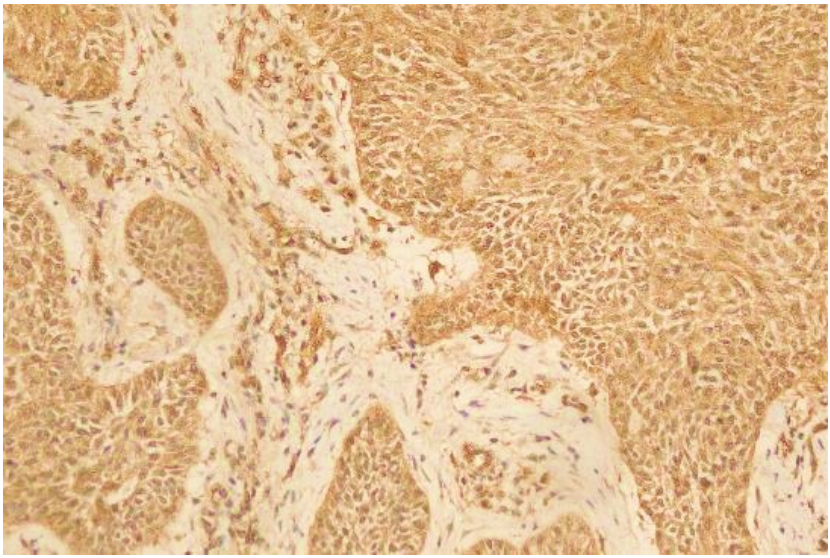


Figure 25. *High cytoplasmic expression of Caspase-3 in basal cell carcinoma. IHC x200.*

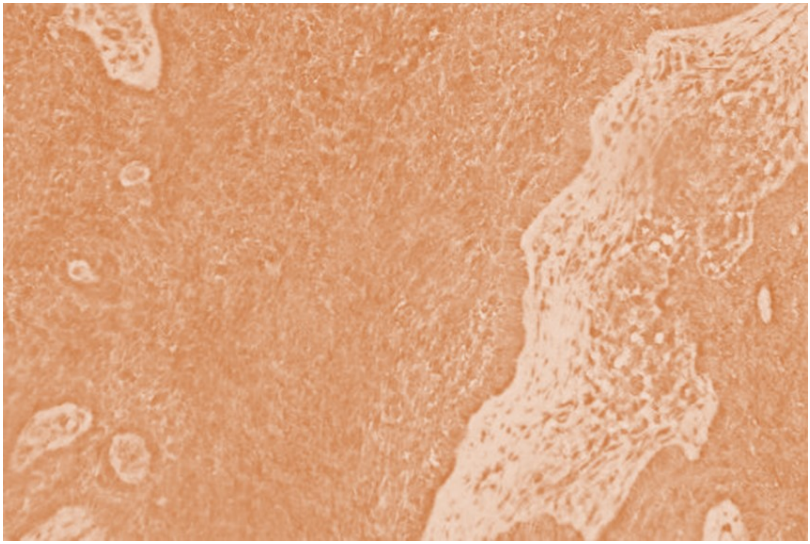


Figure 26. *High nuclear and cytoplasmic expression of Caspase-3 in basal cell carcinoma. IHC x200.*

Cytoplasmic expression of Caspase-3 in non-tumour tissue in BCC has a mean value of 18.02±37.12. Nuclear expression of Caspase-3 in adjacent tissue in BCC has a mean value of 18.27±44.35 (Figure 27).

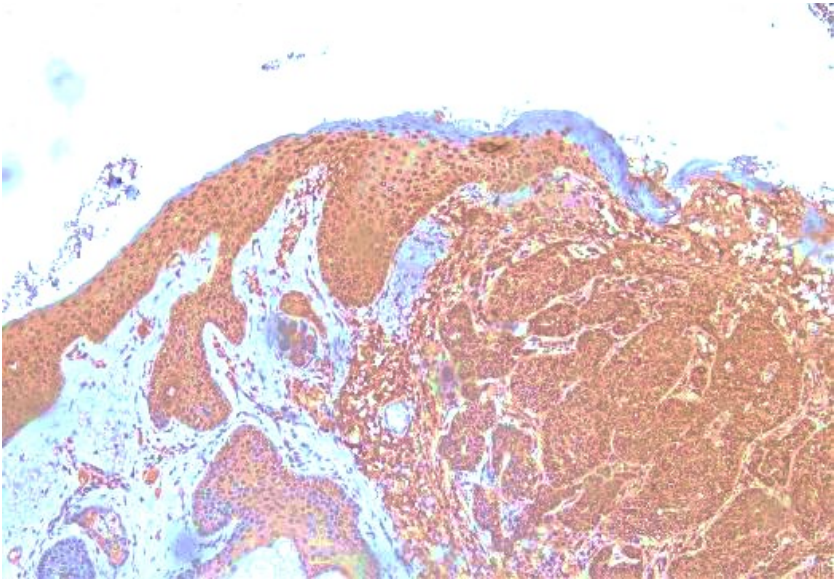


Figure 27. *High nuclear expression of Caspase-3 in adjacent non-tumour tissue and weak cytoplasmic and moderate nuclear expression in tumour tissue in patients with BCC. IHC x200.*

Figure 28 shows moderate cytoplasmic expression of Caspase-3 in non-tumour cells adjacent to BCC. There is no nuclear or cytoplasmic expression in the basal layer.

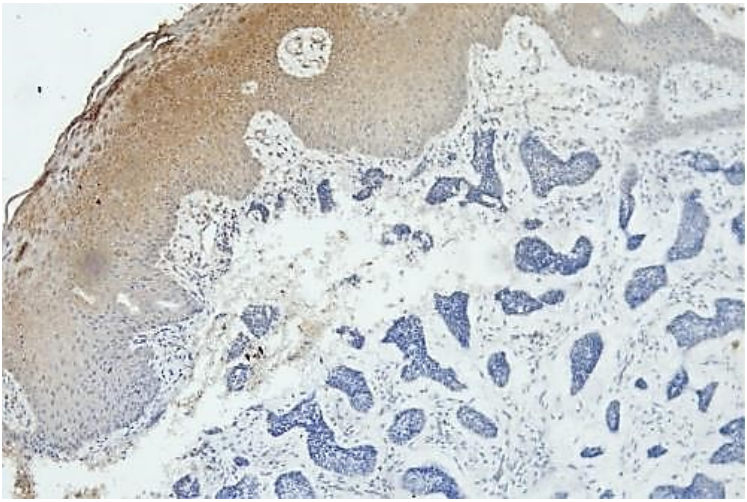


Figure 28. *Expression of Caspase-3 in adjacent non-tumour tissue. Absent in tumour cells in basal cell carcinoma. IHC x200*

Low levels of expression in the basal layer of the epidermis adjacent to the tumour process have also been reported by other authors (Veeran Veeravarmal et al., 2016). According to Heshiki W et al., (2015), this may be due to low levels of apoptosis in non-tumour cells in the basal layer.

4.2.2. Cytoplasmic and nuclear expression of Caspase-3 in tumour tissue in SCC and in adjacent non-tumour tissue.

The mean value of cytoplasmic expression determined by H-score in SCC is 16.86±28.57, with a minimum value of 0 and a maximum value of 110 (Figure 29). The mean value of nuclear expression of Caspase-3 is 6.48±19.58, with a minimum value of 0 and a maximum value of 84.

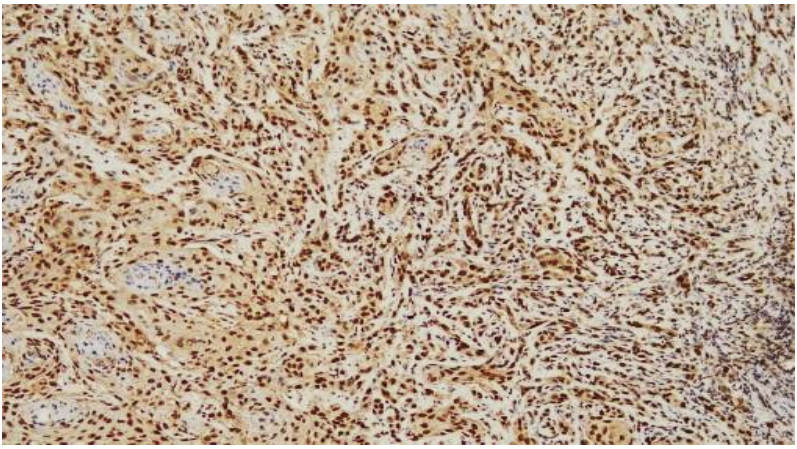


Figure 29. *Weak to moderate cytoplasmic and intense nuclear expression of Caspase-3 in tumour cells in squamous cell carcinoma. IHC x100.*

With regard to nuclear and cytoplasmic expression in the adjacent non-tumour tissue in SCC, it is respectively: 8.05±9.88 for nuclear (Figure 30) and 8.18±9.22 for cytoplasmic.

