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Prognostic factors in melanoma

Witold Kycler[1ABCDEF], Sylwia Grodecka-Gazdecka[2BD], Jan Bręborowicz[2CD], Violetta Filas[2C], Marek Teresiak[1ACDE]

[1] Department of Oncological Surgery II, Great Poland Cancer Centre, Poznań, Poland
[2] Oncology Department, Karol Marcinkowski University of Medical Sciences, Poznań, Poland

Summary

Background There are clinical and pathological factors associated with the clinical course of melanoma. These parameters allow us to predict survival times and establish a course of treatment.

Aim The aim of the study was to assess the significance of immunohistochemical markers in the progression of melanoma, and relate these findings to the influence of clinical and histological factors on survival time.

Materials/Methods In this study, archival histological material obtained from 50 melanoma patients operated on in the Great Poland Cancer Centre between 1990–1995 was analysed. Using immunohistochemistry we detected the presence of markers known to be important in the diagnosis of melanoma, including: HMB-45, PCNA, Ki-67, MMP-2, CD44 and nm23. Following this, univariate logistic regression was performed and clinical, histopathological and immunohistochemical data of statistical significance were correlated with survival time using the Cox nonparametric proportional hazard regression model.

Results The results suggested that the most important prognostic factors correlating with survival time were: the presence of nm23 antigen (p=0.0342) and the thickness of the melanoma, as measured by the Breslow method in mm (p=0.0191). According to the univariate analysis there were correlations between patient death or survival and the histological type, Superficial Spreading Melanoma Malignum (SSMM) (p=0.0187), regional lymphatic metastases (p=0.0030) and positive Ki-67 results (p=0.0282).

Conclusions Taken together, our results allowed us to conclude (a) that there was a correlation between survival time and nm23 antigen expression and the thickness of melanoma, as measured by the Breslow method in mm, (b) that there were correlations between patient survival or death and the histological type of Superficial Spreading Melanoma Malignum, regional lymphatic metastases and positive Ki-67, (c) that other parameters (age, gender, anatomical location of the melanoma, the presence of ulceration, lymphatic infiltration, the existence of satellites, and positive PCNA and MMP-2) showed no significant influence on survival.

Key words **malignant melanoma • prognostic factors • immunohistochemistry**

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Author's address: Witold Kycler, Department of Oncological Surgery II, Great Poland Cancer Centre, Garbary 15 Str., 61-866 Poznań, Poland, e-mail: kycler@interia.pl

Background

There are clinical and pathological factors associated with the clinical course of melanoma. These parameters allow us to predict survival times and establish a course of treatment. Clinical parameters include the following: age, gender, and anatomical localisation of the tumour [1]. Typical histological prognostic factors are: the thickness of melanoma, measured by the Breslow method in mm [2], the status of regional lymph nodes [3], the level of invasiveness measured according to Clark [2] and the histological type of the melanoma [4–8]. Some authors have also reported a correlation between ulceration and vessel invasion with tumour invasion level [6]. Also, Clemente examined the involvement of lymphocyte infiltration [9]. Many authors regarded lesion thickness and the existence of lymphatic metastases to be an independent prognostic factor [3,4,6]. The correlation between other factors is still a matter of some debate and therefore the search for new factors and research in previously unexplored areas is necessary to improve the results of treatment and to gain an improved understanding of melanoma. In this work, despite the factors listed below, we examined a group of markers introduced to diagnostic of melanoma in recent years. Generally these factors fell into two groups, the first represented metastasis-associated factors (MMP-2, CD44 and nm23), the second represented proliferation markers – Ki-67 (MIB1) and PCNA.

