



UNIVERSITY OF SIENA

**PhD PROGRAM IN BIOMEDICINE AND
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CYCLE XXIII**

**EVALUATION OF NUCLEOLIN'S
EXPRESSION IN CUTANEOUS
MELANOCYTIC LESIONS AS SPITZ
NEVI, SPITZOID MELANOMAS,
DYSPLASTIC NEVI AND EARLY
MELANOMAS**

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INDEX

<i>INTRODUCTION</i>	<i>Pag 1</i>
<i>AIM OF THE STUDY</i>	<i>Pag 6</i>
<i>METHODS</i>	<i>Pag 12</i>
<i>RESULTS</i>	<i>Pag 15</i>
<i>DISCUSSION</i>	<i>Pag 20</i>
<i>BIBLIOGRAFIA</i>	<i>Pag 28</i>

INTRODUCTION

Cutaneous Melanoma is a malignant neoplasm that arises from epidermal melanocytes or melanocyte precursor cells which are derived from the neural crest and then migrate to skin during embryonic development¹.

From 1950 to 1990 there was a steady rise in the incidence of cutaneous melanoma with a greater incidence in Australia, New Zealand, Europe, North America and a lower incidence in black person in Africa and in Asia.

Since the mid 1990s there has been a stabilization of the annual incidence of melanoma in women while, on the other hand melanoma's incidence in males continues to increase.

Recent epidemiological studies have suggested as cutaneous melanoma represents 5% of all cancers by incidence in males and 4% in females and that there are every year 160.000 new cases of

melanoma in world population ,62.000 cases in Europe and 6.000 cases in Italy with incidence rates for females aged 30-50 years and for males aged 50-80 years.

In the past decades we observed a steady rise in the mortality for melanoma but in the last years we have observed as melanoma mortality has not risen at the same rate as its incidence².

The steady rise in the incidence of melanoma can be explained considering two different aspects that are the real growth of the cases of melanoma in population and the increasing of the diagnosis of melanoma especially in early stages thanks to campaign of prevention, to periodic examination of patients and to the improvement of clinical detection of melanoma by the use of dermoscopy for the study of cutaneous pigmentary lesions.

Infact in the last years we have observed as there is a steady rise of the number of cases of melanoma diagnosed in early stages (thickness < 1mm measured by the Breslow method) while on the other hand cutaneous melanomas with a thickness greater than 1mm show a steady incidence (this aspect is evident especially in

older people)¹⁻².

These observations are extremely important because demonstrate the importance of the campaign of prevention and education regarding melanoma in population and the importance of early diagnosis of melanoma by a prognostic point of view; on the other hand these observations underline the difficulties that we have in the clinical management of nodular melanoma which represents the worst clinical-pathological subtype of melanoma by a prognostic point of view.

Regarding the prognostic evaluation in patients affected by cutaneous melanoma we have to underline that this neoplasm is usually fatal after tumor cells metastasize and its management, especially of advanced stages of melanoma, is quite difficult because the cells are usually resistant to conventional therapies such as radiation therapy and chemotherapy.

This particular aspect of melanoma explains as in the last years there is a growing interest towards activities of research that aim to discover cellular target that could help us to understand the

resistance of melanoma to conventional therapies and its variable clinical course and that could represent potential cellular target for biochemotherapy and immunotherapy.¹

In the last years some studies have been performed in order to discover and to study some cellular markers as for example the ATP-binding cassette (ABC) transporters that actually are considered as one of the most common cause of multidrug resistance in cancers since these efflux anticancer drugs from cells (melanoma cells express a group of ABC transporters that could explain the resistance of this neoplasm against anticancer drugs)³.

Among the cellular targets that must be investigated we can consider the nucleolin that is an argyrophilic nucleolar protein that, as demonstrated in recent studies, is over-expressed in some types of human cancers as for example Breast Cancer, Chronic Lymphocytic Leukaemia and Retinoblastoma and whose over-expression is investigated for its possible diagnostic and prognostic significance and for therapeutical purpose too⁴⁻⁸.

Recent studies have demonstrated as nucleolin protein is

expressed in cutaneous melanocytic lesions and its expression, valued by immunohistochemistry, is quite different in comparison to the biological behaviour of these lesions.

