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The Presence and Intensity of Melanocytic Stem Cells in Benign and Malign Melanocytic Lesions: Diagnostic and Prognostic Implications

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Melanocytic stem cell, nevi, melanoma, CD133, CD166, Nestin, Ki-67

ABSTRACT • Background and aims: In recent years, the classical treatments - surgery, radiation oncology and medical oncology applications - in oncologic cases failed to provide the expected efficacy. This unsatisfying result has led the scientists to new searches. These investigations have been mainly focused on the understanding of the pathophysiology of cancer facilitating research on stem cell therapy and new targeted therapy modalities. We evaluated the presence and density of melanocytic stem cells in acquired nevi, dysplastic nevi and congenital nevi, primary and metastatic melanoma cases using CD133, CD166 and Nestin, which are reported as stem cell markers in melanocytes during recent studies. Ki-67 proliferation index was also investigated. The directing effect of these stem cells on targeted therapy has been discussed. **Materials and Methods:** A total of 90 cases of acquired melanocytic nevi (dermal, compound, junctional nevus), 5 congenital nevi, 50 dysplastic nevi, 57 malign melanomas, 42 satellite nodules / cutaneous metastases and 16 lymph node metastases referred to the pathology department of our center were included in the study. HE sections of skin biopsies were re-evaluated by light microscopy in accordance with morphological criteria, and immunohistochemistry study by using CD133, CD166, Nestin and Ki-67 antibodies were done on biopsy specimens. **Results:** CD133, CD166 and Nestin positivity rates in primary melanoma cases were determined as 36%, 89.5% and 94.7% respectively. The positivity rates in metastatic melanoma (satellit node / cutaneous metastasis and lymph node metastases) were 46.5%, 96.5% and 96.5%, respectively. The rates were 3.5%, 28.5% and 69.2% for benign melanocytic lesions, respectively. A statistically significant difference was found between the benign and malignant melanocytic lesions in terms of Ki-67 proliferation index. ($p = 0.01$) CD133, CD166 and Nestin stem cell markers support stem cell theory in melanoma development. **Conclusion:** We believe that the presence of cancer stem cell markers may be valuable in developing targeted therapies and in follow up.

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INTRODUCTION

The incidence of melanoma has increased 3-5 times in all western countries over the last 30 years. Cutaneous melanoma accounts for 5.3% of all new cancer cases and 1.5% of cancer deaths (1). Melanoma development is known as a process that occurs in multiple steps after mutations of oncogenes and tumor suppressor genes of mature melanocytes in the epidermis. The majority of melanomas develop on the surface of melanocytic nevi, primarily the dysplastic nevi (2). In a recent meta-analysis, however, only 29.1% of melanomas have been shown to be associated with nevi (3). It is necessary to consider that, unlike the linear tumor progression model showing the development of melanomas from normal skin and nevi, melanomas may originate from melanocytic stem cells or progenitor cells (4).

According to the cancer stem cell hypothesis, malignant tumors can develop from two different routes: 1. cancer stem cells (CSC) and 2.more differentiated tumor cells, developed from these stem cells. According to this hypothesis, the main characteristic that differentiates CSC from other malignant cells is the ability to self-renew and differentiate. Cells with these two characteristics are also called “Cancer-initiating Cells”. Tumor-initiating cells were first demonstrated in acute myeloid leukemia, and then in malignant tumors of different solid organs and systems such as colon, breast, over, brain, bone (5-11).

The main markers of CSC, carried by melanocytes have also been described, mainly CD166, an active leucocyte adhesion molecule of the immunoglobulin family, CD133, also called human prominin-1, and Nestin, an intermediate filament (12). The exact physiological function of CD133 (human prominin-1 / AC 133) is not yet known, but has been detected in primary melanomas and human melanocytes (13). CD166 is found in the human melanoma cell group, and expression of this transmembrane protein on the mesenchymal stem cell surface has also been demonstrated. However, the expression of this protein was found to be related to the primary tumor thickness in the melanoma (14). Nestin has been found to be positive in both tumoral and endothelial cells in melanoma patients and recently it has been reported that the detection of this protein in the vessel wall and melanoma cells could be evaluated as a poor prognostic indicator in the course of the disease (15). Ki-67 is a core protein found in proliferating cells and malignancies and may be helpful in distinguishing melanoma and nevi. It has been reported that staining is usually observed in <5% in nevi and 13-30% in melanomas.

