ORIGINAL ARTICLE

Extracellular matrix proteins, neural differentiation and melanoma progression

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Abstract

Background: Highly invasive melanoma cells traverse the extracellular matrix (ECM) by cell adhesion, ECM proteolysis, and cell migration. Ln5 and Integrin α5β1 play a key role in regulating melanoma growth, invasion, migration, angiogenesis and tissue remodeling. The present work utilizes the in situ 3D tumor matrix of melanomas to study their role in the formation of tumor-vascular-complexes (TVCs), vasculogenesis and tumor invasion.

Material and Methods: Serial frozen and paraffin sections of nodular melanomas were subjected to histochemistry, enzyme histochemistry: dopa oxidase and immunohistochemistry using the monoclonal antibodies HMB45, GFAP, NFP, Syn, Ln5 (laminin 5) & integrin (α5β1) by the avidin/biotin system and assessed in general tumor areas, infiltrating margins, TVCs, vasculogenesis.

Results: General areas: Ln5 shows a range of 75%-100% positivity in pigmented and 5.9%-29.8% cells in amelanotic areas. Integrin α5β1: shows a range of 69.4%-94.8% positivity in pigmented and 4%-55.2% in amelanotic areas. Infiltrating margins: Ln5/Integrin α5β1, are both required for preparing the ECM for spread of the tumor cells and angiogenesis. The cells at the infiltrating margins are positive for both. TVCs: Ln5/Integrin α5β1 expression is highest in the outer layers. Vasculogenesis (VM): Irregular spaces lined by Syn, Ln5/Integrin α5β1 positive tumor cells expand into islands of hemopoeisis, associated with the lining tumor cells. Combined TVCs and VM: In the mixed tumors a combination of TVCs and VM are seen. Irregular crevices appear lined by Ln5/Integrin α5β1 positive cells which flatten to form endothelial tubes which connect with the vascular network. Ln5/Integrin α5β1 positive cells co-express neural markers, NFP and Syn in all areas.

Discussion: In conclusion, Ln5/Integrin α5β1 form the substrate for the advancing cell mass in the outer layers of the TVCs, infiltrating tumor margins and vasculogenic spaces. The architecture and remodeling of the TVCs is maintained with integrin α5β1 providing attachment and dynamic force for tissue compaction, cohesion and migration aided by Ln5. Neural differentiation is associated with migratory ability as indicated by NFP and Syn coexpression with Ln5/Integrin α5β1.

Key words

Integrin α5β1, Laminin-5, Tumor-vascular-complexes, Neural markers, Vasculogenesis

1 Introduction

Melanoma is a highly invasive tumor arising from melanocytes, derivatives of the neural crest. Malignant growth and metastasis is a "multistep" process. Invading tumor cells traverse the extracellular matrix (ECM) and the altered chemical reactions between normal cells and matrix in neoplasia influence tumor invasion [1]. Tumor invasion of ECM barriers involves tumor cell adhesion, ECM proteolysis, and cell migration [2]. Cell surface receptors for the ECM molecules, such as the integrins, play key roles in the regulation of normal and tumour cell migration and survival.

Invasion through ECMs and subsequent metastasis are dependent on co-operation between adhesive and proteolytic systems. Matrix metalloproteinases (MMPs) hydrolyze ECM components such as collagen, laminin, fibronectin, proteoglycans and contribute to the spreading of tumor cells by eliminating the surrounding ECM and basement membrane (BM) barriers. Integrins, laminins and matrix metalloproteinases (MMPs) are important mediators of the metastatic process in many malignant tumors [2].

Ln5 and integrin α5β1 are important for the invasion of the stroma. Integrins are a super family of transmembrane heterodimeric surface receptors and subunits involved in cell-matrix and cell-cell adhesion [3, 4]. Integrins link fibronectin (FN) fibrils to the actin cytoskeleton for fibrillogenesis. Integrins stimulate cell migration by activating GTPases and by anchoring actin filaments to the membrane and thereby activate the invasiveness of cells (Figure 1).