More precisely Mourmouras et al have demonstrated as nucleolin expression in benign nevi is strongly different respect to melanoma; moreover among melanomas they observed that the expression of abnormal pattern of positivity for nucleolin in malignant cell accompanied melanoma progression with more and more evidence from early melanoma to metastatic melanoma.

AIM OF THE STUDY

Nucleolin is an argyrophilic protein codified by a gene sited on chromosome 2 and it represents, both to nucleoplasmin, one of the most important nucleolar protein.

Nucleolin can be found not only in the nucleolus but in cytoplasm and in plasma membrane too and this DNA and RNA-binding protein can be considered a multi-functional cellular component since it's involved in several cellular activities; in the nucleolus it controls many aspects of DNA and RNA metabolism, in the cytoplasm it shuttles proteins into the nucleus and provides a post-transcriptional regulation of mRNA and on cell surface it serves as an attachment protein for several ligands as for example growth factors⁹⁻¹³.

Recent studies have suggested as this multi-functional protein is highly expressed in cancer cells and in proliferating cells⁴⁻⁸.

More in detail nucleolin seems to be involved in neoplastic cells in activities as apoptosis, cellular proliferation and differentiation, neoangiogenesis, cell cycle control and tumoral cells invasiveness⁵⁻¹⁶

Moreover nucleolin works as a transcriptional regulator and during oncogenesis it can activate and stabilize oncogenes and on the other hand it can interact with tumor suppressor genes too.

As suggested by studies regarding the relationship between nucleolin and some human cancers this protein seems be deregulated and over-expressed in malignant neoplasms^{4-8,16-23}.

Usually the changes of the normal nuclear morphology are considered an important marker of neoplastic changes by a cellular point of view and an altered pattern of nucleolin positivity in the cell can be considered as a deregulated activity of this protein in tumoral disease.

Recent studies regarding nucleolin expression in tumoral cells demonstrates as it can be observed as single or multiple nuclear dots while in the cytoplasms there is a weak presence of nucleolin

as dots^{5-11,24}

Since nucleolin is highly expressed on neoplastic plasma membrane and because of its oncogenetic properties its expression must be investigated in order to establish its prognostic significance; moreover the correspondance observed between nucleolin's over-expression and tumoral clinical course create an interest towards nucleolin not only for its prognostic significance but for diagnostic and therapeutical purposes too^{4-11,25-30}

Recently some studies have demonstrated by immunohistochemistry as nucleolin is expressed in cutaneous melanocytic lesions and its expression changes in relationship to the type of lesion; more precisely considering benign congenital or acquired nevi, dysplastic nevi and primary and metastatic melanomas melanocytic cells exhibith different pattern of positivity for nucleolin¹⁵.

On this basis we aimed with our study to investigate nucleolin expression by immunohistochemistry in some melanocytic

lesions that often create important diagnostic difficulties in clinicians and dermatopathologists with prognostic and medical-legal relevance consequences.

More in detail we focused our attention on Spitz nevus and on a subtype of melanoma that resembles by a clinical and histopathological point of view Spitz Nevus which is called Spitzoid Melanoma or Melanoma with Spitz nevus like features.

In some cases the distinction between the two is impossible; despite detailed characterization over the past few decades of the histopathological features of these tumors, studies have revealed a lack of objective criteria for differentiating Spitzoid melanoma from Spitz nevi, even when reviewed by expert pathologists³².

Cases in which metastatic melanomas initially were misdiagnosed as Spitz nevi, leading to final outcome, are well documented in literature.

Because of the difficulties associated with its diagnosis, these subtype of melanoma are problematic for dermatologists, dermatopathologists and oncologists.

Nowadays it remains unclear as to whether Spitz nevus and Spitzoid melanoma reside at opposite ends of a biologic spectrum or represent two separate entities.

Our study aim to understand if nucleolin evaluation by immunohistochemistry in spitzoid lesions can represent an useful tool in order to discriminate between these lesions and the diagnostic and prognostic meaning of nucleolin expressions in these melanocytic tumors.

For a similar reason we investigated the expression of nucleolin in Dysplastic nevi with severe atypical aspects and early melanomas in order to assess if nucleolin expression could represent or less an additional diagnostic parameter and can help dermatopathologists and clinicians in the right management of these melanocytic lesions.