In this study, we aimed to find the presence and intensity of melanocytic cancer stem cells, -which are thought to be responsible for cancer initiation, relapses and metastases in recent years- by using the CSC markers CD133, CD166 and Nestin, in benign melanocytic lesions and also in primary or metastatic melanoma cases. And we also aimed to compare these findings with Kİ -67 proliferation index (16).

MATERIALS and METHODS

This study included 90 acquired nevi (43 compounds, 37 intradermal, 10 junctional), 5 congenital nevi, 50 dysplastic nevi, 57 melanomas (14 superficial spreading, 30 nodular, 7 acral lentiginous, 3 lentigo maligna, 1 blue nevus-derived melanoma, 2 unclassified), 16 lymph node metastases and 42 satellite nodule / cutaneous metastases.

Ventana Benchmark immunohistochemical automated staining procedure using CD133, CD166, Nestin polyclonal and Ki-67 monoclonal antibodies was performed. Ventana i-View DAB Detection kit from 4-µm sections of paraffin-embedded tissues of benign and malignant melanocytic lesions using appropriate positive controls was used. (Ventana Medical Systems, Inc., Tucson, Ariz.) The clone names, dilutions and commercial sources of the antibodies used are set forth in Table 1. All antibod-

ies were titrated by following the manufacturer's instructions and using positive and negative control tissues. Antibody titrations were determined for each antibody.

Immunohistochemically stained sections were evaluated by two pathologists. Klein et al. scoring system was used. It was interpreted as focal (<50%) and diffuse (≥50%) according to positive cell ratio. In addition, the staining grade was scored as weak, moderate or strong compared to the internal control (12).

MS-Excel 2003 and SPSS fo Win Ver.15.0 (SPSS INC., Chicago, IL., USA) package programs were used for all statistical analysis and calculations. In statistical decisions, $p \leq 0.05$ value was accepted as significant. The statistical analyses were performed at the Department of Biostatistics, Ankara University Faculty of Medicine.

The study was conducted in accordance with the Helsinki Declaration. Since our study was non-invasive, observational, and retrospective, no patient's consent was obtained.

RESULTS

Of the benign lesions 55.6% belonged to females, 44.4% belonged to males and of the malign lesions 40.2% belonged to females and 59.8% belonged to males. The youngest patient with a benign melanocytic lesion was 1 year old, the oldest patient was 68 years old and the mean age was 31.71 years. The youngest patient with a malignant melanocytic lesion was 16 years old, the oldest patient was 88 years old and the mean age was 63.22 years.

In immunohistochemistry examinations the cytoplasmic / membranous staining with CD133, CD166 and Nestin antibodies were accepted as positive. Endothelial cells, lymphocytes and mesenchymal cells for CD133; peripheral nerve sections, draining and sebaceous gland structures, lymphocytes and fibroblasts for CD166; ecrin and sebaceous gland structures, peripheral nerve sections, dermal mesenchymal cells and hair follicles were used for Nestin as internal positive controls. The results of the investigations of 145 benign melanocytic lesions and 115 malignant melanocytic lesions are shown in Table 2 and Table 3.

CD133 expression was observed in 4/37 melanocytic dermal nevi, 1/5 congenital nevi, 21/57 primary melanoma, 19/42 cutaneous metastatic melanomas and 8/16 metastatic lymph nodes (Figure 1A, B). Most of the positive stained nevi were focal and weakly stained. None of the benign melanocytic lesions showed moderate, strong or diffuse staining. Positive staining in primary melanoma, cutaneous metastatic melanoma, and metastatic lymph nodes was observed focally in a spectrum ranging from weak- moderate to strong (4/21, 19/19 and 7/16, respectively). Focal staining in primary melanomas, cutaneous metastatic melanomas, and metastatic lymph nodes was rare, irrespective of density (17/21, 0/19 and 1/16 respectively).