The ability of tumour cells to adhere is a crucial step in the metastatic process, altering the clinical prognosis of some human tumours such as melanoma. The prognostic value of proteolytic enzymes in melanoma have been studied and MMP-2 belongs to this group [10]. Such enzymes allow tumour cells, via changes to their adhesive properties, to migrate and form remote metastases. Differences in MMP-2 expression between primary and metastatic melanomas have been demonstrated [10,11]. Increased expression of MMP-2 has been shown to correlate with an invasive phenotype. Moreover, significant prognostic parameters which correlate with the expression of this protein include: the presence of cathepsin B and D, gender, lesion location, tumour thickness according to Breslow, the level of invasion according to Clark and presence of ulceration [12–14]. CD44, a member of the hyaluronan binding-proteins family, has been implicated as a contributor in tumour progression and metastasis [15,16]. It has been demonstrated, that the expression of this glycoprotein on the surface of melanoma cells increases its migration ability, and in consequence, increases its metastatic potential [17–19]. It has been suggested that the nm23 gene represents a class of metastatic associated genes [20–22]. The expression of nm23 was found to be inversely related to local recurrence, the level of invasion and tumour thickness [23], but its prognostic role in the case of melanomas of the skin is highly controversial owing to differing results obtained from gene and protein expression [23,24]. Nevertheless, a potential correlation has been found between nm23 expression and the survival of melanoma patients [25,26]. The presence of proliferation markers may suggest an influence on tumour invasion potential [26]. Karbowniczek et al. [27] described a correlation between MIB-1 (Ki-67) expression, the area and shape of melanoma cell nuclei, the thickness of the lesion according to Breslow, the level according to Clark and the metastatic potential. Other authors have emphasized the importance of Ki-67, its influence on tumour invasion level, and its correlation with survival probability [28]. Other markers applied in melanoma diagnosis, such as HMB-45, do not seem to have any prognostic value.

During the evolution of prognosis in cases of melanoma, several markers have been used but none of them was powerful alone. Certain prognostic factors, however, correlate highly with the biological behaviour of this type of cancer.

Aim

The aim of our study was to assess the significance of immunohistochemical markers in melanoma progression compared with the influence of clinical and histological factors on survival time.

Materials and Methods

Clinical, histopathological and immunohistochemical data are presented in Tables 1–3. The analysed data represents the cases of 50 patients operated for melanoma in the Second Oncological Surgery Ward of the Great Poland Cancer Centre, Poznań. The average age of the examined patients was 53.4 years (range 27–83 years, standard deviation 14.01). The group included 34 women (68%) and 16 men (32%). Clinical data such as: age, gender, the occurrence of tumour spread, anatomical location of the tumour and survival time were assessed. Reassessment of archived haematoxylin & eosin

Table 1. Clinical characteristics of patients.

Characteristic		No	Percentage
Patient number		50	100%
Mean age, range		53.5	27–83 years
Gender	male	16	32%
	female	34	68%
Five year survival		24	48%
Dissemination		23	46%
Site of metastases	lymph node	8	16%
	skin	21	42%
	internal organs	16	32%
Lesion location	trunk, shoulder girdle, neck or head	25	50%
	limbs	25	50%
Extent of first operation			
Lymphadenectomy carried out along with tumour excision		21	42%
Tumour excision only		29	58%

Table 2. Histological characteristics of tumour data.

Characteristic		No	Percentage
Histological type	SSMM	42	84%
	NMM	8	16%
Thickness according to Breslow	0–0.75	9	18%
	0.76–1.5	10	20%
	1.51–4.0	10	20%
	>4.0	21	42%
Level of invasion according to Clark	I	–	–
	II	6	12%
	III	24	48%
	IV	14	28%
	V	6	12%
Metastases to lymph nodes		21	42%
Lymphocyte infiltration		21	42%
Regression symptoms		18	36%
Presence of ulceration		24	48%
Satellites		0	0%
Preexisting naevus		5	10%
Melanin pigment		45	90%

Table 3. Immunohistochemical data.

Immunohistochemical factor		No	Percentage
HMB45	positive	50	100%
PCNA	positive	38	76%
nm23	positive	30	60%
MMP-2	positive	23	46%
CD44	positive	34	68%
Ki-67	positive	35	70%