As well known in the last years some controversials exist regarding these pigmentary lesions; infact some authors believe that dysplastic nevi and early melanoma (melanoma in situ according to Clark classification) can represent two different

phases of a same pathological continuum while on the other hand some authors believe that dysplastic nevi are histopathological entities well distinguished from melanoma in situ that probably will never evolve in melanoma.

Surely for clinicians this controversial aspect regarding these pigmentary lesions create some difficulties in the therapeutical management of patients.

On this basis we have considered the change of nucleolin expression revealed by immunohistochemistry in benign nevi, dysplastic nevi and early melanoma and we have tried to understand if is possible to find some useful difference in nucleolin expression between dysplastic nevi with severe atypical manifestations and melanoma in situ too.

METHODS

We studied 192 cutaneous melanocytic lesions that were surgically excised and later histologically revised by expert dermatopathologists.

More precisely we studied two different groups of melanocytic cutaneous lesions: in the first one we had 103 spitzoid cutaneous lesions (39 Spitzoid Melanomas -5 of these were metastatic ones- and 64 Spitz Nevi) while in the second group we had 44 Dysplastyc Nevi and 45 Early Melanomas (Melanomas in situ according Clark classification).

In each lesions we cut from paraffin blocks 4 micron thick sections and later the sections were deparaffined, rehydrated and incubated with 3% H₂O₂.

Then after microwave treatment in buffer-citrate for 5 minutes the

sections were incubated for 60 minutes with monoclonal antibody anti-nucleolin at room temperature, while for negative controls we used non-immune serum immunoglobulins.

As chromogen it was used fuchsin.

Then sections were counterstained with Harris hematoxylin, and after dehydrating in alcohol and clearing in xylene these were coverslipped.

Then sections were studied using a microscope at high resolution that was connected to a digital camera and interfaced with a PC and quantitative expression of nucleolin was performed using an image analysis program.

In each case several fields were photographed at high resolution and later we evaluated positive and negative nuclei for nucleolin; than for positive nuclei we evaluated separately the presence of nucleolin in dot-like structures, in nucleoplasm or in both these conditions in relationship to the total nuclear area. All results were expressed as mean values \pm SD.

Then a statistical evaluation through a monovariate and a

multivariate analysis was performed.

In this study we have evaluated the expression of nucleolin positivity and its pattern of positivity in relationship to the different types of melanocytic lesions above mentioned and its diagnostic and prognostic significance.

RESULTS

In our study we observed, as already known, that in the skin the expression of nucleolin positivity is different among different cellular types and in particular between melanocytic and not-melanocytic cells.

Infact among not-melanocytic cutaneous cells we observed that epithelial cells (keratinocytes and adnexal cells) presented negative or homogeneously stained nuclei with or without one to three positive dots for nucleolin.

A similar aspect could be observed in endothelial and lymphocytic cells while on the other hand we could underline the presence of a steady negativity for nucleolin in adypocytes, fibroblasts, nervous and smooth muscle cells.

Regarding melanocytic cells we observed that, in normal condition, they showed the presence of one (and sometimes two),

small and round dot positive for nucleolin inside a negative nucleus and a negativity for nucleolin in the cytoplasm; this aspect could be described for melanocytes inside benign nevi and for cutaneous melanocytes far from nevi.

Moreover was observed the presence of melanocytes with positivity or negativity for nucleolin in all the melanocytic lesions examined (Spitz nevi, dysplastic and malignant ones).

As known in benign nevi melanocytic cells showed the presence of little, regular, roundish dots positive for nucleolin and/or an homogeneous positivity in the nucleoplasm.

Melanocytes found in dysplastic nevi and in malignant melanoma showed the presence of single or multiple irregular (for shape and size) dots positive for nucleolin in the nucleus; this aspect could be associated or less to an irregular positivity in the nucleoplasm (that represent another atypical aspect) defining an abnormal pattern of nucleolin positivity.

Our observations demonstrates as abnormal pattern of positivity for nucleolin in melanocytic cells is represented by irregular

nuclear dots, irregular positivity for nucleolin in nucleoplasm or both these conditions; these aspects are never or rarely evident in benign nevi and this condition surely represents a distinctive peculiarity between benign and malignant melanocytic lesions.

Moreover in malignant melanocytes there was a weak positivity for nucleolin in the cytoplasm while this last aspect was strongly evident in mytotic cells.