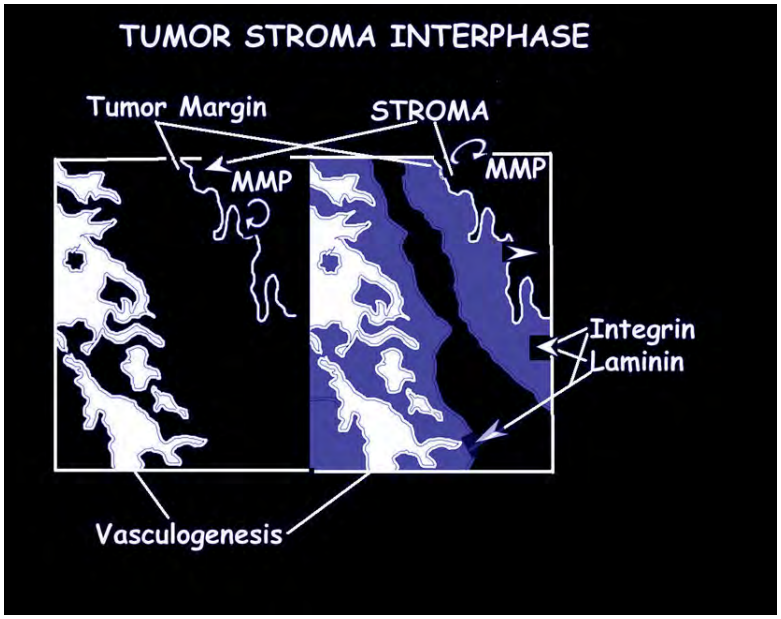

Figure 1. Diagramatic representation of interactions of the matrix protein and the ECM at the tumor stroma interphase. Integrin and LN5 positivity at the margin activates the MMPs in the stroma to facilitate tumor infiltration of the stroma

In a three-dimensional (3D) environment that more closely reflects in vivo tissue architecture, FN fibrils that link adjacent cells contribute to tissue compaction and cohesion. Integrin α5β1 mediates strong intercellular cohesion of 3D cellular aggregates [5]. Tissue compaction and cohesion are essential to a variety of important biological processes. In the developing embryo, for example, they play vital roles in early and late morphogenetic processes, including blastomere formation [6]. In the vertical growth phase (VGP) melanomas, tumor vascular interaction at the infiltrating margins results in the formation of tumor vascular complexes (TVCs) which show organized neural differentiation [7-9]. The present work utilizes the in situ 3D tumor matrix of melanomas to study the role of Ln5 and integrin α5β1 in TVC formation, tumor invasion and vasculogenesis.

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2 Material and methods

A random series nodular melanomas in the vertical growth phase (VGP) were received from the Cancer Surgery Unit of Safdarjung Hospital, New Delhi, fixed in cold (4°C) 10% formol glutaraldehyde for overnight cold fixation. The formaldehyde-glutardehyde cold fixation retains the morphology, gives crisp and efficient immunohistochemical staining both in frozen as well as paraffin sections. The same blocks can be subjected to electron microscopy as well. 10 blocks were taken from each tumor. Nodules were sampled in the ratio of pigmented to amelanotic areas in the entire tumor. As the specimen were received and sampled the blocks were arranged in a grid, according to the pigment level which varied between 7% to 95% as ascertained by pigment cell counts (Figure 2).