stained histopathological specimens was carried out and the findings were described in terms of: the thickness of melanoma infiltration in millimeters, as measured by the Breslow method, the level of invasion according to Clark, histological type, the presence of lymphocyte infiltration, regression features, the existence of a preexisting naevus, the presence of ulceration, the existence of satellites and lymphatic metastases. Lesions located on the trunk, head, neck, shoulder girdle, of original focus, were found in 25 patients (50%) and were located on the limbs of 25 patients (50%). Melanoma spread during the observation period was noted in 23 patients (46%). Metastases were most frequently found in the lymph nodes – 21 cases (42%), as well as in the internal organs – 16 cases (32%) and the skin – 8 cases (16%). With regards to the thickness of lesions, according to Breslow, in the range <1mm there were 9 patients (18%); from 1.01 to 2.0mm – 10 patients (20%); from 2.01 to 4.0mm – 10 patients (20%) and >4.0mm – 21 patients (42%). Level II invasion according to Clark was found in 6 patients (12%), level III in 24 cases (48%), level IV in 14 cases (28%) and level V in 6 patients (12%). In the examined group of patients, regional lymphatic metastases were found in 21 patients (42%) at the beginning of treatment. Superficial forms of SSMM type melanomas were seen in 42 patients (84%), and nodular types in 8 patients (16%). The existence of ulceration was found in 24 patients (48%). Regression symptoms were found in 18 patients (36%). Lymphocyte infiltration occurred in 21 patients (42%). The existence of a preexisting naevus was observed in 5 patients (10%). Amelanotic type melanomas were present in 10% of the examined group (5 patients). No satellite changes were found in the examined patients.

Immunohistochemical staining was performed-using the EnVision system (DAKO). Immunoreagents used were from the DAKO and CHEMICON Corporations. Tests for the expression of immunohistochemical markers were performed on tissue material derived from formalin-fixed, paraffin embedded specimens. Sections were cut at 4–5 µm, mounted on APES coated glass slides and incubated for 20 minutes at 58°C. Sections were de-parafinized overnight in xylene and subsequently rehydrated through graded alcohols. Sections were washed in Tris-buffered-saline for 15' and incubated in citrate buffer (pH=6,0; acid monohydrate 10 mmol/L adjusted with 2N sodium hydroxide) in a microwave for 30 minutes at 250 W (microwave treatment applied to CD44, MMP-2 and Ki67 tests). The slides were rinsed in TBS for 15 minutes. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 minutes. After rinsing in water for 10 minutes and in TBS for 15 minutes, sections were incubated with primary antibodies for 60 minutes (HMB45: 1/50 – DAKO – Code M 634, PCNA: 1/100 – DAKO – Code M 0879, Ki-67: 1/100 – DAKO – Code M 7240, CD44: 1/100 – DAKO – Code M 70 82 and MMP-2: 1/200 – CHEMICON – Code MAB 13405) or with polyclonal rabbit antibodies: (nm23: 1/100 – DAKO – Code A 0096). After rinsing in water for 15 minutes and rinsing in TBS, sections were incubated with EnVision+™/HRP (Mouse) complex (DAKO – Code: K 4001) or with EnVision™/HRP (Rabbit) complex (DAKO – Code: K 4003) for 30 minutes. The peroxidase reaction was visualized for all examined markers using 3,3-diaminobenzidine as a chromogen. The sections were counterstained in Mayer's haematoxylin, dehydrated through a series of graded alcohols, cleared in xylene and mounted under coverslips. As positive controls, confirmed melanoma tissue was used for HMB45 and Ki-67, breast carcinoma material for PCNA and nm23, vesicle cancer was used for MMP-2 and lymph node sections were used for CD44.