On the other hand in our study we observed that abnormal pattern of nucleolin (nuclear irregular dots, nucleoplasm positivity or both these conditions) were strongly described in melanoma and the presence of these patterns is more and more evident in relationship to melanoma's progression.

Infact in some cases of metastatic spitzoid melanoma we analyzed the expression of abnormal patterns of nucleolin in neoplastic cells in the primitive melanoma and in the cutaneous metastasis; in this case we observed as the expression of abnormal patterns of nucleolin was greater in the cutaneous metastasis than in the primary melanoma revealing as these abnormalities are expressed

more and more in relationship to melanoma progression.

The statistical analysis revealed as existed a significant difference in the expression of nucleolin positivity in malignant lesions on the basis of cancer development.

More in detail we observed that these abnormal patterns of nucleolin positivity in melanocytic lesions are expressed with a growing evidence when we refer respectively to dysplastic nevi, primary and metastatic melanomas while we know that we don't observe normal nucleolin pattern in cases of melanoma.

On these basis we can underline as probably the expression of abnormal patterns of nucleolin in these malignant neoplasms could be considered as a new prognostic factor after Breslow Method that is based on the evaluation of melanoma thickness.

At last we have to underline as we observed the existence of similarities in cellular positivity for nucleolin in melanocytic cells in some nevi with severe dysplasia and melanoma in situ; this aspect confirm the great difficulties that dermatopathologist find to differentiate these types of melanocytic lesions by a

histopathological point of view with all the forensic consequences that a misdiagnosis can create .

A different condition was observed regarding spitzoid melanocytic lesions; infact in Spitz nevi we observed an aspect similar to benign nevus with the presence of regular dots in the nucleus and lower levels of nucleolin positivity in relationship to total nuclear area

But in Spitzoid Melanomas the nucleolin expression was really different with an abnormal pattern represented by the presence of irregular nuclear dots and/or positivity in nucleoplasm so that nucleolin expression helped to discriminate among these last lesions.

At last in cutaneous metastasis of spitzoid melanoma we observed a deeper expression of abnormal pattern of positivity for nucleolin inside the nucleus of cells in comparison with primary lesion.

DISCUSSION

Cancer chemotherapeutic efficacy is frequently impaired by either intrinsic or acquired tumor resistance to multiple, structurally unrelated therapeutic drugs with different mechanisms of action, a phenomenon termed multidrug resistance³.

Cutaneous Melanoma, which rank 6th in incidence of all cancers, represent a type of human cancer whose management in advanced stages is strongly difficult since its cell are usually resistant to conventional therapy as chemotherapy¹⁻³.

This multidrug resistance can explain the reason why melanoma is usually fatal after tumor cells metastasize.

For this reason in the last decade, in order new open therapeutic avenues in the treatment of melanoma several study have aimed to discover cellular mutations, signaling pathways that can

explain this multidrug resistance.

The causes that underlie multidrug resistance in melanoma are not well known and probably are to be mediated by different mechanisms.

Those mechanisms are represented by increased DNA repair in response to DNA-damaging agents, altered expression of oncogenes such as the tumor suppressor p53, increased levels of endogeneous nitric oxide and over-expression of anti-apoptotic protein such as Bcl-2 or the expression of one or more ATP-binding cassette transporters (ABCA9, ABCB1, ABCB5, ABCB8, ABCC1, ABCC2 AND ABCD1) in melanoma can justify the efflux of antitumoral drugs from neoplastic cells and consequently melanoma resistance to chemotherapy³.

In the last years studies regarding human tumors such as Retinoblastoma, Breast Cancer, Chronic Lymphocytic Laekemia have demonstrated as in cancer cells can be observed an over-expression and a deregulated activity of a nucleolar protein called nucleolin⁴⁻⁸.

This argyrophilic protein in human cells can be found not only in the nucleolus, where both to nucleoplasmin represent a major nucleolar protein, but is present in nucleoplasm, cytoplasm and cell surface and can shuttle among these cellular sections.