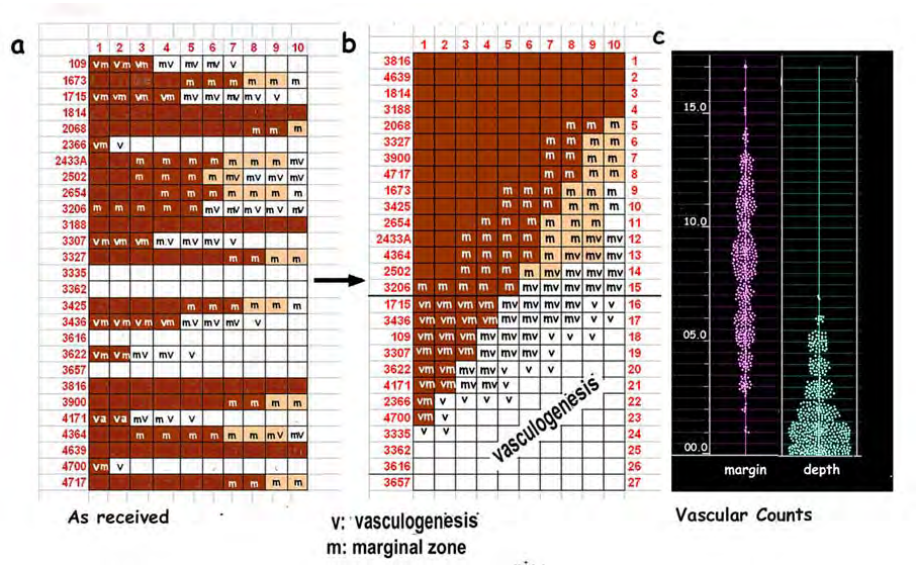

Figure 2. Composite diagram showing sampling of nodules in the ratio of pigmented to amelanotic areas in the each tumor. As the specimen were received and sampled the blocks were arranged in a grid (a). In grid (b) cases are arranged according to the pigment level which varied between 7% to 95%, as ascertained by pigment cell counts. The areas showing the infiltrating margin are marked 'm' and those with vasculogenesis 'v'. TVCs are seen in the marginal zone. (c) Scatter diagram comparing vascular counts at the marginal two high power fields and within the tumor

Each block was subjected to 20-40, 5µm thick serial, frozen and paraffin sections and maintained under refrigeration at 4°C for routine Histochemistry (HE, reticulin+gold impregnation for aurophilia [10], PAS to assess the vascular pattern); Enzyme histochemistry: dopa oxidase, dopamine oxidase; immunohistochemistry, using the monoclonal antibodies(mAb) HMB45, GFAP, NFP, Syn, catecholamines, from BioGenex, LN5 (laminin 5) from Kappa Zymed & integrin α5β1 from Dakopats, by the avidin/biotin system [11-14]. All mAbs used are of high sensitivity and specificity. The same mAb were used simultaneously against known positive sections from human skin as positive controls. As negative control all slides included a serial section stained with no mAb.

[*Immuno Chemicals:* GFAP: BioGenex Lab: Polyclonal; NFP: BioGenex Labs: Monoclonal; Synaptophysin: BioGenex Labs; Monoclonal; Dako Pat kits: Polyclonal; LN5 (Laminin 5): Kappa Zymed: Monoclonal; integrin α5β1: Dako Pat kits: Monoclonal.]

2.1 Comparative quantification

Comparative quantification of studied proteins was done by positive Cell Counts. Cells positive for each mAb were counted in 1000 cells from a random 10hpf per block in serial sections. Tables 1-4 highlight the comparative expression as well as the co-expression of the different markers. Cytospectrophotometric analysis gave erratic results since individual cells show great variations in positivity.

2.2 Angiogenesis from normal stromal vessels at the advancing margin of tumor

103 blocks from mixed tumors, 51 blocks from pigmented and 52 from poorly pigmented nodules (Figure 2) at the interphase between the tumor and surrounding preexisting stroma, have been included for the study. A large number of angiogenic vessels are seen within 2hpf of the tumor margin with a maximum of 20 vessels per HPF associated with TVCs. Vascular channels are counted at the tumor margins in each of the 103 blocks to a depth of two high power fields (HPF) and at a depth of 5 to 6 HPF within the tumor in 10 HPF (1030 HPF marginal and 1030 HPF within tumor) (Figure 2c).

2.3 Tumor-Vascular-Complexes

Endothelial tubes from normal stromal vessels interact with tumor cells to form tumor-vascular-complexes (TVCs). The TVCs from both pigmented and amelanotic nodules are counted in 5 random HPF in each block along the tumor margin at the tumor-stromal interphase (out of a possible maximum of 100 vessels). A total of 1030 TVCs formed around vessels in cross section were included in the study.