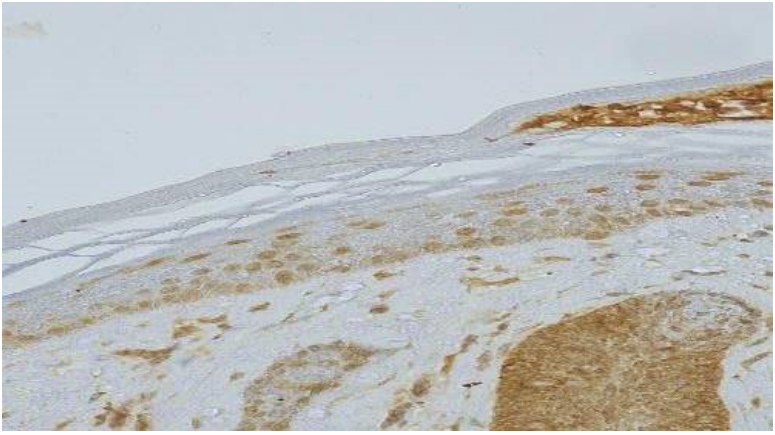


Figure 30. *Moderate nuclear expression of Caspase-3 in non-tumour cells and intense cytoplasmic expression in squamous cell carcinoma. IHC x200*

4.2.3. Comparative analysis between the mean values of Caspase-3 expression in tumour tissue and adjacent non-tumour tissue in patients with BCC and SCC

4.2.3.1. Caspase-3 expression in BCC.

When analyzing the mean values of cytoplasmic and nuclear Caspase-3 expression in tumor and non-tumor cells, no statistically significant difference was found for cytoplasmic (p= 0.74) and nuclear expression (p= 0.94) (Tables 7 and 8).

Table 7. *Mean values of cytoplasmic expression of Caspase-3 in BCC tumour tissue and in adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
In tumour cells	46	20.2	60.44	**0.74**
In non-tumour cells	46	18.02	37.12	

Table 8. *Mean values of nuclear expression of Caspase-3 in the tumour tissue of BCC and in the adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
In tumour cells	46	17.87	56.74	**0.94**
In non-tumour cells	46	18.27	44.35	

Increased expression of Caspase-3 has been found in various types of carcinomas: stomach carcinoma, hepatocellular carcinoma, prostate carcinoma, etc. High levels of expression have been found in glial tumours, malignant melanoma, acute myeloid leukaemia and oral cavity SCC (Huang J et al., 2017). In eyelid skin BCC, Koyun E et al. (2020) found low levels of expression of the protein involved in the process of apoptosis. Such low levels have also been found in other neoplasms such as cervical and mammary gland carcinoma (Devarajan E et. 2002). According to some authors, reduced expression of Caspase-3 can lead to "escape" from apoptosis and acceleration of the process of tumorigenesis, as tumor cells acquire resistance to stress factors in the microenvironment. (Huang Q et al., 2011). On the other hand, overexpression of Caspase-3 by dead tumour cells may lead to the release of signals that stimulate the growth of neoplastic cells, allowing them to multiply and survive under stressful conditions.

Taken together, the data from the literature suggest that the expression of the apoptotic marker Caspase-3 may be increased or decreased depending on the histological variant of the tumour and its location.

We found that Caspase-3, the marker for apoptosis, is expressed to the same extent in tumour and non-tumour tissue of BCC. In the present study, we used pro-Caspase-3, which is the inactive form of Caspase-3. Our results correspond to the data obtained from the analysis of pro-Caspase-3 in gastric carcinoma, where it was found that only pro-Caspase-3 is present in tumour and adjacent non-tumour tissue, while the active form is absent (Kania J et al., 2003). It is assumed that during tumorigenesis, Caspase-3 disappears and apoptosis is

blocked. Similar results of reduced to absent expression of Caspase-3 in the cytoplasm of epithelial cells in premalignant lesions and gastric carcinoma have been observed by other authors (Sun Y et al., 2006). Analysis of apoptosis in the buccal mucosa using the TUNEL method shows the opposite - a gradual increase in the number of apoptotic cells, with the lowest number in normal buccal mucosa, increasing in dysplasia and carcinoma in situ, and highest in carcinoma (Huang JS et al. 2017).

Other studies (Huang Q et al., 2011) have found that Caspase-3 promotes carcinogenesis in both in vivo and in vitro studies. Higher levels of Caspase-3 expression were found in invasive breast cancer compared to non-tumour breast tissue, and the increased level is a marker for poor prognosis in these patients. Huang Q et al. (2011) found that high levels of Caspase-3, through the activation of prostaglandin E2, have a powerful stimulating effect on the growth of tumour cells that have survived radiotherapy, resulting in a significant increase in the frequency of recurrence and an increased risk of death. A deficiency of Caspase-3 in tumour- e cells significantly increases the sensitivity of the tumour to radiotherapy. In addition to apoptosis, Feng X et al. (2015) show that after irradiation in vivo (HT-29 and CASP3DN cell lines) and in vitro models, Caspase-3 mediates the proangiogenic properties of dying tumour cells. Inactive Caspase-3 significantly suppresses tumour angiogenesis in a xenograft model of tumour growth.

Huang YL et al (2021) found a statistically significant difference between the expression of Caspase-3 in tumour tissue compared to normal tissue. A similar relationship was also found for gasdermin E (GSDME) in patients with lung cancer.

GSDME is a protein that plays a key role in pyroptosis, a form of programmed cell death associated with the release of inflammatory cytokines. Cells with high levels of GSDME expression redirect Caspase-3-mediated apoptosis to pyroptosis. Cousin F. et al. (2000) found lower Caspase-3 expression in BCC compared to normal tissue. However, studies are still few in number and further analysis of the role of Caspase-3 in tumour genesis is needed (Koyun E et al., 2020). Low levels of Caspase-3 expression in tumour tissue compared to normal tissue have been found in patients with prostate adenocarcinoma and some low-grade carcinomas (Winter RN et al., 2001). According to Devarajan et al. (2002), low levels of Caspase-3 expression are associated with chemoresistance in tumour tissue.

The data in the literature on Caspase-3 expression vary considerably, which, in our opinion, may be at least partly due to the different methods of investigation and evaluation of the immunohistochemical expression of the antibody. In the present study, the assessment of Caspase-3 is based on two indicators: staining intensity and percentage of cells with the corresponding intensity, which, in our opinion, gives a fairly accurate idea of its tissue level in tumour and non-tumour tissue.

4.2.3.2. Caspase-3 expression in SCC.

In SCC, the analysis between the mean values of Caspase-3 cytoplasmic expression in tumour and non-tumour cells found a statistically significant difference ($p=0.03$). In tumour tissue, the cytoplasmic expression level of Caspase-3 is higher compared to the adjacent non-tumour tissue (Table 9).

No statistically significant difference was found in the nuclear expression of Caspase-3 in tumour and non-tumour tissue (p=0.58) (Table 10).

Table 9. *Mean values of cytoplasmic expression of Caspase-3 in the tumour tissue of SCC and in the adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
In tumour cells	45	16.86	28.59	**0**
In non-tumour cells	45	8.18	9.22	

Table 10. *Mean values of nuclear expression of Caspase-3 in the tumour of the SCC and in the adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
In tumour cells	45	6.48	19.58	**0.58**
In non-tumour cells	45	8.05	9.88	

Caspase-3 is a key executor of apoptosis, which exists as a proenzyme in the cytoplasm of cells (Yu You et al., 2017). During apoptosis, it cleaves, followed by activation and translocation to the nucleus. In the nucleus, activated caspase-3 cleaves specific nuclear substrates, leading to characteristic apoptotic changes such as chromatin condensation, DNA fragmentation and nuclear destruction, thus performing its effector functions.

We found cytoplasmic and nuclear expression of Caspase-3

in both tumour and non-tumour tissue in SCC. The level of Caspase-3 is elevated in the cytoplasm of tumour cells. The increased level may indicate that the apoptotic pathway is active in SCC. It is important to note that active Caspase-3 can be found in the cytoplasm and even in the nucleus of cells that do not show obvious signs of apoptosis (Krajewska M et al. 1997). This may mean that caspase-3 activity may not always be strictly limited to the apoptotic pathway.

Huang JS et al. (2017) analysed squamous cell carcinoma of the buccal mucosa and found increased expression of Caspase-3. According to the authors, low Caspase-3 expression is associated with better overall survival, earlier disease stage, and absence of lymph node metastasis. According to the same authors, Caspase-3 is actively involved in tumorigenesis (Huang JS et al. 2017). In our opinion, increased tissue cytoplasmic expression of Caspase-3 may be one of the reasons for the more aggressive biological behavior of SCC compared to BCC.

4.2.4. Comparative analysis of Caspase-3 expression in BCC and SCC depending on clinical and morphological characteristics.

4.2.4.1. Relationship between Caspase-3 expression and patient age

In order to analyse the dependence between cytoplasmic expression of Caspase-3 and the age of the patients, they were divided into two age groups (Table 11). The table shows the mean values of cytoplasmic expression of Caspase-3 in tumour cells in BCC. Using dispersion analysis, we found no correlation between Caspase-3 expression and patient age ($p=0.44$).

Table 11. *Mean values of cytoplasmic expression of Caspase-3 in BCC tumour tissue in relation to age.*

Age	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
31-60	27	11.61	44.39	**0.44**
61-90	19	25.93	69.34	

Table 12 shows the mean values of Caspase-3 nuclear expression in BCC tumour tissue in relation to age. Dispersion analysis showed that there is no statistically significant correlation between the Caspase-3 nuclear expression index and the age of patients in BCC tumour tissue (p=0.37).

Table 12. *Mean values of nuclear expression of Caspase-3 in BCC tumour tissue in relation to age.*

Age	Number of cases, N	Mean values of Caspase-3 nuclear expression in tumour cells	Standard deviation (SD)	P-value
31-60	27	8.44	32.65	**0.37**
61-90	19	24.15	68.19	

SCC patients were also divided into two groups: up to 60 years and over 60 years. Table 13 shows the mean values of cytoplasmic expression of Caspase-3 in the tumour tissue of SCC patients in relation to age. Dispersion analysis showed that there is no statistical correlation between the cytoplasmic expression of Caspase-3 and the age of patients with SCC (p=0.87).