Nucleolin is a multi-functional DNA and RNA-binding protein and is involved in different cellular activities such as ribosome and nucleolus biogenesis, gene-expression and DNA metabolism^{7-9,13-14}

This protein, that as demonstrated for the human tumors above mentioned, is overexpressed and deregulated in cancer cells is able to activate and stabilize oncogenes and to interact with tumor suppressor genes; moreover nucleolin is involved in apoptosis, neoangiogenesis, cell cycle control, cellular proliferation and differentiation and in tumor cells invasiveness.

Recent studies have demonstrated as nucleolin inside cancer cells is able after interacting with cellular peptides to activate intracellular signaling pathways and as is involved in nuclear changes thanks to its interaction with chromatin¹⁴⁻¹⁶

Last but not least nucleolin expression on tumoral cell surface and its expression in endothelial cells during neoangiogenesis observed in neoplastic development can easily explain the growing interest toward this protein that could represent an hypothetical target of anti-tumoral therapies ¹⁹

It's well-known as changes in nuclear morphology can represent a marker of neoplastic development and an altered pattern of positivity towards nucleolin could be a signal of its over-expression and of its deregulated activity during oncogenesis²⁴⁻²⁶.

On this basis our study confirmed the expression of nucleolin in cutaneous malignant melanocytic lesions including benign nevi, early and advanced melanoma and metastatic melanomas.

In our study we assess nucleolin expression in melanocytic lesions that can arouse great controversies by a diagnostic point of view such as spitzoid lesions (Spitz nevus and Spitzoid Melanoma) or as nevi with severe dysplasia that can be considered histologically borderline with melanoma in situ.

In our study we have observed as melanocytic cell can be positive

or negative for nucleolin; in positive cell we have verified the presence in nucleus of a single, roundish, homogeneous dot and a diffuse stain of nucleoplasm while cytoplasm is usually weakly stained and this aspect is typically described in benign melanocytic lesions^{5-7,22,23}.

On the other hand we have observed an abnormal pattern of nucleolin in melanocytic cells as multiple dots irregular for size and shape inside nucleus and/or the presence of a disomogeneous positivity in nucleoplasm in dysplastic and malignant primary and metastatic melanomas.^{15,25-30}

On the basis of our observations we can underline as the expression of nucleolin positivity in melanocytic cells is quite distinctive between benign and malignant melanocytic lesions.

Moreover the percentage of expression of abnormal pattern in comparison to the different melanocytic lesions and especially in the different phases of growth of melanoma could lead us to assess that nucleolin could represent a sort of index of progression and because of this nucleolin expression could represent a sort of

prognostic marker for melanoma progression.

Therefore nucleolin expression in melanocytic lesions can help to discriminate between benign and malignant ones and its more and more evident in relationship to melanoma progression.

However in our study we observed as nucleolin expression cannot be considered a reliable parameter to discriminate between dysplastic nevi with severe atypical aspects and melanoma in situ; infact we observed a sort of overlapping in the results that we justified considering as these two type of lesions are often considered two distinct phases of a same pathological continuum.

On this basis nucleolin expression cannot be considered a diagnostic parameter that can help us to discriminate without any doubt between dysplastic nevi and early melanoma.

At last in our study we didn't find abnormal pattern of positivity for nucleolin in Spitz nevi, and on the other hand we find an abnormal pattern of positivity for nucleolin in Spitzoid melanoma. Moreover the expression of abnormal pattern of nucleolin was more evident in metastasis of spitzoid melanoma

than in the same primary melanoma.

On this basis we can say that as demonstrated for melanoma in radial growth phase, vertical growth phase and metastatic melanoma, in spitzoid lesions (Spitz nevi and spitzoid melanoma with or without metastasis) nucleolin could represent an index of progression.

This last observation must be underlined since a great number of pigmentary melanocytic lesions misdiagnosed are usually represented by Spitzoid lesions³¹.

In conclusion we can say that the different pattern of positivity for nucleolin showed by spitzoid lesions, if confirmed in study with a greater number of patients, could open new perspectives in the diagnostic assessment of these cutaneous neoplasms

By a clinical point of view the different expression of nucleolin in spitzoid melanoma and in metastatic spizoid melanoma could underline, if confirmed, the prognostic and therapeutic value of nucleolin expression.

At last if we consider the multidrug resistance that melanoma

shows against conventional chemotherapy, a deeper knowledge of nucleolin activity in melanoma cells could induce us to consider nucleolin as a possible target for alternative anti-tumoral therapies.

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