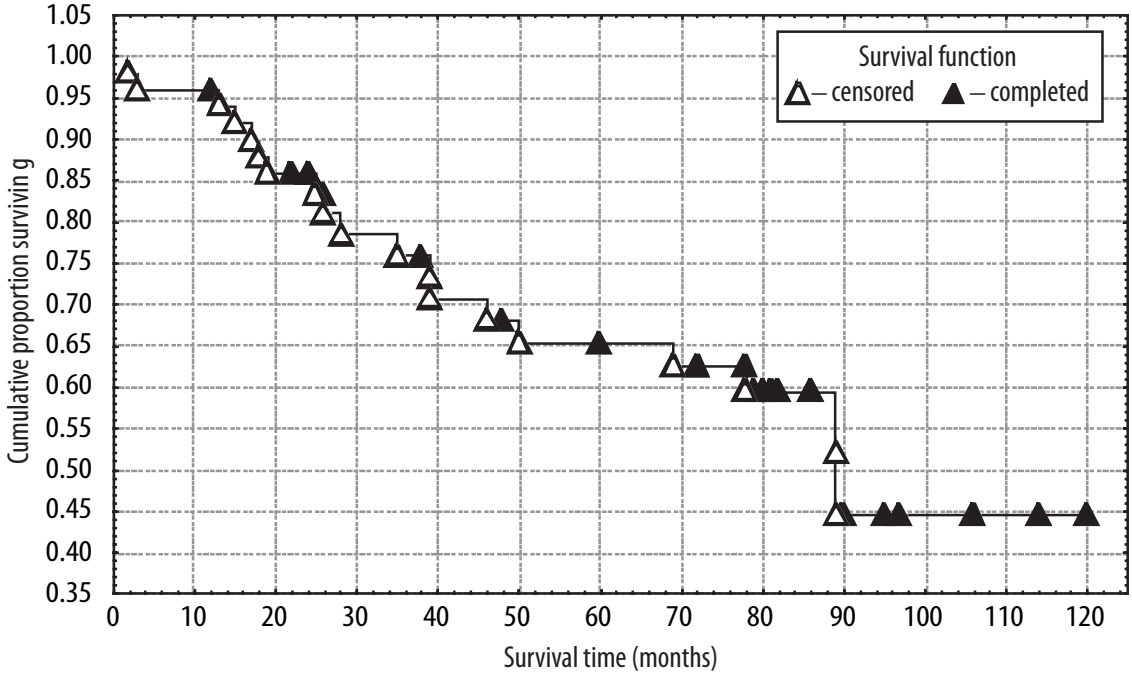

Figure 1. Graph of survival times vs. the cumulative proportion surviving, according to the Kaplan-Meier method.

Tumours were scored by assessing the site of staining (nucleus for Ki-67 and PCNA, membrane for CD44 and cytoplasm for HMB45, MMP-2 and nm23) and the proportion of positive cells. All markers were considered as negative (–), when no staining or very weak staining in single cancer cells (<10%) was found or as positive (+) when at least 50% of examined cell structures were at least weakly stained or when more than 10% of examined cells showed intensive staining.

Statistical analysis was performed using univariate logistic regression. The Shapiro-Wilks and Lilliefors test was used, followed by the t-Student and χ^2 square tests, with Yates' modifications, and Fisher's exact test. The Mann–Whitney U-test served as the nonparametric method. The statistical significance of analysed factors on patient survival or death was assessed. Survival probability was estimated by use of the Kaplan-Meier method. Subsequently, clinical, histopathological and immunohistochemical data of statistical significance were correlated with survival time. The statistical significance of prognostic factors on survival time was assessed by the Cox nonparametric proportional hazard regression model. A p value <0.05 was considered statistically significant for all procedures. The statistical analysis was performed using Statistica for Windows version 6.0.

Results

The average age in the examined group of patients was 53.4 years (age range 27-83 years, standard deviation 14.01). The group included 34 women (68%) and 16 men (32%). 24 patients (48%) survived for over 5 years.

The HMB-45 marker reacted positively in 50 cases (100%), PCNA in 38 cases (76%), Ki-67 expression was detected in 35 cases (70%), MMP-2 in 23 patients (46%), nm23 was positive in 30 patients (60%) and finally, CD44 was observed in 34 patients (68%).

The median survival time, according to the Kaplan-Meier analysis, was 89 months – lower quartile (25th percentile) 36.46 months. The curve of survival is presented in Figure 1.

The statistical significance of the examined prognostic factors was assessed using the Cox proportional hazards regression model of survival.

Due to the lack of satellite changes and 100% positive reaction to HMB-45 antigen, these indicators were excluded from further statistical analysis in the examined group of patients.

Table 4. The statistical significance of analysed factors, as assessed by univariate logistic regression.