Table 13. *Mean values of cytoplasmic expression of Caspase-3 in the tumour tissue of SCC in relation to age.*

Age	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
31-60	20	17.12	32.15	**0.87**
61-90	25	15.70	23	

Table 14 presents the mean values of nuclear expression of Caspase- -3 in the tumour tissue of SCC in patients in both age groups. No statistically significant correlation was found between nuclear expression of Caspase-3 in tumor tissue in SCC in relation to patient age (p=0.58).

Table 14. *Mean values of nuclear expression of Caspase-3 in the tumour tissue of SCC in relation to age.*

Age	Number of cases, N	Mean values of nuclear expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
31-60	20	7.76	22.28	**0.58**
61-90	25	4.55	15.39	

No correlation between Caspase-3 expression in tumour tissue and age has been observed in a number of tumours. Dozic B et al. (2016) found no correlation between the two indicators in adenoid cystic carcinoma of the parotid gland. Tanimoto T et al. (2005) also found no correlation between protein expression and age in patients with head and neck SCC. The authors reported both cytoplasmic and nuclear immunohistochemical expression of Caspase-3, with the study group including squamous cell carcinomas of the oral cavity, nasal cavity, pharynx, larynx and paranasal sinuses. In squamous cell

carcinoma of the buccal mucosa, no correlation was found between Caspase-3 expression in tumour tissue and patient age (Huang JS et al., 2017). In contrast to the above authors, Oudejans JJ et al. (2005) found a statistically significant correlation between Caspase-3 expression in tumour tissue of nasopharyngeal carcinoma and patient age. The absence of Caspase-3 is found in older patients.

We found that cytoplasmic and nuclear expression of Caspase-3 in the two skin carcinomas, BCC and SCC, did not show any dependence on age.

4.2.4.2. Dependence between Caspase-3 expression and patient gender

When analysing the mean values of cytoplasmic and nuclear expression of Caspase-3 in tumour cells in relation to patient gender (Tables 15 and 16), no statistically significant dependence was found, either in terms of cytoplasmic expression (p=0.14) and nuclear expression (p=0.16) in BCC.

Table 15. *Relationship between cytoplasmic expression of Caspase-3 in tumour cells and the sex of patients with BCC.*

Gender	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
Men	20	31.69	15.05	**0.14**
Women	26	4.47	3.94	

Table 16. *Correlation between the nuclear expression of Caspase-3 in tumour cells and the gender of patients with BCC.*

Gender	Number of cases, N	Mean values of nuclear expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
Men	20	28.08	14.18	**0.16**
Women	26	3.89	3.63	

When comparing the mean values of cytoplasmic and nuclear expression of Caspase-3 in tumour cells according to the patient's gender in SCC, no statistically significant correlation was found for cytoplasmic (p=0.17) and nuclear expression (p=0.55) (Tables 17 and 18). Our results show that the expression of Caspase-3 in tumour tissue is not influenced by the patient's gender.

Table 17. *Relationship between cytoplasmic expression of Caspase-3 in tumour cells and the sex of patients with SCC.*

Gender	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
Men	25	21.44	33.83	**0.17**
Women	20	10.3	18.60	

Table 18. *Correlation between nuclear expression of Caspase-3 in tumour cells and the gender of patients with SCC.*

Gender	Number of cases, N	Mean values of nuclear expression of Caspase-3 in tumour cells	Standard deviation (SD)	P-value
Men	25	7.68	20.65	**0.55**
Women	20	4.5	14.98	

Our results show that cytoplasmic and nuclear expression of Caspase-3 in the tumour tissue of BCC and SCC is not influenced by the gender of the patients.

Similar results have been reported by other authors for different types of carcinomas: colorectal (de Heer P et al. 2007, Koelink P et al. 2009, Ahmed S et al. 2022), gastric, ovarian and cervical carcinoma (Hu Q et al. (2014)

4.2.4.3 Relationship between Caspase-3 expression and the BCC risk group.

The different histological variants of BCC in the present study are represented by a relatively small number of cases, therefore the dependence of Caspase-3 on the histological variant was not analysed. The dependence of Caspase-3 on the risk group was determined. BCCs were divided into two categories: low and high risk. The low-risk group includes: nodular, fibroepithelial and superficial BCC. Micronodular BCC belongs to the high-risk group. Tables 19 and 20 present the mean values of cytoplasmic and nuclear expression of Caspase-3 depending on the risk. No statistically significant correlation was found for either cytoplasmic (p=0.41) or nuclear expression (p=0.32) of the apoptosis marker in the risk group for BCC.

Table 19. *Relationship between cytoplasmic expression of Caspase-3 and low- and high-risk BCC.*

Group	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Low risk	40	17.55	56.37	**0.41**
High risk	5	41.40	92.57	

Table 20. *Correlation between Caspase-3 nuclear expression and low- and high-risk BCC.*

Group	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Low risk	40	14.8	51.52	**0.32**
High risk	5	41.8	93.47	

There is no statistically significant correlation between the different histological variants of SCC and the two expressions of Caspase-3: cytoplasmic (p=0.39) and nuclear expression (p=0.45) (Tables 21 and 22).

Table 21 *. Correlation between cytoplasmic Caspase-3 expression and histological variant of SCC.*

Histological type	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Conventional SCC	3	13.22	23.21	**0**
Verucose SCC	5	25.00	47.68	
Keratoacanthoma	7	27.71	35.98	

Table 22. *Correlation between nuclear expression of Caspase-3 and histological variant of SCC.*

Histological type	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Conventional SCC	3	4.25	15.67	**0.45**
Verucose SCC	5	14.8	33.09	
Keratoacanthoma	7	10.71	25.39	

O' Donovan N et al. (2003) found higher levels of Caspase-3 expression in ductal compared to lobular breast cancer. According to other authors, Caspase-3 expression is higher in patients with grade G4 glioma than in tumours with grade G1 and G2 (Feng X et al. 2023). High levels of cleaved Caspase-3 expression have been found in patients with glial tumours (Feng X et al. 2023). The authors found that high levels of cleaved Caspase-3 expression are associated with higher stage, malignant histological type, wild-type IDH, and high microvascular density in the tumour. Data obtained from cell cultures of irradiated glioma cells show that Caspase-3 mediates the formation of proangiogenic complexes and stimulates cell division via COX-2. Patients with gliomas with high levels of cleaved caspase-3 and COX-2 expression have the lowest survival rates. According to the authors, the results of the study of the Caspase-3/COX-2 signalling pathway may be useful in the treatment and prediction of therapeutic effects in glial tumours (Feng X et al., 2023).

Ahmed S et al. (2022) found a statistically significant difference between caspase-3 expression and the histological type of colorectal carcinoma. Antibody expression is higher in conventional adenocarcinoma than in mucinous adenocarcinoma of the colon. According to the authors, Caspase-3 acts as a tumour suppressor in colorectal carcinoma because it is associated with favourable clinical and morphological characteristics.

The lack of correlation between cytoplasmic and nuclear expression of Caspase-3 in relation to risk stratification in BCC and histological variant of SCC may, in our opinion, be tissue-dependent.

4.2.4.4. Relationship between Caspase-3 expression and the degree of differentiation of SCC

We found a statistically significant difference in cytoplasmic (p=0.01) and nuclear (p=0.0001) expression of Caspase-3 in relation to the degree of differentiation of SCC (Tables 23 and 24). In low- and moderately differentiated carcinomas taken together, the expression of the anti- e antibody is higher than in carcinomas with G1 differentiation.

Table 23. *Correlation between cytoplasmic Caspase-3 expression and tumour differentiation grade in patients with SCC.*

Grade	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Highly differentiated	29	13.21	21.33	**0**
Moderately differentiated	14	18.43	33.86	
Low differentiation	1	101.00		

Table 24. *Correlation between Caspase-3 nuclear expression and tumour differentiation grade in patients with SCC.*

Grade	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Highly differentiated	29	4.10	13.69	**0.0001**
Moderately differentiated	14	5.86	19.73	
Low differentiated	1	84.00		

Our results show an inverse relationship between cytoplasmic and nuclear expression of Caspase-3 and SCC differentiation. As tumour differentiation decreases, Caspase-3 expression increases. Our results differ from the data obtained for colorectal carcinoma regarding nuclear expression (Ahmed S et al. 2022). The authors found that there is a reduction in the nuclear expression of Caspase-3 in low-differentiated tumours compared to highly differentiated ones. In their study, cytoplasmic expression of Caspase-3 was not found.

In Hodgkin's lymphoma, classic variant, Caspase-3 expression is pronounced, but absent in lymphocyte-dominant disease (Izban KF, 1998). Analysis of the tissue expression of Caspase-3 in prostate carcinoma has shown that cytoplasmic expression is reduced in low-grade adenocarcinoma compared to high-grade carcinoma and normal prostate gland (Winter RN, 2001).

4.2.4.5. Relationship between cytoplasmic expression of Caspase-3 and area of necrosis in patients with BCC and SCC

Tables 25 and 26 show the mean values of cytoplasmic and nuclear expression of Caspase-3 in relation to the area of necrosis in patients with BCC. No statistically significant correlation was found between cytoplasmic and nuclear Caspase-3 expression and tumour necrosis area in BCC (p=0.43 for cytoplasmic and p=0.46 for nuclear expression).

Table 25. *Relationship between cytoplasmic expression of Caspase-3 and tumour necrosis area in patients with BCC.*

Area of necrosis	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Absent	40	22.73	63.74	**0.43**
Below 10%	5	00	0	

Table 26. *Correlation between Caspase-3 nuclear expression and tumour necrosis area in patients with BCC.*

Area of necrosis	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Absent	40	20.10	59.89	**0.46**
Below 10	5	0	0	

The mean values of cytoplasmic and nuclear expression of Caspase-3 in relation to the area of tumour necrosis in patients with SCC are shown in Tables 27 and 28. When analysing the cytoplasmic and nuclear expression of Caspase-3 in relation to the area of tumour necrosis in SCC, no statistically significant correlation was found (p=0.58 for cytoplasmic and p=0.54 for nuclear expression).