Table 1 Details of antibodies used for immunohistochemical staining

Antibody	Clone	Dilution	Target Protein Description	Cellular Location	Most Commonly Used as a Marker for	Manufacturer
CD133 (Prominin-1)	Polyclonal	1:150	Glycoprotein in cellular protrusions	Cell membrane / cytoplasmic	Neural, glial and adult stem cells	Abnova
CD166 (ALCAM)	Polyclonal	1:100	Transmembran protein	Cell membrane / cytoplasmic	Leukocytes, fibroblasts, epithelial and nerve cells.	Sigma
Nestin	Polyclonal	1:200	Intermediate filament protein	Cell membrane / cytoplasmic	Neural stem cells, other stem cells	Sigma
Ki-67	SP6	1:200	Necessary for cell proliferation	Nuclear	Proliferation index	Cellmarque

Table 2 CD133, CD166, Nestin and Ki-67 staining of 145 benign melanocytic lesions

	CD133 pos/total (%)	CD166 pos/total (%)	Nestin pos/total (%)	Ki-67 (%)
JN	-	-	1/10 (10)	0.10
MCN	-	24/43 (55,8)	37/43 (86,2)	0.16
MDN	4/37 (10.8)	12/37 (32.4)	32/37 (86.4)	0.08
CN	1/5 (20)	-	4/5 (80)	0.00
DN	-	5/50 (10)	23/50 (44)	0.48

JN: Junctional nevi, MCN: Melanocytic compound nevi, MDN: Melanocytic dermal nevi, CN: Congenital nevi, DN: Dysplastic nevi.

Table 3 CD133, CD166, Nestin and Ki-67 staining of 115 malign melanocytic lesions

	CD133 pos/total (%)	CD166 pos/total (%)	Nestin pos/total (%)	Ki-67 (%)
PM	21/57 (36.8)	51/57 (89.5)	54/57 (94.7)	27.95
CM/SN	19/42 (45.3)	40/42 (95.3)	40/42 (95,2)	29.83
LNM	8/16 (50.1)	16/16 (100)	16/16 (100)	34.56

PM: Primary Melanoma, CM: Cutaneous metastasis/Satellite nodules, LNM: Lymph node metastasis.

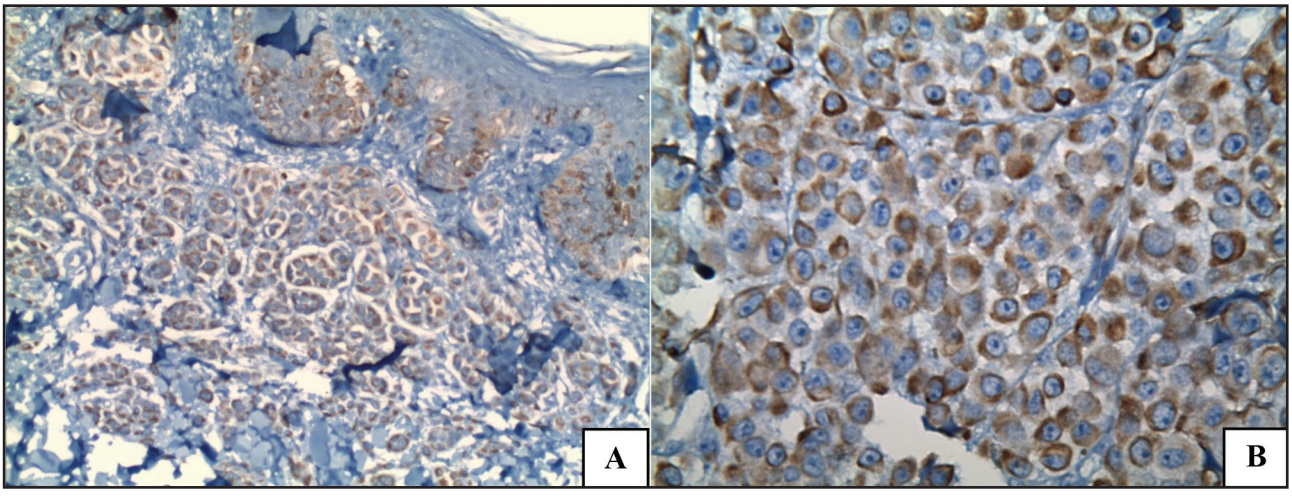

Figure 1 **A.** Benign melanocytic lesions showing focal staining for CD133. **B.** Malign melanocytic lesions showing strong and diffuse staining for CD133.