Histochemistry; HE, PAS, combined reticulin + gold impregnation for aurophilia [10] Gold chloride sublimate specifically and exclusively stains radial glia (confirmed by GFAP and nestin positivity) which stain intensely black. Reticulin is seen as a network of fine fibrils.

2.4 Immunohistochemistry

Marker positivity has been examined in the layers of the perimantle zone (PMZ) of tumor-vascular complexes (TVC) formed during angiogenesis and compared in pigmented and amelanotic nodules as well as in the TVCs. The layers are numbered 1 to 5 with layer 1 being closest to the vessel and layer 5 being furthest. Camera Lucida diagrams were used to localize integrin α5β1and Ln5 positivity in comparison with neural markers. For the purpose of analysis, the distribution pattern of the antibody is quantitated in the layers of the lobules by layer and cell positivity.

Pigment, DOPA and HMB-45 positivity are criteria for diagnosis. Positive dopa reaction, HMB45 positivity and the presence of premelanosomes on electron microscopy is diagnostic of amelanotic melanomas.

Statistical analysis has been done using Mann-Whitney Rank Sum Test, with Holm-Sidak Multiple Comparison Procedures, and Pearson Product Moment Correlation has been performed on the data.

3 Results

Melanomas present great variation in their morphological picture and pigment content. The expression of Laminin 5 (Ln5) the ‘fibronectin receptor’ and integrin α5β1 in association with pigmentation and morphological variations have been examined in this section. These matrix proteins are related to invasion. They have been studied in:

- General tumor areas;
- Infiltrating margins, at the stroma-tumor interphase;
- TVCs;
- Vasculogenesis;
- Mixed nodules: Combined TVCs and VM.

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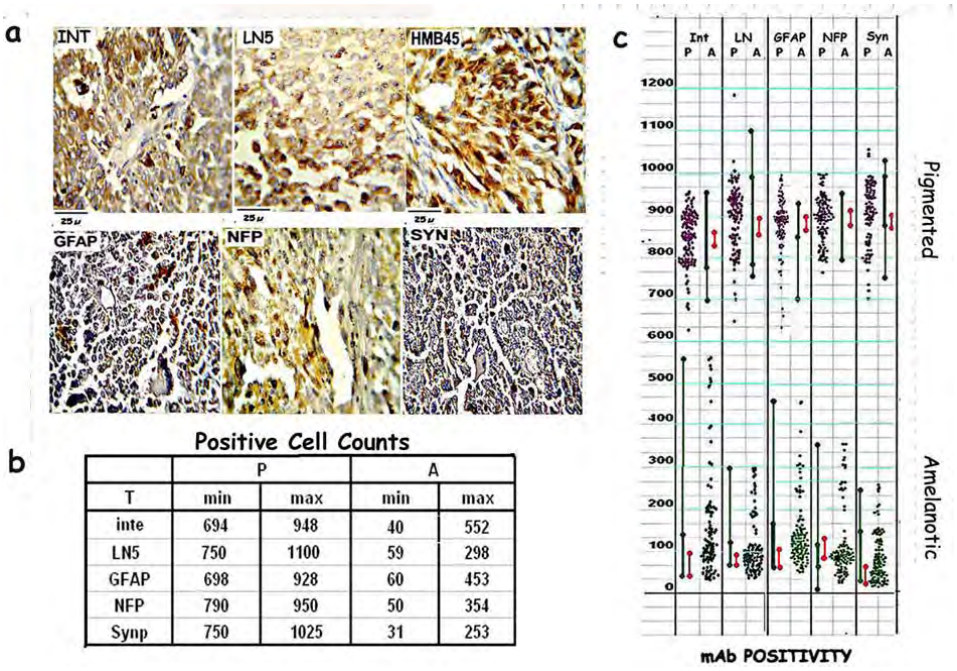
3.1 General tumor

Cell counts:

Positive cells were counted in 1000 cells from a random 10hpf per block. All pigmented areas were positive for the matrix proteins Ln5 and integrin α5β1 (Figure 3a, Table 1).