Variable		0		1		P
Age		50.89±13.97		55.06±14.03		ns
Gender	K	n=14	73.7%	n=20	64.5%	ns
	M	n=5	26.3%	n=11	35.5%	
Limb lesion location		12	63.1%	13	41.9%	ns
SSMM		13	68.4%	29	93.5%	p=0.0187*
Breslow	<1.0 mm	1	5.2%	8	25.8%	p=0.0183*
	1.01–2 mm	3	15.8%	7	22.6%	
	2.01–4 mm	3	15.8%	7	22.6%	
	>4 mm	12	63.2%	9	29.0%	
Clark	I level					ns
	II level	1	5.3%	5	16.1%	
	III level	8	42.1%	16	51.6%	
	IV level	8	42.1%	6	19.4%	
	V level	2	10.5%	4	12.9%	
Regression factors		5	26.3%	13	41.9%	ns
Lymphocyte tumour infiltration		9	47.4%	12	38.7%	ns
Preexisting naevus		3	15.8%	2	6.4%	ns
Melanin		15	78.9%	30	96.8%	ns
Ulceration		13	68.4%	11	35.5%	ns
Regional lymph metastases		13	68.4%	8	25.8%	p=0.0037*
Elective lymphadenectomy		9	47.4%	12	38.7%	ns
HMB 45		19	100.0%	31	100.0%	ns
PCNA		15	78.9%	23	74.2%	ns
Nm23		16	84.2%	14	45.2%	p=0.0107*
MMP-2		8	42.1%	15	48.4%	ns
CD44		12	63.2%	22	70.9%	ns
Ki-67		19	61.3%	16	84.2%	p=0.0282*

* $p<0.05$; ns – not significant.

The following parameters were analysed: age, gender, lesion location, the extent of surgery (lymphadenectomy carried out along with tumour excision), the depth of invasion in mm according to Breslow, the level of invasion according to Clark, the existence of lymphatic metastases, histological type of SSMM, regional lymph metastases, ulceration, regression factors, the presence of a preexisting naevus, lymphocytic tumour infiltration, the presence of melanin, expression of PCNA, Ki-67, metalloproteinase 2 (MMP-2), CD44 glycoprotein and nm23 diphosphate nucleoside kinase. The statistical significance of analysed factors on patient survival or death was assessed. The results of this analysis are presented in Table 4. The statistical significance of prognostic factors on survival time was assessed by the Cox nonparametric proportional hazard regression model. These final results are presented in Table 5.

There were correlations between patient survival or death and the thickness of the melanoma, as measured by the Breslow method in mm (p=0.0188), histological type of Superficial Spreading Melanoma Malignum (SSMM)

Table 5. Statistically significant factors associated with survival time, as assessed by the Cox nonparametric proportional hazard regression model.

Statistical survival analysis	Depended variable: survival – (months) X^2=28.18 ; df=6 ; p=0.00009 (p<0.05)				
Prognostic factor	**Beta**	**Statistical error**	**Exponent beta (relative risk)**	**Wald statistic**	**p**
Lymph node metastases	0.82	0.56	2.27	2.14	ns
Breslow thickness	0.71	0.30	2.03	5.49	p=0.0190*
SSMM type	–1.56	0.92	0.21	2.86	ns
nm23	2.11	0.99	8.24	4.48	p=0.0342*
Ki-67	0.07	1.09	1.07	0.004	ns

* p<0.05; ns – not significant.

(p=0.0187), regional lymphatic metastases (p=0.0030), positive Ki-67 (p=0.0282) and nm23 antigen expression (p=0.0107).

The expression of nm23 diphosphate nucleoside kinase (p=0.0342) and the thickness of the melanoma, as measured by the Breslow method in mm (p=0.0191), also showed a statistical correlation with survival time.

Analyses carried out on the following selected parameters: age, gender, lesion location, whether or not lymphadenectomy was carried out along with tumour excision, positive reaction to PCNA, metalloproteinase 2 (MMP-2), the expression of CD44 glycoprotein, the presence of a preexisting naevus, the level of invasion according to Clark, the presence of ulceration, regression symptoms, lymphocyte infiltration and expression of melanin showed no statistically significant influence on patient survival.

Discussion

An important element in planning treatment is to determine which groups of patients are at increased risk of disease progression. Correlations between the investigated factors in this study allowed us to determine such a group of persons.

The prognostic value of molecular markers

A prognostic factor in the examined group turned out to be a positive reaction for the presence of nm23 diphosphate nucleoside kinase. Its importance has been emphasized in the literature by some authors, while others have not observed such a correlation, such that the prognostic value of nm23 expression remains uncertain. Betke et al. performed an assessment for the presence of nm23 protein in benign naevi, malignant melanomas and melanoma metastases to lymph nodes and internal organs [21] and found that reduced nm23 expression was related to a strong correlation with patient survival time and metastases (p=0.0003). Similar correlations for nm23 expression in melanoma were confirmed by Florenes et al. [22] and Lee et al. [20] while such authors as Van den Oord et al. [26], Saitoh et al. [23] and Holmes et al. [24] observed no correlation between nm23 expression in melanoma and survival time or metastasis formation. Many authors have studied nm23 expression in other human cancers. The correlation between reduced levels of nm23 protein and survival time has been described in ductal breast cancer with regional lymphatic metastases [29]. In colon cancer the correlation between reduced nm23 levels and the occurrence of metastases in the liver was shown, but not related to the condition of regional lymphatic nodes [30]. In our group of patients the detection of nm23 expression in melanoma cells correlated with a prolonged survival time.