CD166 expression was observed in 24/43 melanocytic compound nevi, 12/37 melanocytic dermal nevi, 5/50 dysplastic nevi, 51/57 primary melanomas, 40/42 cutaneous metastatic melanomas and 16/16 metastatic lymph nodes (Figure 2A, B). In the majority of positive stained nevi, focal weakness and sometimes moderate diffuse staining were observed. No strong diffuse staining was observed in any of the benign melanocytic lesions. On the other hand, positive staining in primary melanoma, cutaneous metastatic melanoma, and metastatic lymph nodes was diffuse in a spectrum ranging from weak-to-strong (25/51, 16/40 and 6/16, respectively). Focal staining in cutane-

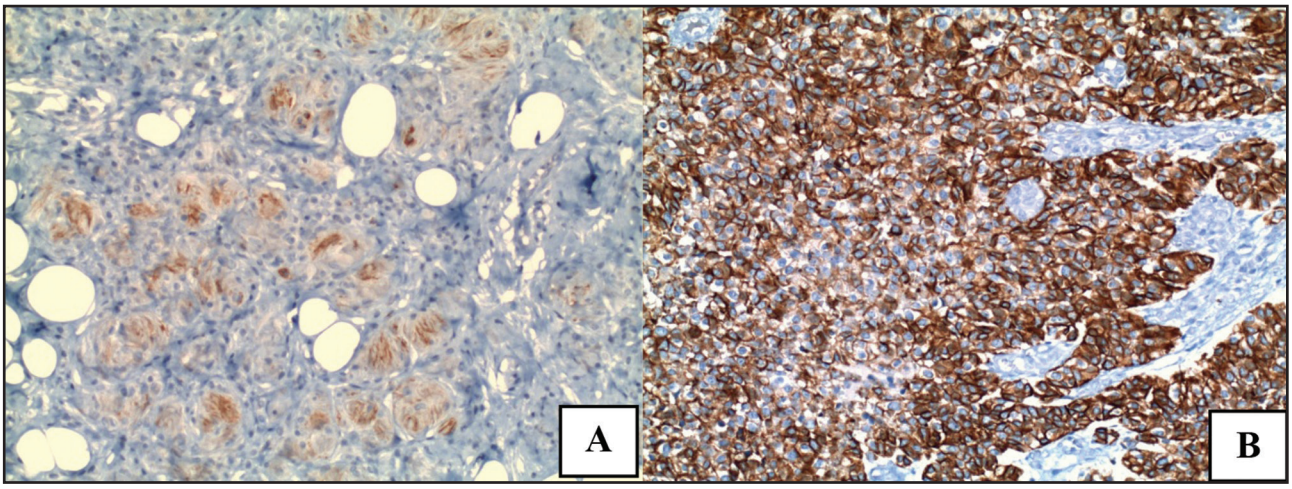

Figure 2 **A.** Benign melanocytic lesions showing focal staining for CD166. **B.** Malign melanocytic lesions showing strong and diffuse staining for CD166.

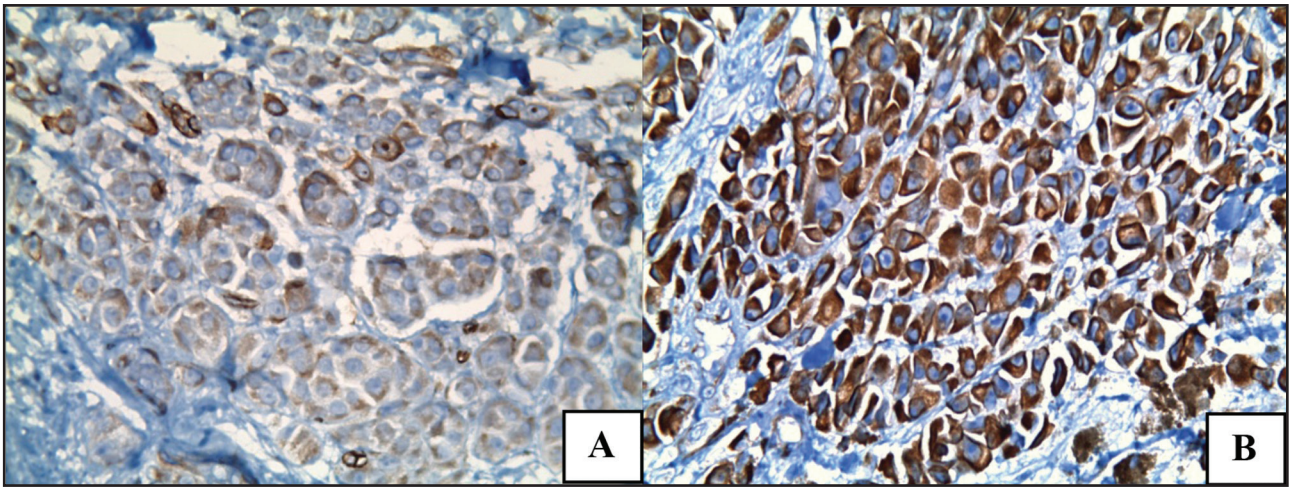

Figure 3 **A.** Benign melanocytic lesions showing focal staining for Nestin. **B.** Malign melanocytic lesions showing strong and diffuse staining for Nestin.

ous metastatic melanomas, in primary melanomas and metastatic metastatic lymph nodes was infrequent, irrespective of density (26/51, 24/40 and 10/16, respectively).