	P		A	
T	min	max	min	max
inte	694	948	40	552
LN5	750	1100	59	298
GFAP	698	928	60	453
NFP	790	950	50	354
Synp	750	1025	31	253

Figure 3. Composite diagram showing differentiation patterns in general tumor areas including (a) histology showing immunopositivity for integrin α5β1, Ln5, HMB 45, and the neural markers GFAP, NFP and Syn; [mAbX400] and (b) table showing counts of cells positive for each mAb. (c) Scatter diagram showing positivity in pigmented and amelanotic tumors. Positivity of all markers is high in the pigmented nodules and low in amelanotic nodules

Table 1. General tumor areas: Differentiation in melanomas

	T	Pigmented				Amelanotic			
	mAb	min	%	max	%	min	%	max	%
Matrix	LN5	750	75	1100	100	59	5.9	298	29.8
	Inte	654	65.4	948	95	40	4	552	55.2
Neural	NFP	790	79	950	95	50	5	354	35.4
	Synapt	750	75	1025	99.2	31	3.1	253	35.3
Biogenic	DA/NA	814	81.4	1066	99.5	23	2.3	443	44.3
	SER	688	70	905	90.5	41	4.1	849	64.9
	MLT	698	70	989	98.9	40	4	553	55.3

Note. Differentiation patterns in general tumor areas show the co-expression of matrix proteins and neural markers, and biogenic amines. Cell counts give a quantitative comparison of the matrix proteins with the neural markers.

Ln5: Positive cell counts showed a range of 750-1100 (75%-100%) positive cells per block in 84 blocks from pigmented nodules as shown in the scatter diagram and values. Positivity for all the markers is low or absent in amelanotic areas. Positive cells counted in 83 blocks from amelanotic nodules showed a range of 59-298 (5.9%-29.8%) cells per block. Thus

the total range of immunopositivity is 70% to 100% in pigmented areas and 0% to 65% in amelanotic areas (Figure 3b&c; Table 1).

Integrin α5β1: Positive cell counts showed a range of 694-948 (69.4%-95%) positive cells per block in 84 blocks from pigmented nodules as shown in the table and scatter diagram (Figure 3b&c). Positivity for all the markers is low or absent in amelanotic areas. Positive cells counted in 83 blocks from amelanotic nodules showed a range of 40-552 (4%-55.2%) cells per block. Thus the total range of immunopositivity is 70% to 95% in pigmented areas and 4% to 55% in amelanotic areas (Figure 3b&c; Table 1).

3.2 Infiltrating margins

The tumor cells along the margin at the stroma-tumor interphase are positive for Ln5 (98.4%) and integrin (α5β1) (85.7%), which are required for preparing the ECM for infiltration of the tumor cells and vascular network (Figure 4; Table 2&3). Endothelial buds arise from the adjacent normal stromal vessels, which cannelise, and enter the tumor substance. Tumor cells ensheath the neovasculature and grow out into several layers as seen on auro-reticulin staining. The margins of amelanotic areas, abutting on the surrounding tissue show Ln5 (71.4%) and integrin α5β1 (57.1%) positivity. The reactivity of the stromal vasculature is similar to that seen in the more differentiated areas.

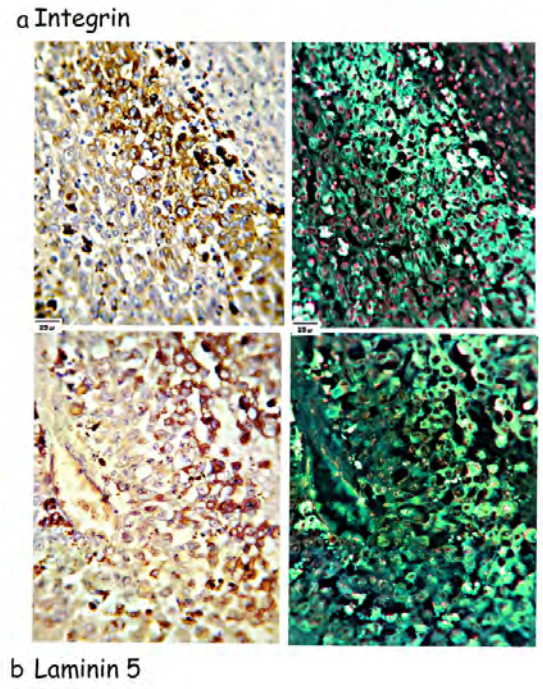


Figure 4. Serial sections showing Integrin $\alpha_5\beta_1$ (a) and Laminin5 (b) positivity in cells adjacent to the infiltrating margins of the tumor well highlighted in the invert images [mAbX400]. The cells are well compacted at the tumor-stroma interphase.