Table 27. *Relationship between cytoplasmic expression of Caspase-3 and tumour necrosis area in patients with SCC.*

Area of necrosis	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Absent	33	17.91	30.09	**0.58**
Below 10%	8	8.63	11.61	
10-30	3	27.33	46.48	

Table 28. *Correlation between nuclear expression of Caspase-3 and tumour necrosis area in patients with SCC.*

Area of necrosis	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Absent	3	8.39	22.32	**0.54**
Below 10	8	1	2.83	
10-30	3	0	0	

In malignant skin tumours, areas of coagulation necrosis may occur as a result of inadequate vascularisation and metabolic stress (hypoxia). Necrosis in the tumour is often associated with more aggressive behaviour and may be an unfavourable prognostic factor for overall survival in patients with colorectal (Stefanova N, 2018) and renal cell carcinoma (Yanulova N, 2024). We found that in the majority of cases of BCC and SCC, necrosis is absent. No statistically significant correlation between cytoplasmic and nuclear expression of Caspase-3 in relation to tumour necrosis in the two carcinomas was found. With the exception of the three cases of SCC with higher cytoplasmic expression of Caspase-3 in areas of necrosis between 10% and 30%, higher antibody expression is observed when necrosis is absent. This may mean that necrosis in tumour tissue in BCC and SCC develops in a different way from apoptosis.

4.2.4.6. Relationship between Caspase-3 expression and tumour-infiltrating lymphocytes in BCC and SCC

Tables 29 and 30 show the mean values of cytoplasmic and nuclear expression of the apoptotic marker in relation to TILs in BCC. The expression of Caspase-3 in the cytoplasm and nuclei of tumour cells does not show a dependence on TILs (p=0.55 for cytoplasmic and p=0.58 for nuclear expression).

Table 29. *Dependence between cytoplasmic expression of Caspase-3 and TILs in tumour tissue in patients with BCC.*

TILs	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Absent	18	22.28	70.82	**0.55**
Slightly pronounced	15	7.40	19.82	
Moderately pronounced	12	33	77.13	

Table 30. *Correlation between nuclear expression of Caspase-3 and TILs in tumour tissue in patients with BCC.*

TILs	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Absent	18	20.17	68.87	**0.58**
Slightly pronounced	15	6.2	17.93	
Moderately pronounced	12	29	69.35	

With regard to the mean values of cytoplasmic expression of Caspase-3 in tumour tissue and TILs in SCC, no statistically significant correlation was found (p=0.06) (Table 31). There was also no correlation with regard to nuclear expression (p=0.91) (Table 32).

Table 31. *Correlation between cytoplasmic expression of Caspase-3 and TILs in tumour tissue in patients with SCC.*

TILs	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Absent	14	13.79	26.93	**0**
Slightly pronounced	22	11.05	17.68	
Moderately pronounced	8	38.25	45.6	

Table 32. *Correlation between nuclear expression of Caspase-3 and TILs in tumour tissue in patients with SCC.*

TILs	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Absent	14	6.0	22.45	**0.91**
Slightly pronounced	22	5.77	15	
Moderately pronounced	8	9.25	26	

Although there is no significant correlation between cytoplasmic and nuclear expression of Caspase-3 by TILs in the two carcinomas, it is noteworthy that the values of the apoptotic factor are higher in moderately expressed TILs.

In colorectal carcinoma, patients with highly expressed TILs have the highest overall survival compared to patients without TILs (Stefanova N, 2018). A significant difference was found in overall survival between patients with a strong antitumour inflammatory response and those with a mild response, in whom overall survival was lowest. In renal cell carcinoma, no correlation was found between cytoplasmic and nuclear expression of apoptosis-inducing factor (AIF) – another marker for apoptosis – and TILs (Yanulova N, 2024).

In our opinion, the different data may be due to different criteria for determining TILs, different antibodies, and may also be tissue-dependent.

4.2.4.7. Relationship between cytoplasmic and nuclear expression of Caspase-3 and localisation of BCC and SCC

The mean values of cytoplasmic and nuclear expression of Caspase-3 in relation to the localisation of BCC and SCC are shown in Tables 33 and 34. In the present study, we did not find a statistically significant difference between protein expression and tumor localization in BCC for both cytoplasmic ($p=0.90$) and nuclear expression ($p=0.91$).

Table 33. *Correlation between cytoplasmic expression of Caspase-3 and BCC localisation.*

Localisation	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Chapter	36	22.47	66.47	**0.90**
Trunkus	4	25.00	35.36	
Limbs	2	0	0	
Genitals	3	0	0	

Table 34. *Correlation between Caspase-3 nuclear expression and BCC localization.*

Localisation	Number of cases, N	Mean values of Caspase-3 nuclear expression	Standard deviation (SD)	P-value
Head	36	19.94	62.49	**0.91**
Trunkus	4	21.50	32.66	
Limbs	2	0	0	
Genitals	3	0	0	

In SCC, no correlation was found between the expression of the apoptosis antibody and the localisation of the tumour process (p=0.13 for cytoplasmic expression and p=0.81 for nuclear expression). The data are presented in Tables 35 and 36.

Table 35. *Correlation between cytoplasmic expression of Caspase-3 and SCC localisation.*

Localisation	Number of cases, N	Mean values of cytoplasmic expression of Caspase-3	Standard deviation (SD)	P-value
Chapter	31	11.71	22.51	**0.13**
Neck	2	57.00	33.94	
Trunkus	2	4.50	6.36	
Limbs	8	30.25	43.02	
Genitals	1	14		

Table 36. *Correlation between Caspase-3 nuclear expression and SCC localization*

Localisation	Number of cases, N	Mean values of nuclear expression of Caspase-3	Standard deviation (SD)	P-value
Chapter	31	5.65	19.13	**0.81**
Neck	2	0	0	
Trunkus	2	0	0	
Limbs	8	13.75	26.33	
Genitals	1	0		

Data from the literature show that in esophageal SCC, Caspase-3 expression, determined immunohistochemically and by Western blot, is found in 60% of cases and correlates with a favorable prognosis in patients who have undergone resection (Hsia JY et al., 2003). Similar results were obtained when analysing Caspase-3 expression in neuroblastoma tumour tissue, where high expression was associated with younger age at the time of diagnosis, lower tumour stage and better prognosis (Nakagawara A et al., 1997). According to other authors, high Caspase-3 expression is associated with a higher risk of recurrence, but no association with the prognosis of patients with colorectal and hepatocellular carcinoma (Jonges LE et al., 2001, Persad R et al., 2004). Caspase-3 positivity has been found in non-small cell lung cancer, and this positivity is an independent prognostic factor for survival in these patients. Caspase-3-positive patients have a higher median survival than patients who are negative for the protein (Yoo J et al., 2004)**.** These data are consistent with the findings of Koomagi R, Volm M (2000), who found that there is expression in 72% of non-small cell lung carcinomas in patients with a favourable clinical prognosis.

The data obtained by Takata T et al. (2001) differ

significantly from those of Koomagi R, Volm M (2000) and Yoo J et al. (2004). Retrospective studies of resected non-small cell lung carcinomas in pathological stage I have shown that the 5-year survival rate of patients with strong Caspase-3 expression is significantly lower than that of patients with weak expression (Takata T et al. 2001). The authors also studied Caspase-3 in relation to apoptosis, because it is known to play a key role in apoptotic cell death. They found that caspase-3 expression did not correlate with the frequency of apoptotic cells determined by TUNEL, with tumour proliferative activity examined with the proliferative cell nuclear antigen PCNA, or with p53 status in non-small cell lung carcinoma. Takata T et al. (2001) concluded that increased expression of uncleaved caspase-3, i.e. inactive caspase-3, is associated with an unfavourable prognosis in patients with resected non-small cell lung carcinoma.

The results of Takata T et al. (2001) regarding non-small cell lung carcinoma do not differ from those of Huang JS et al. (2017) regarding squamous cell carcinoma of the buccal mucosa. High immunohistochemical expression of Caspase-3 is associated with advanced pathological stage, larger tumour size and poor survival in patients with resected squamous cell carcinoma of the oral cavity and postoperative radiotherapy. In addition to uncleaved Caspase-3, the authors also studied cleaved Caspase-3 and found that low expression of both: Caspase-3 and cleaved Caspase-3 is associated with better overall survival, early pathological stage and absence of lymph node metastasis compared to high expression. Thus, T et al. (2001) conclude that cleaved Caspase-3 and/or Caspase-3 may be prognostic biomarkers in oral mucosa SCC.

Comparative analysis of the immunohistochemical expression of Caspase-3 in tissues of prostate carcinoma and prostate hyperplasia shows lower expression in carcinoma (Gu J

et al. 2015). Low expression is found in advanced stages of the disease, low tumour differentiation (Gleason score), but the expression of Caspase-3 in prostate carcinoma tissues does not show dependence on PSA (prostate-specific antigen) values and distant metastases. Gu J et al. (2015) sought a relationship between the pro-apoptotic signalling pathway, assessed by Caspase-3, and the apoptosis inhibitors: livin and survivin. The study data indicate that the two inhibitory proteins are negatively correlated with Caspase-3 expression. According to the authors, the three proteins: Caspase-3, livin and survivin are closely related to the onset and development of prostate cancer.