Nestin expression was detected in 1/10 junctional nevi, 37/43 melanocytic compound nevi, 32/37 melanocytic dermal nevi, 4/5 congenital nevi, 23/50 dysplastic nevi, 54/57 primary melanomas, 40/42 cutaneous metastatic melanomas and 16/16 metastatic lymph nodes (Figure 3A, B). Benign melanocytic lesions frequently had focal moderate and weak diffuse staining. No diffuse strong staining was observed in benign melanocytic lesions. Medium-strong and diffuse staining were frequently observed in primary melanomas, cutaneous metastatic melanomas, and metastatic lymph nodes (29/57, 29/42 and 11/16, respectively). Focal staining in primary melanoma, cutaneous metastatic lesions, and metastatic lymph nodes was infrequent, irrespective of its density (25/57, 11/42 and 5/16, respectively)

Staining with CD133, CD166, and Nestin was generally observed in nevic nests in the superficial papillary dermis and was less common in the junctional region. The rates of CD133, CD166 and Nestin staining in malign lesions were 14.79 (4.10-53.31), 15.74 (7.51-32.96) and 8.00 (3.55-17.99) times higher than in benign lesions, respectively, and was found to be statistically significant (P = 0.01).

Proliferation index was calculated by counting 1000 cells and finding the percentage of Ki-67 (MIB-1) specific nuclear staining cells. Ki-67 proliferation index was 0% (0-1%) in benign melanocytic lesions and 21% (1-96%) in malignant melanocytic lesions. The mean Ki-67 proliferation indices of malignant melanocytic lesions were significantly higher than benign melanocytic lesions. (p = 0.01) (Figure 4A, B). The mean Ki-67 proliferation indices of benign melanocytic lesions (acquired nevi, congenital nevi, dysplastic nevi) are found in Table 2. A statistically significant difference was found in terms of Ki-67 proliferation index when compared to other nevi. (P = 0.01) Ki-67 proliferation index was higher in the dysplastic nevi than in the other nevus groups.

DISCUSSION

Melanoma is the most common cutaneous fatal cancer in the world with an increasing incidence in the last 50 years. Although the incidence varies with the region, sex, ethnicity and anatomical location, it is more common in the elderly population (17). In our study, benign melanocytic lesions were seen more frequently in the 20-30 years of life, melanoma was more in the 60-70 age group, and benign melanocytic lesions were more common in women and malignant melanocytic lesions were more common in males, and the difference was statistically significant.

In a study with CD133, CD166 and Nestin stem cell markers, it was determined that stem cell markers increased in melanomas compared to benign melanocytic lesions (12). In this study, stem cell markers such as CD133, CD166 and Nestin were found increased in primary or metastatic melanomas compared to benign melanocytic lesions in accordance with the literature and support the stem cell theory in melanoma development.

In a study by Klein et al., The junctional region and nevi nests in superficial papillary dermis were found stained with CD166 and it was discussed by the authors that it may play an important role in melanoma progression and invasion (12). In our study, we also found that, staining in nevi nests of superficial papillary dermis is more spesific than junctional region and staining with Nestin is more