Table 2. Infiltrating margins in pigmented and amelanotic tumors

Polar	Pigmented				Amelanotic			
	main		margin		main		margin	
mAb	/1000	%	/1000	%	/1000	%	/1000	%
LN5	694	69.4	984	98.4	325	32.5	714	71.4
Inte	756	75.6	857	85.7	220	22	571	57.1
NFP	550	55	950	95	200	20	570	57
Synapt	700	70	980	98	250	25	670	67

Note. Matrix proteins are concentrated at the tumor margins in the purely pigmented and amelanotic tumors and are co-expressed with neural markers.

Table 3. Infiltrating margins in Mixed tumors

Mixed	Pigmented				Amelanotic			
	main		margin		main		margin	
mAb	/1000	%	/1000	%	/1000	%	/1000	%
LN5	833	83.3	1100	100	101	10	298	29
Inte	777	78	867	86.7	102	10	552	55
NFP	790	79	950	95	90	9	354	35
Synapt	900	90	1025	100	61	6	253	25

Note. Mixed tumors show a similar trend with a higher positivity as compared with the polar group seen in Table 2.

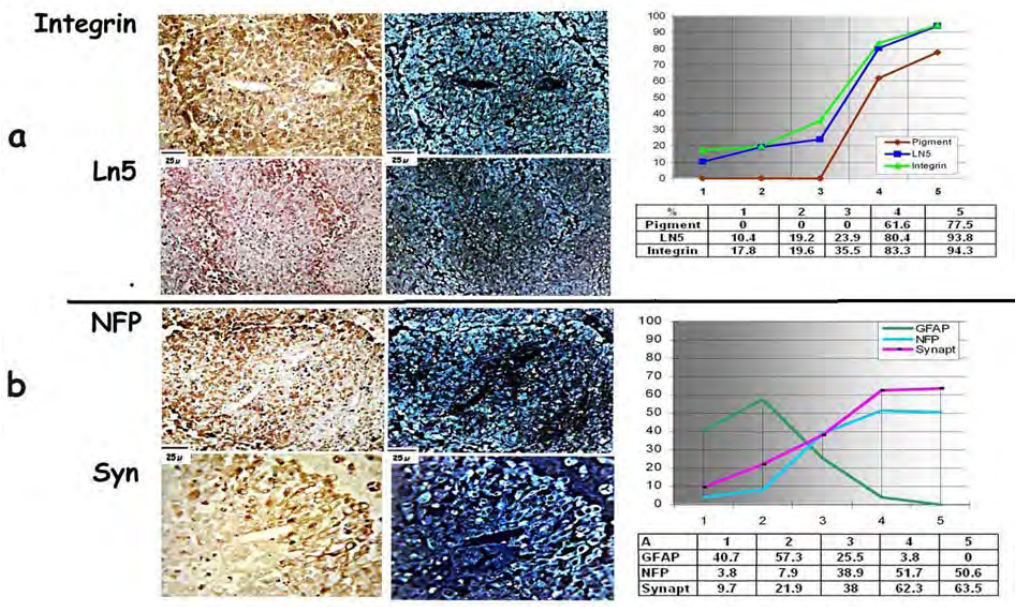


Figure 5. Ln5 and integrin α5β1 expression in tumor-vascular complexes (TVCs). (a) Shows mAb positivity for Ln5 and integrin α5β1 in the outer layers of the TVC, the positive cell counts are given as a graph and table.[mAbX200] (b) Comparative expression of NFP and Syn with attached graph and table (mAbX200).