Some authors have emphasized the importance of Ki67 in the differentiation of melanoma cells. Its proliferative effects and indirect influence on tumour invasion level, however, meant that they could not always confirm correlations with survival rate [27,28,31]. Karbowniczek et al. [27] described an increased median nuclear diameter with positive results for Ki67 and a correlation with the depth of invasion, in mm, according to Breslow and the level of metastatic potential according to Clark. Talve et al. [28] stressed the importance of proliferative parameters such as: Ki67 and mitotic index, but did not show an influence on survival

rate. Rudolph et al. [31] demonstrated the value of Ki67 in the diagnosis and differentiation of benign melanocytic changes and melanoma.

In our study Ki67 antigen was detected in the nuclear area of primary melanoma tumour cells, in 35 (70%) melanoma cases, and showed a statistical correlation with the probability of survival or death. Possibly, expression levels for Ki67 would be helpful in the determination of the aggressiveness and metastatic potential of melanomas. We demonstrated no correlation between Ki67 expression and patient survival time.

Immunohistochemical studies confirmed the diagnostic value of the HMB45 marker but showed that it has no prognostic value because of its nearly 100% specificity for melanoma. The influence of PCNA, CD44, MMP-2 on the probability of survival or death was not determined.

Analysis of histopathological factors

Most studies have indicated that the single best predictive attribute for clinical outcome for a given patient is the depth of tumour invasion (Breslow), and that this is the most powerful independent prognostic factor [2,32]. It has become the gold standard for stratifying patients according to the risk of metastatic disease. These studies showed that, as we expected, tumour thickness in our patients was an accurate indicator for the biological behaviour of melanoma. In the analysed material, the depth of invasion, in mm according to Breslow, showed statistically significant influence on patient survival, but the level of invasion according to Clark showed no influence on survival.

In the examined group of patients, we observed improved prognosis for patients with the SSMM histological type of melanoma. A similar correlation has also been recognized by other authors [1,3,6].

The existence of lymphatic metastases is one of the most predictive negative factors affecting the clinical course for patients with cutaneous melanoma [33]. In our material, univariate analyses disclosed that this factor had an independent negative impact on patients' risk of death. We could not confirm an influence on overall patient survival.

No other prognostic factors, such as the presence of ulceration, lymphatic infiltration, the existence of satellites or preexisting melanocytic naevi could be demonstrated by statistical correlation in our studies.

Analysis of clinical parameters

Our studies showed that 25% of patients died during a period of 36.46 months and that 50% lived longer than 89 months. None of the clinical parameters associated with survival time displayed significant influence on survival, in the studied group of patients.

The markers analysed in this article, have been previously shown to be useful prognostic factors, or potential candidates, for determining prognosis in the context of melanoma disease progression. Most of these markers correlated with clinical or histological factors. Clinical prognostic significance, however, was demonstrated mostly in deeply invasive or metastatic stages of tumour progression, meaning that the predictive value for the clinical outcome was valid only for that subgroup of patients. It was found that no single histological, immunohistochemical, serological or molecular marker can provide a precise predictive value for aggressive behavior in melanoma patients, regardless of clinical stage or tumour size. However, combinations of markers with histological parameters would help to identify patients at high risk of developing metastases and who would thus benefit from more aggressive treatment.

Conclusion

We concluded that: (a) the expression of nm23 diphosphate nucleoside kinase showed a statistical correlation with survival time, meaning improved prognosis for patients with skin melanoma, and (b) that a second significant prognostic factor in this analysis was the thickness of the melanoma, as measured by the Breslow method in mm, and (c) that, according to a univariate analysis, there were correlations between patient survival or death and the histological type of Superficial Spreading Melanoma Malignum, regional lymphatic metastases and positive Ki-67. (iv) Other parameters displayed no significant influence on survival time in the examined group.

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