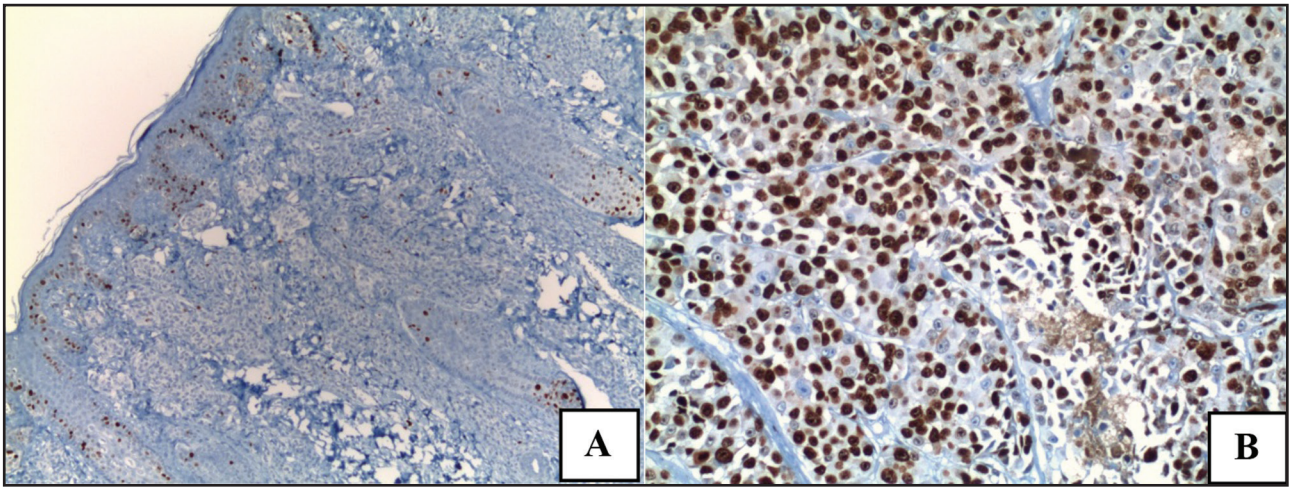

Figure 4 **A.** Benign melanocytic lesions showing for Ki-67. **B.** Malign melanocytic lesions showing for Ki-67

profound than CD133, CD166. Staining in the junctional region is limited. It supports the role of stem cells in the nevus-melanoma progression.

In a meta-analysis of 299 melanoma cases obtained from five studies, CD133 expression levels were determined. High CD133 expression was shown in 47.9% of melanoma cases. It has been concluded that the use of CD133 together with other CSC markers may be useful, since it is not a suitable marker for determining cancer stem cells alone (18). Similarly, in our study, staining was observed in 41.7% of melanoma cases with CD133. While more staining was observed compared to the nevi, those were focal and positivity was less compared to other stem cell markers.

Studies conducted on colorectal cancer suggest that high CD133 expression may be used as an independent prognostic factor in follow-up of this disease and may be associated with poor prognosis, more tumorigenic and / or metastatic capacity (19). As seen in our study, reaching 100% staining with these CSC markers, especially in metastatic lymph nodes, suggests that these markers may correlate with the metastatic capacity of the tumor. Diffuse and strong staining was observed especially in metastatic lymph nodes with CD166 and Nestin. These findings suggest that CD166 and Nestin stem cell markers may be valuable prognostic parameters in malignant melanoma together with classical pathologic parameters.

Nestin expression was detected in primitive neuroectodermal tumors of the central nervous system, glioblastoma, triple negative breast cancer and cutaneous metastatic melanoma. Evidence is growing between Nestin expression enhancement and pathogenesis in triple negative breast cancers, and it has also been reported that vessels around the tumor may be a sign of angiogenesis with Nestin positivity on the wall of newly formed capillaries (20). In this study, melanoma lesions were positive by Nestin in vascular structures, suggesting that it supports angiogenesis.

Ki-67 proliferation index can be used as a reliable marker in the discrimination of melanoma and nevi (16). In our study, it was found to be higher in melanoma and there was no significant difference between primary or metastasis cases, but a significant difference was observed between benign melanocytic lesions in themselves in dysplastic nevi. In primary and metastatic melanomas there is an increase in proliferation parallel to the presence of increased stem cells markers such as CD133, CD166 and Nestin.

In our study, we found more CD133, CD166 and Nestin stem cell markers in melanoma lesions. Most of the cases are under treatment, suggesting that current therapies do not affect the stem cells.

Recent efforts to determine CSC and to kill them with targeted treatment have intensified. It is thought that new and more CSC markers may be identified and used for diagnosis and treatment. Today's therapies provide temporary regression in primary or metastatic tumors, but they do not provide a desired increase in patient survival. One of the reasons for not achieving the targeted level of success is considered to be resistance to treatment in cancer cells or that current treatment does not effectively kill CSCs. This suggests that more effective treatments should be developed to kill CSC to combat cancer.

We think that studies on cancer stem cells may be the focus of novel therapeutic modalities which are related to targeted therapy and treatment because there are evidences that these stem cells may be responsible for cancer initiation, promotion, metastases, resistances and relapses.

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