Table 4. Differentiation in TVC

	P	1	2	3	4	5
Matrix Proteins	LN5	10.4	19.2	23.9	80.4	93.8
	Inte	17.8	19.6	35.5	83.3	94.3
Neural	NFP	3.8	7.9	38.9	51.7	50.6
	Synapt	9.7	21.9	38	62.3	63.5
Biogenic Amines	DA/NA	17	50	75	84.9	96.9
	Pigment	0	0	12.5	75	87.5
	SER	19.1	71.8	62.8	9.4	0.9
	MLT	27.5	60.9	64.1	8.6	1.6

Note. In TVCs matrix proteins LN5 and integrin are expressed in the outer layers. neural markers NFP and Syn and the catecholamines DA/NA are co-expressed in the same layers while indoleamines are expressed in the middle layers. The prominent areas of expression are highlighted.

3.3 TVCs

Ln5: In the layer 1, Ln-5 expression is seen in 10.4% tumor areas. Ln5 expression increases in layer 2 (19.2%) increasing again in the layers 3 (23.9%) and 4 (80.4%) where peak expression is seen. In the layer 5 the expression is 93.8% (Figure 5a, Table 4)

Integrin α5β1 expression increases from the inner layers of the lobule to the outer layers. Integrin α5β1 expression is seen in 17.8%, 19.6% and 35.5% tumor areas in the layers 1, 2 and 3 respectively. Maximum tumor areas express integrin α5β1 in the layers 4 and 5 (83.3% & 94.3%).

NFP/Syn: Neural marker positivity shows a similar trend with high positive counts in the peripheral layers of the TVCs. (NFP: 51.7% &50.6%; Syn: 62.3% & 63.5% in L4&L5 respectively) (Figure 5b; Table 4).

3.4 Vasculogenesis

Amelanotic areas

42.9% tumor areas show scattered positivity for Syn, Ln5 and integrin α5β1 which form the nidus for VM. A network of Ln5/integrin α5β1 positive cells, give rise to irregular spaces which are lined by these positive tumor cells (Figure 6). The spaces expand into islands of hemopoeisis, blood filled spaces with megakaryocytes, myeloid and erythroid series of cells which can be seen associated with the lining tumor cells [15].

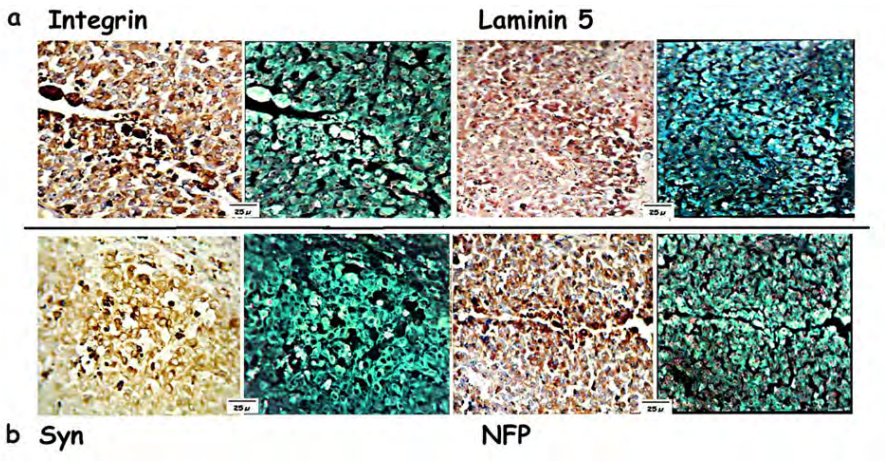


Figure 6. Vasculogenic channels appear as spaces in sheets and solid columns of tumor cells which ultimately connect with the existing capillary network. Ln5 and integrin α5β1 positivity is seen (a) related to cells bordering vasculogenic channels and associated (b) Syn and NFP positivity (mAbX400)

3.5 Mixed nodules

Combined TVCs and VM

In the pigmented nodules 9.3% and in the poorly pigmented nodules 31.7% show a combination of classical TVCs as well as VM. Ln5 and integrin (α5β1) positive cells are seen within the sheets and chords of tumor cells surrounding the TVCs. Irregular crevices, lined by 93.8% Ln5 and 91.4% integrin α5β1 positivity, appear within groups of tumor cells to form channels indicating that these are aggressive and invasive cells (Figure7). Some small groups of tumor cells separate from the main mass to spill into these spaces. The lining tumor cells flatten to form capillary channels which then connect with the vascular network. Ln5 and integrin α5β1 positive cells co-express neural markers, NFP and Syn.