In the present study, no analysis was performed between the T stage of the tumour and the expression of Caspase-3 because, with the exception of two patients who were in stage II, the rest were in stage I. No comparative analysis of Caspase-3 expression versus lymphovascular invasion and lymphatic metastasis was performed because no tumour emboli were found in the vessels and no data on the lymphatic status of the patients studied were available.

The research by Zhang Z. et al. (2015) focuses on the causes of recurrence after radiotherapy for colorectal cancer. After irradiation, colorectal tumour cells undergo apoptosis and necrosis, synthesise cleaved Caspase-3 and High-mobility group box 1 (HMGB1) protein, and when cultured with even a small number of live tumour cells, they stimulate their proliferation. The authors conclude that both apoptotic and necrotic cells can stimulate the proliferation of cells that have survived radiotherapy.

Devarajan E et al. (2002) found that about ¾ of mammary gland tumours and adjacent non-tumour tissues do not contain Caspase-3 transcripts and do not express the Caspase-3 protein. Despite the absence of Caspase-3, the levels of Caspase-8 and

Caspase-9 remain unchanged. According to the authors, the loss of Caspase-3 expression contributes to the survival of neoplastic cells in breast cancer.

4.3.1. Cytoplasmic and nuclear expression of RIPK3 in BCC tumour tissue and adjacent non-tumour tissue

Cytoplasmic and nuclear expression of RIPK3 was examined in the tumour tissue of all 91 patients. The mean value of cytoplasmic expression determined by H-score in BCC was 121.72±98.66, with a minimum value of 0 and a maximum value of 300 (Figure 31A, 31B, 32A). The mean value of nuclear expression of RIPK3 in BCC was 117.56±109.12, with a minimum value of 0 and a maximum value of 300 (Figure 32B). The mean value of cytoplasmic expression of RIPK3 in non-tumour tissue in BCC is 68.35±40.28 (Figure 32A). The nuclear expression of RIPK3 in non-tumour tissue has a mean value of 101.04±77.09 (Figure 33B).

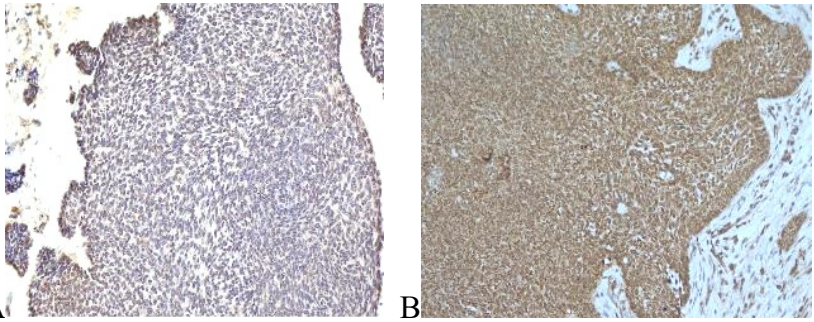


Figure 31. *A.-Low to absent cytoplasmic expression of RIPK3 in BCC. IHC x200.B. Intense cytoplasmic expression of RIPK3 in BCC. IHC x200.*

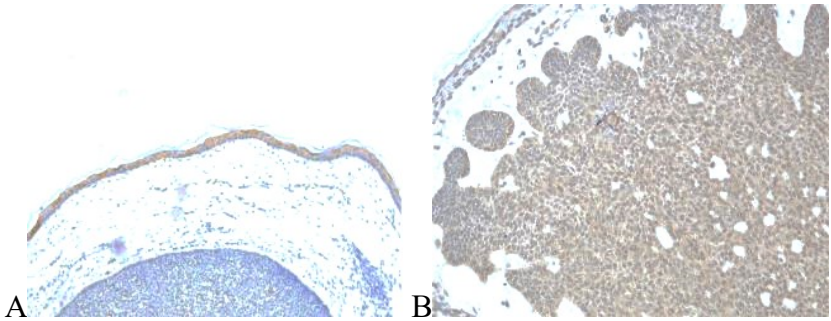


Figure 32. *A. Absent cytoplasmic expression of RIPK3 in tumour tissue and weakly expressed in non-tumour tissue in BCC. IHC x100. B. Nuclear expression in tumour and non-tumour tissue in BCC. IHC x200.*

The mean value of cytoplasmic expression in tumour tissue in the present study hardly differs from the mean value of cytoplasmic expression of RIPK3 in breast carcinoma, also determined by H-score (Stoeva M, 2022). With regard to the average nuclear value in breast carcinoma, the values are significantly higher in compared to the present study (Stoeva M, 2022). The cytoplasmic expression of RIPK3 in renal carcinoma is slightly higher than that in BCC, but the nuclear expression is four times lower (Yanulova N, 2024). Using human in vitro colorectal carcinoma lines, Feng X et al. (2015) found that overexpression of RIPK3 is associated with inhibition of tumour proliferation, migration and invasion.

4.3.2. Cytoplasmic and nuclear expression of RIPK3 in tumour tissue in SCC and in adjacent non-tumour tissue

The mean value of cytoplasmic expression, determined by H-score in SCC, is 88.93±74.23, with a minimum value of 0 and a maximum value of 222. The mean value of nuclear expression

of RIPK3 is 119.00±99.01, with a minimum value of 0 and a maximum value of 300. The mean value of RIPK3 expression in the nuclei of non-tumour cells is 45.95±79.13, while cytoplasmic expression in non-tumour tissue is 34.23±42.42.

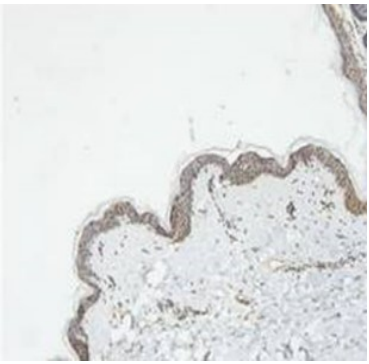
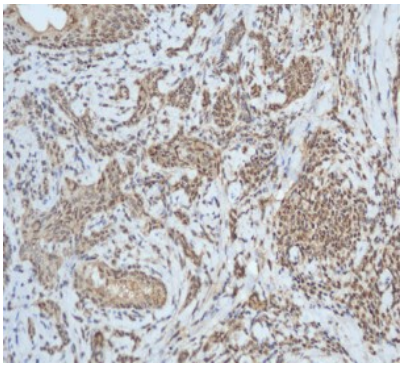
A  B 

Figure 33. *A. Weak cytoplasmic expression of RIPK3 in adjacent non-tumour tissue in SCC. IHC x 100. B. Moderate to strong nuclear expression of RIPK3 in SCC tumour tissue. IHC x100.*

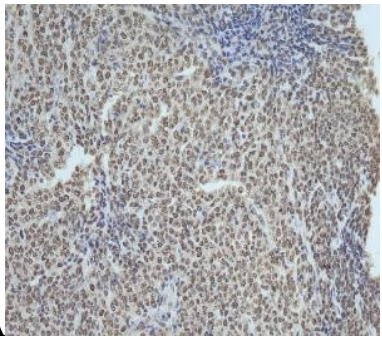
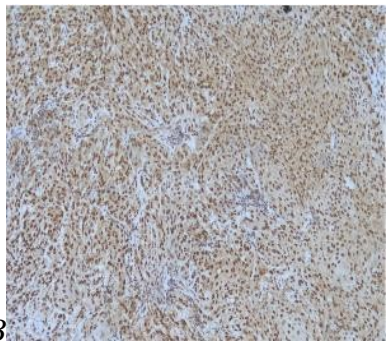
A  *B* 

Figure 34. *A. Pronounced nuclear and B. pronounced nuclear and weakly pronounced cytoplasmic expression in tumor cells of RIPK3 in SCC. IHC x200.*

In CRC (Stefanova N, 2018), the analysis of cytoplasmic expression of RIPK3 in adjacent non-tumour tissue, based on average values, is almost four times higher compared to the current results. Stoeva M (2022) determines the cytoplasmic expression of RIPK3 in 19 cases of proliferative and non-proliferative fibrocystic disease. The average value in the proliferative type fibrocystic disease group is more than five times higher than in the non-tumour tissue of the SCC. Five times higher values are reported by Yanulova N (2024) in the cytoplasm of cells adjacent to renal cell carcinoma (Yanulova N, 2024).

Kalchev K (2020) found that RIPK3 expression in prostate carcinoma tumour cells is higher than in glands without PIN (prostate intraepithelial neoplasia). The expression of the antibody is highest in tumour glands and decreases in dysplastic and benign glands.

Taken together, our results and those from the literature show that RIPK3 expression is different in both tumour and non-tumour tissue.

4.3.3. Comparative analysis between the mean values of *RIPK3* expression in tumour tissue and in adjacent non-tumour tissue in patients with BCC and SCC.

Table 37 shows the mean values of cytoplasmic RIPK3 expression in tumour and adjacent non-tumour tissue. The mean value of cytoplasmic expression of RIPK3 in BCC tumour tissue is higher compared to non-tumour tissue, and the difference is statistically significant (p=0.0007) in BCC. No such difference was found in nuclear expression (p=0.31) (Table 38).

Table 37. *Correlation between cytoplasmic expression of RIPK3 in tumour and non-tumour tissue in patients with BCC.*

Group	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
In tumour cells	46	121.72	98.66	**0.0007**
In non-tumour cells	46	68.35	40.28	

Table 38. *Correlation between nuclear expression of RIPK3 in tumour and non-tumour tissue in patients with BCC.*

Group	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
In tumour cells	46	117.57	109.12	**0.31**
In non-tumour cells	46	101.04	77.09	

Tables 39 and 40 present the mean values of cytoplasmic and nuclear expression of RIPK3 in tumour tissue in patients with SCC and in adjacent non-tumour tissue. The mean values of cytoplasmic and nuclear expression of RIPK3 in tumour tissue are higher than in non-tumour tissue, and this difference is significant (for cytoplasmic p=0.00001 and nuclear p=0.00006).

Table 39. *Cytoplasmic expression of RIPK3 in tumour tissue in patients with SCC and in adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
In tumour cells	45	88.93	74.23	**0**
In non-tumour cells	45	34.23	42.42	

Table 40. *Correlation between nuclear expression of RIPK in tumour tissue in patients with SCC and in adjacent non-tumour tissue.*

Group	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
In tumour cells	45	119.00	99.01	**0.00006**
In non-tumour cells	45	45.95	79.13	

We found that cytoplasmic and nuclear expression of RIPK3 in the tumour tissue of SCC is higher compared to non-tumour tissue, while in BCC this applies only to cytoplasmic positivity.

Yanulova M (2024) also reports a statistically significant difference between cytoplasmic expression in tumour and non-tumour tissue in renal cell carcinoma, but in tumour tissue, RIPK3 expression is lower than in adjacent non-tumour tissue. Our results also differ from those of Feng X et al. (2015), who evaluated the immunohistochemical cytoplasmic expression of RIPK3 in colorectal carcinoma and compared it with expression in preserved tissue using a three-point scale to assess intensity. They found higher cytoplasmic expression levels of RIPK3 in normal tissue compared to tumour tissue. Moriwaki et al. (2015) also found reduced tissue expression of RIPK3 in CRC compared to adjacent non-tumour tissue, while a similar relationship was not found by Stefanova N (2018), who also studied CRC. Stoeva M (2022) studied the cytoplasmic expression of RIPK3 in breast cancer tumour tissue and in a control group with fibrocystic disease and found that expression in non-tumour tissue was higher compared to tumour tissue.

4.3.4. Comparative analysis of RIPK3 expression in BCC and SCC depending on clinical and morphological characteristics

4.3.4.1. Relationship between cytoplasmic expression of RIPK3 and patient age.

Tables 41 and 42 show the mean values of cytoplasmic and nuclear expression of RIPK3 in tumour tissue in BCC in relation to patient age. Patients were grouped into two age groups: up to 60 years inclusive and over 60 years. No statistically significant dependence was found between cytoplasmic expression of RIPK3 and the age of patients with BCC (p=0.44). A similar correlation was also absent in nuclear expression (p=0.48).

Table 41. *Mean values of cytoplasmic expression of RIPK3 in tumour tissue of patients with BCC depending on age.*

Age	Number of cases, N	Average values of cytoplasmic expression of RIPK3 in tumour cells	Standard deviation (SD)	P-value
31-60	27	135.16	93.64	**0.44**
61-90	19	112.26	102.72	

Table 42. *Average values of nuclear expression of RIPK3 in tumour tissue of patients with BCC depending on age.*

Age	Number of cases, N	Average values of nuclear expression of RIPK3 in tumour cells	Standard deviation (SD)	P-value
31-60	27	131.26	104.86	**0.48**
61-90	19	107.93	112.91	

The mean values of cytoplasmic expression of RIPK3 in the tumour tissue of SCC in relation to the age of the patients are

shown in Table 43. There is a statistical difference between the cytoplasmic expression of RIPK-3 in the tumour tissue of SCC and the age of the patients, but it is minimal (p=0.048).

In SCC, no statistically significant correlation was found between nuclear expression and age (p=0.89).

Table 43. *Mean values of cytoplasmic expression of RIPK3 in the tumour tissue of patients with SCC in relation to age.*

Age	Number of cases, N	Mean values of cytoplasmic expression of RIPK3 in tumour cells	Standard deviation (SD)	P-value
31-60	25	78.70	72.83	**0.048**
61-90	20	118.76	68.19	

Table 44. *Average values of nuclear expression of RIPK3 in the tumour tissue of SCC patients in relation to age.*

Age	Number of cases, N	Average values of nuclear expression of RIPK3 in tumour cells	Standard deviation (SD)	P-value
31-60	25	123.84	104.86	**0.89**
61-90	20	119.90	96.33	

The figure shows the dependence of cytoplasmic expression of RIPK3 in the tumour tissue of patients with SCC in relation to age. With increasing age, cytoplasmic expression of RIPK3 in the tumour tissue of patients with SCC increases.

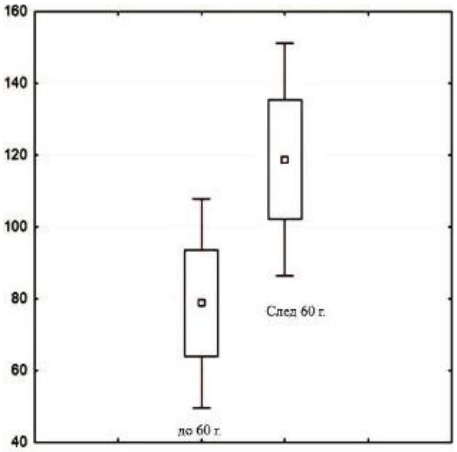


Figure 35 *Graphical representation of the mean values of cytoplasmic expression of RIPK3 ( ) in the tumour tissue of patients with SCC in relation to age.*

Our results show that with increasing age of patients with skin SCC, cytoplasmic expression of RIPK3 in tumour tissue increases. The data obtained do not differ from those for renal cell carcinoma (Yanulova N, 2024). The expression of RIPK3 in tumour tissue in renal cell carcinoma increases with the advancing age of patients. In CRC, Feng X et al. (2015) found no correlation between the cytoplasmic expression of RIPK3 in tumour tissue and the age of patients. In their study, patients were divided into two groups: < 65 years and > 65 years. In breast cancer, no correlation was found between the two indicators: tissue expression of RIPK3 and patient age (Stoeva M, 2022). In lung carcinoma, Chung J. et al. (2019) also found no correlation between RIPK3 expression in the cytoplasm of tumour cells and patient age.

With regard to BCC, there is no correlation between cytoplasmic expression of RIPK3 and patient age.

Taken together, the present results and data from the literature

suggest that cytoplasmic expression of RIPK3 in tumour tissue may depend not only on the organ location of the tumour, but also on its histological variant.

We did not find a correlation between the nuclear expression of RIPK3 in the two carcinomas: BCC and SCC in relation to the age of the patients. A similar lack of correlation has been found in renal cell carcinoma (Yanulova N, 2024). N. Yanulova divided the patients into three groups: the first group up to 44 years of age, the second between 45 and 64 years of age, and the third over 65 years of age. In colorectal carcinoma, the nuclear expression of RIPK3 also does not show a correlation with age. N. Stefanova N (2018) divides the nuclear expression of RIPK3 in tumour cells into two categories: low and high according to the cut-off value, and patients are divided into two groups: over and under 65 years of age.

4.3.4.2 Relationship between cytoplasmic expression of RIPK3 and patient gender

No significant difference was found between nuclear and cytoplasmic expression of RIPK3 in BCC tumour tissue in relation to gender (p=0.35 for cytoplasmic and p=0.42 for nuclear expression). Tables 45 and 46 show the mean values of cytoplasmic and nuclear expression of RIPK3 in BCC tumour tissue.

Table 45. *Correlation between cytoplasmic expression of RIPK3 and gender in patients with BCC.*

Gender	Number of cases, N	Average values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Men	20	137.50	92.57	**0.35**
Women	26	109.58	103.23	

Table 46. *Correlation between nuclear expression of RIPK3 and gender in patients with BCC.*

Gender	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Men	20	132.6	103.08	**0.42**
Women	26	106.03	114.18	

The mean values of cytoplasmic and nuclear expression of RIPK3 in the tumour tissue of SCC are given in Tables 47 and 48. The sex of patients with SCC does not show a correlation with the cytoplasmic and nuclear expression of RIPK3 (p=0.38 for cytoplasmic expression and p=0.43 for nuclear expression).

Table 47. *Correlation between cytoplasmic expression of RIPK3 and gender of patients with SCC.*

Gender	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Men	25	88.08	73.38	**0.38**
Women	20	99.75	73.75	

Table 48. *Correlation between RIPK3 nuclear expression and gender in patients with SCC.*

Gender	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Men	25	105.76	98.04	**0.43**
Women	20	132.1	98.55	

We found that the two localisations of RIPK3: cytoplasmic and nuclear in both skin carcinomas: BCC and SCC do not show

gender dependence. A similar lack of dependence between cytoplasmic and nuclear expression of RIPK3 on gender has been established in renal cell carcinoma (Yanulova N, 2024). Cytoplasmic expression of RIPK3 in tumour tissue does not show gender dependence in patients with CRC (Feng X. et al., 2015; Stefanova N, 2018). Similar results have been reported by Chung J et al. (2019) in patients with lung adenocarcinoma, in whom both cytoplasmic and nuclear expression of RIPK3 was analysed in tumour tissue using a four-point scale (0, 1+, 2+, 3+). The lack of dependence between nuclear expression of RIPK3 and the sex of patients with BCC and SCC does not differ from the data for renal cell carcinoma and breast carcinoma, in which no dependence was also found ( ) (Stoeva M, 2022, Yanulova N, 2024).

4.3.4.3. Relationship between RIPK3 expression and the risk group for BCC.

Table 49 shows the mean values of cytoplasmic expression of RIPK3 in the different histological variants of BCC, grouped according to risk. There is a statistical difference between the cytoplasmic expression of RIPK3 and low- and high-risk BCC, but it is minimal (p=0.048). The same was found for nuclear expression (p=0.047). In high-risk BCC, cytoplasmic and nuclear expression of RIPK-3 are higher compared to the low-risk group.

Table 49. *Correlation between cytoplasmic expression of RIPK3 and BCC risk group.*

Group	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Low risk	41	112.59	93.74	**0.048**
High risk	5	196.60	117.39	

Table 50. *Correlation between nuclear expression of RIPK3 and BCC risk group.*

Group	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Low risk	41	108.22	105.17	**0.047**
High risk	5	204.20	128.80	

4.3.4.4. Correlation between RIPK3 expression and histological variant of SCC

The mean values of cytoplasmic expression of RIPK3 in relation to the histological variant of SCC are shown in Table 51. There is no statistically significant dependence between cytoplasmic expression of RIPK3 and the histological variant of SCC (p=0.10). Table 52 presents the data on nuclear expression of RIPK3 in SCC in relation to the histological variant. No statistically significant correlation was found (p=0.25).

Table 51. *Correlation between cytoplasmic expression of RIPK3 and histological variant of SCC.*

Group	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Conventional SCC	32	74.38	73.81	**0.10**
Verucose SCC	5	128.20	50.64	
Keratoacanthoma	7	127.43	74.06	

Table 52. *Correlation between nuclear RIPK3 expression and histological variant of SCC.*

Group	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Conventional SCC	32	103.59	102.24	**0.25**
Verucose SCC	5	157.6	76.28	
Keratoacanthoma	7	161.86	87.87	

The results for BCC show that the cytoplasmic and nuclear expression of RIPK3 in the BCC risk group show a statistical difference, but it is minimal. In SCC, there is no correlation between cytoplasmic and nuclear expression in relation to the histological variant of SCC. Cytoplasmic expression of RIPK3 in lobular breast cancer is higher compared to ductal breast cancer, but the statistical significance is minimal (Stoeva M, 2022). Nuclear expression of RIPK3 does not differ between ductal and lobular carcinoma. In renal cell carcinoma, cytoplasmic and nuclear expression of RIPK3 do not show dependence on the histological variant: papillary, chromophobe, and clear cell (Yanulova N, 2024).

4.3.4.5. Dependence between RIPK3 expression and degree of differentiation of SCC

Table 53 presents the mean values of cytoplasmic expression of RIPK3 in relation to the degree of differentiation of SCC. There is no statistically significant difference in cytoplasmic expression (p=0.48). No statistically significant difference was found for nuclear expression (p=0.30) (Table 54).

Table 53. *Correlation between cytoplasmic expression of RIPK3 and the degree of differentiation of SCC.*

Grade	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Highly differentiated	29	92.69	68.88	**0.48**
Moderately differentiated	14	87.50	85.80	
Low differentiated	1	0		

Table 54. *Correlation between nuclear expression of RIPK3 and degree of differentiation of SCC.*

Grade	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Highly differentiated	34	108.47	100.34	**0.30**
Moderately differentiated	12	147.50	136.20	

Our results do not show a statistically significant correlation between cytoplasmic and nuclear expression of RIPK3 depending on the degree of differentiation of SCC. Our results are consistent with data on colorectal carcinoma in , which also show no significant correlation between cytoplasmic and nuclear expression of RIPK3 and the degree of differentiation (Feng et al., 2015, Stefanova N, 2018). Unlike us, in renal cell carcinoma, a correlation has been established between cytoplasmic expression of RIPK3 and the degree of differentiation. Tumours with G2 differentiation have higher RIPK3 expression than those with G4 (Yanulova N, 2024). Similar data have been obtained for breast carcinoma. High

cytoplasmic expression is found in better differentiated tumours (Stoeva M, 2022).

No correlation between nuclear expression of RIPK3 and the degree of differentiation was found in breast and renal cell carcinoma (Stoeva M, 2022, Yanulova N, 2024).

4.3.4.6. Dependence between RIPK3 expression and the area of necrosis in BCC and SCC.

Table 55 shows the mean values of cytoplasmic expression of RIPK3 and the area of necrosis in BCC. There is a statistically significant correlation between cytoplasmic expression of RIPK3 and the presence of necrosis (p=0.011). Cytoplasmic expression of RIPK3 in BCC tumour tissue is higher when necrosis is present compared to the group where it is absent.

Table 55. *Correlation between cytoplasmic expression of RIPK3 and tumour necrosis area in patients with BCC.*

Area of necrosis	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
No necrosis	41	109.1	93.95	**0.01**
Necrosis up to 10% of the area	5	225.2	78.8	

The mean values of RIPK3 nuclear expression in relation to the area of necrosis in patients with BCC are presented in Table 56. A significant difference was found between RIPK3 nuclear expression and the presence of necrosis (p=0.006). In the presence of necrosis, RIPK3 expression was significantly higher than in the group without necrosis.

Table 56. *Correlation between nuclear expression of RIPK3 and*

tumour necrosis area in patients with BCC.

Necrosis area	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
No necrosis	41	103.41	105.32	**0.006**
Necrosis up to 10% of the area	5	243.6	68.1	

The cytoplasmic and nuclear expression of RIPK3 in the tumour tissue of SCC are shown in Tables 57 and 58. There is no statistically significant difference between cytoplasmic expression and the area of necrosis in tumour tissue in patients with SCC (p=0.06). With regard to nuclear expression in SCC, the values between the two indicators are borderline (0.0498).

Table 57*. Correlation between cytoplasmic expression of RIPK3 and tumour necrosis area in patients with SCC.*

Area of necrosis	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
No necrosis	33	76.00	73.99	**0.06**
Necrosis up to 10% of the area	8	111.38	59.5	
Necrosis from 10% to 30% of the area	3	171.33	59.37	

Table 58. *Correlation between nuclear expression of RIPK3 and tumour necrosis area in patients with SCC.*

Area of necrosis	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
0	33	99	99.18	**0.0498**
1	8	166.50	80.20	
2	3	212.33	46.72	

We found that cytoplasmic and nuclear expression of RIPK3 in tumour tissue in BCC is higher when necrosis is present compared to the group in which it is absent. Our results for BCC differ from those for renal cell carcinoma, in which no significant difference was found between RIPK3 expression in relation to necrosis for the two antibody localisations (Yanulova N, 2024). Unlike BCC, in SCC there is no significant correlation between cytoplasmic RIPK3 expression and tumour necrosis, while in nuclear expression the values are borderline.

4.3.4.7. Relationship between RIPK3 expression and TILs in BCC and SCC

Table 59 shows the mean values of cytoplasmic expression of RIPK3 in BCC in relation to TILs. We found that there is a statistically significant difference between cytoplasmic expression of RIPK3 and the varying degrees of TILs expression (p=0.036). The intensity of the necrotic marker is higher in cases of more pronounced tumour infiltration by TILs.

A similar relationship was found for nuclear expression (p=0.025) (Figure 60).

Table 59. *Correlation between cytoplasmic expression of RIPK3 and TILs expression in patients with BCC.*

TILs	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Absent	18	114.00	94.13	**0.036**
Slightly pronounced	16	86.31	97.95	
Moderately pronounced	12	180.50	85.77	

Table 60. *Correlation between nuclear expression of RIPK3 and degree of TILs expression in patients with BCC.*

TILs	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Absent	18	109.89	102.30	**0.025**
Slightly pronounced	16	76.81	105.40	
Moderately pronounced	12	187.58	103.96	

The mean values of cytoplasmic and nuclear expression of RIPK3 in SCC are shown in Tables 61 and 62. We found that there was a significant difference between cytoplasmic and nuclear expression of RIPK3 in SCC and the degree of TILs expression (p=0.00007 for cytoplasmic and p=0.006 for nuclear expression). High RIPK3 expression is associated with more pronounced TIL infiltration.

Table 61. *Correlation between cytoplasmic expression of RIPK3 and degree of TILs expression in patients with SCC.*

TILs	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Absent	14	53.29	57.74	**0.00007**
Slightly pronounced	22	78.05	68.03	
Moderately pronounced	8	181.25	33.42	

Table 62. *Correlation between nuclear expression of RIPK3 and degree of TILs expression in patients with SCC.*

TILs	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Absent	14	77.50	80.93	**0.006**
Slightly pronounced	22	112.09	104.06	
Moderately pronounced	8	210.63	48.81	

Our results show that in BCC and SCC, cytoplasmic and nuclear expression of RIPK3 is directly correlated with TILs. High antibody expression is associated with higher TIL intensity in tumour tissue. Our data differ in part from those for colorectal carcinoma (Stefanova N, 2018). N. Stefanova found an inverse relationship between TILs and cytoplasmic expression of RIPK3 in tumour tissue; with high antibody expression, the number of TILs is low, while high TIL intensity is usually associated with a negative reaction to RIPK3. In the analysis of nuclear expression, she found a positive correlation, with high RIPK3 expression correlating with a strong immune response.

In renal cell carcinoma, no correlation was found between cytoplasmic and nuclear expression of RIPK3 and TILs (Yanulova N, 2024).

The role of TILs in tumour tissue is the subject of intense debate. It is known that apoptosis does not lead to an inflammatory response, unlike necroptosis. There is evidence that TILs are increased in the tumour tissue of human hepatocellular carcinoma, and this is associated with increased expression of genes related to necroptosis, such as *RIPK1, RIPK3, MLKL (*Nicolè L et al. 2022). According to other studies, there is a positive correlation between necrotic proteins: RIPK3,

MLKL, pMLKL and CD163+ M2 macrophages, while an inverse relationship with CD8+ T lymphocytes has been found in non-small cell lung carcinoma (Duangthim N et al. 2024). The authors believe that the tumour microenvironment in non-small cell lung cancer has a suppressive effect and that key necroptotic proteins, especially RIPK3, may be associated with therapeutic resistance (Duangthim N et al. 2024).

Mediators and inflammatory cells are important components of the local tumour microenvironment (Mantovani A et al., 2008). In some malignant processes, the inflammatory response precedes the appearance of the tumour, while in other tumours it induces an inflammatory microenvironment. According to Mantovani A (2008), inflammation in the tumour environment has a stimulating effect on the malignant process because it promotes angiogenesis, cell proliferation and survival, and stimulates metastasis.

Our results support the notion of the immunogenic properties of necroptosis because the necroptosis marker RIPK3 is elevated in both skin carcinomas when TIL intensity is highest.

Data obtained from immunohistochemical analysis of RIPK3 in CRC tumour tissue show that high cytoplasmic expression of the antibody inhibits the immune response, while RIPK3 localised in the nucleus activates innate immunity (Stefanova N, 2018). In our opinion, in skin carcinoma, regardless of the histological variant, high cytoplasmic and nuclear expression of RIPK3 stimulates TILs.

4.3.4.8. Relationship between RIPK3 expression and BCC and SCC localization

Tables 63 and 64 show the mean values of cytoplasmic and nuclear expression of RIPK3 in BCC tumour tissue in relation to its localisation. There is no statistically significant difference

between RIPK3 expression in relation to localisation (p=0.66 for cytoplasmic and p=0.66 for nuclear expression).

Table 63. *Correlation between cytoplasmic expression of RIPK3 and BCC localization.*

Localisation	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Chapter	37	127.46	98.55	**0.66**
Trunkus	4	132.00	125.64	
Limbs	2	49	69.30	
Penis/Vulva	3	85.67	100.38	

Table. 64. *Correlation between nuclear expression of RIPK3 and BCC localization.*

Localisation	Number of cases, N	Mean values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Chapter	37	124.35	110.53	**0.66**
Trunkus	4	128.00	122.63	
Limbs	2	47.50	67.18	
Penis/Vulva	3	66.67	115.47	

The mean values of cytoplasmic and nuclear expression of RIPK3 in SCC tumour tissue in relation to tumour localisation are shown in Tables 65 and 66. A significant correlation was found between cytoplasmic expression of RIPK3 and SCC localization (p=0.02). The highest expression was found in tumours located in the neck and extremities, and the lowest in the head. The localisation of SCC in the genital area was not included in the analysis of mean values because there was only one patient.

Table 65. *Correlation between cytoplasmic expression of RIPK3 and SCC localization.*

Localisation	Number of cases, N	Mean values of cytoplasmic expression of RIPK3	Standard deviation (SD)	P-value
Chapter	31	72.26	69.71	**0.02**
Neck	2	198.50	33.23	
Trunkus	2	76.50	10.61	
Limbs	8	140.38	63.12	
Penis/Vulva	1	-		

Table 66 presents the mean values of RIPK3 nuclear expression depending on the location of the SCC. No statistically significant difference was found between the two indicators (p=0.08).

Table 66. *Correlation between nuclear expression of RIPK3 and SCC localization.*

Localisation	Number of cases, N	Average values of nuclear expression of RIPK3	Standard deviation (SD)	P-value
Chapter	31	91.06	89.83	**0.08**
Neck	2	236.50	13.44	
Trunkus	2	168.50	47.38	
Limbs	8	200.38	87.15	
Penis/Vulva	1	-		

Our results show that cytoplasmic expression of RIPK3 in SCC in tumour tissue depends on the localisation of SCC. With regard to BCC, there is no significant dependence of cytoplasmic and nuclear expression of RIPK3 on tumour localisation. No statistical dependence was found between the

nuclear expression of RIPK3 in the tumour tissue of SCC and its localisation.

Our results for the cytoplasmic expression of SCC correspond to the data obtained by N. Stefanova (2018), who found a significant difference between RIPK3 expression and CRC localisation. When the carcinoma is located in the right half of the colon, there is low expression of the antibody, while in left-sided localisation, expression is high. With regard to the nuclear expression of RIPK3 in the two histological variants of skin carcinoma, similar to us, she did not find a significant correlation between the two localisations. No correlation between nuclear expression of RIPK3 in tumour tissue and localisation has been established in renal cell carcinoma (Yanulova N, 2024).

The lack of correlation between cytoplasmic expression and the location of BCC does not differ from the data obtained for colorectal and renal carcinoma (Feng X. et al. 2015; Yanulova N, 2024).

RIPK3 is found in the nucleus and cytoplasm of both types of skin carcinomas: BCC and SCC. Studies show that after activation of necroptosis, RIPK3 translocates from the nucleus to the cytoplasm of cells, and this export is important for necroptotic cell death (Weber K et al. 2018).

V. CONCLUSION

In conclusion, the study provides new information on the expression and localization of RIPK3 and Casp-3 in BCC and SCC, highlighting the possibility of using these markers as auxiliary diagnostic and prognostic tools in the future. Further study with larger cohorts and integration of genomic analysis data is needed to more accurately define their role in the pathogenesis of non-melanoma skin carcinomas and their potential in personalized oncology.

VI. CONCLUSIONS

1. No statistically significant difference was found for the cytoplasmic and nuclear intensity of Caspase-3 in tumour and non-tumour tissue of BCC.
2. Caspase-3 cytoplasmic expression in SCC tumour cells is higher compared to non-tumour tissue, unlike nuclear expression, where there is no statistically significant difference.
3. Cytoplasmic and nuclear expression of Caspase-3 in BCC and SCC tumour tissue does not correlate with patient age.
4. In BCC and SCC, cytoplasmic and nuclear expression of the apoptotic protein does not show dependence on gender, tumour necrosis area, TILs in tumour tissue, or tumour location.
5. The expression of Caspase-3 in the cytoplasm and nuclei of BCC and SCC does not show dependence on the risk stratification of BCC and the histological variants of SCC.
6. Moderately and poorly differentiated carcinomas (G2+G3) have higher levels of cytoplasmic and nuclear Caspase-3 compared to highly differentiated SCCs.
7. Cytoplasmic expression of RIPK3 in the tumour tissue of BCC and SCC is higher compared to the adjacent non-tumour tissue.
8. Nuclear expression of RIPK3 in the tumour tissue of SCC is higher than in non-tumour tissue. A similar relationship is not observed in BCC.
9. With increasing age, the cytoplasmic expression of RIPK-3 in the tumour tissue of patients with SCC increases.

10. There is no correlation between cytoplasmic and nuclear expression of RIPK3 in the tumour tissue of BCC and SCC and the gender of patients.
11. In BCC, cytoplasmic and nuclear expression of RIPK3 is higher in high-risk compared to low-risk histological variants. In SCC, there is no correlation between RIPK3 expression and histological variant for both antibody localisations.
12. The expression of RIPK3 in the cell cytoplasm and nucleus does not show a dependence on the degree of differentiation of SCC.
13. Cytoplasmic and nuclear expression of RIPK3 in BCC is higher in tumours with necrosis compared to those without.
14. The intensity of the necrotic marker is high in the cytoplasm and nucleus of BCC and SCC with marked tumour infiltration by TILs.
15. The expression of RIPK3 in the cell cytoplasm and nucleus does not show dependence on the location of BCC.
16. Cytoplasmic expression of RIPK3 is highest in SCCs located in the neck and extremities and lowest in the head.

VII. CONTRIBUTIONS OF THE DISSERTATION

7.1. Original scientific contributions

- A comprehensive clinical-morphological and immunohistochemical analysis of Caspase-3, a marker for apoptosis in patients with BCC and SCC, was performed.
- The expression of RIPK3, a marker for necroptosis, was analysed in relation to the clinical and morphological characteristics of patients with BCC and SCC.

7.2. Scientific contributions of a practical nature

- It has been proven that BCC and SCC differ in the cytoplasmic expression of Caspase-3 in tumour tissue compared to non-tumour tissue.
- It has been established that the cytoplasmic and nuclear expression of Caspase-3 in the tumour tissue of BCC and SCC does not depend on the following clinical and morphological indicators: age, gender, tumour necrosis area, BCC risk stratification, histological variant of SCC, TILs, and tumour location.
- It has been proven that with a decrease in the differentiation of SCC, the cytoplasmic and nuclear

expression of the apoptosis marker Caspase-3 increases.

- The preserved nuclear and cytoplasmic expression of Caspase-3 in tumour tissue in BCC, as well as the increased cytoplasmic and unchanged nuclear expression of the antibody in SCC, suggest an active apoptotic form of cell death that can be induced after chemotherapy and radiotherapy.
- It has been established that cytoplasmic expression of RIPK3 in the tumour tissue of BCC and SCC is higher compared to the adjacent non-tumour tissue.
- Cytoplasmic and nuclear expression of RIPK3 in high-risk groups of BCC has been assessed.
- It has been proven that cytoplasmic and nuclear expression of RIPK3 in BCC is higher in tumours with necrosis and pronounced tumour infiltration by TILs.
- The increased cytoplasmic expression of RIPK3 in BCC and SCC, as well as the increased nuclear expression of the antibody in SCC and unchanged in BCC, support the presence of an active necrotic cell death pathway that can be induced by applied therapy.

VIII. PUBLICATIONS RELATED TO THE DISSERTATION

Full-text publications

1. **Vasilev P**, Bakardzhiev I. Expression of apoptosis and necroptosis markers caspase-3 and receptor-interacting protein kinase-3 in malignant neoplasms. Varna Medical Forum, 2023; Vol 12 (1):122-133

2. **Vasilev P.** Updating the classification of cutaneous squamous cell carcinoma. Varna Medical Forum, 2023; Vol 12 (2):25-38

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