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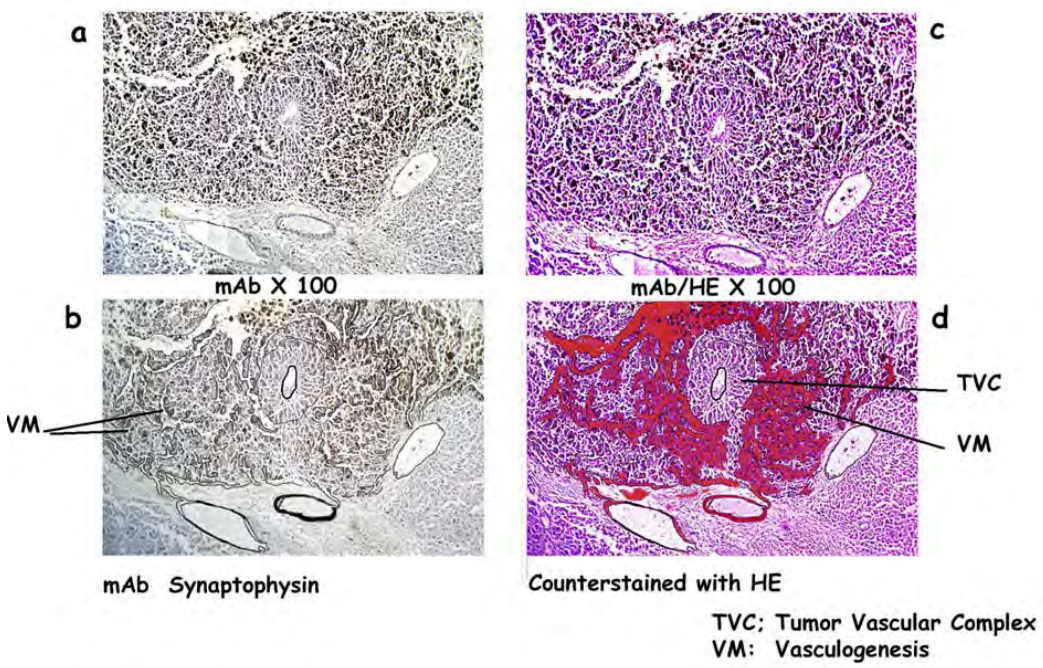

Figure 7. Mixed nodules showing both TVC and vasculogenesis (VM). (a, b) Sections stained with mAb Synaptophysin to locate positive cells (SynX100). TVC shows positivity in the peripheral layers and the VM channels are lined by positive cells as outlined in (b). (c, d) Same sections counterstained with (HE Syn/HEX100). TVC is surrounded by sheets of tumor cells with vasculogenic channels (highlighted in red) (d) connected with the pre-existing stromal vessels

4 Discussion

The invasion and metastasis of tumors is a highly complex, multistep process that requires a tumor cell to modulate its ability to adhere, degrade the surrounding extracellular matrix, migrate, proliferate at a secondary site and stimulate angiogenesis [16]. The growth and dissemination of tumors depends on the establishment of an adequate blood supply to provide nutrients sufficient for their proliferation. It is widely accepted that development of a neovasculature or angiogenesis through various angiogenic mechanisms is critical for tumor development [17].

Proteins of the ECM form a non-cellular compartment to the tumor microenvironment that is extensively modified and remodeled by proteases secreted by neoplastic or non-neoplastic cells. Proteolysis of the ECM proteins allows for migration of neoplastic cells through the BM into the interstitial stroma [18]. As a result important changes in cell-cell and cell-ECM interactions occur, and new signals are generated from the cell surface. These signals affect gene expression and ultimately influence critical cell behaviors such as proliferation, survival, differentiation, and motility [19].

Matrix metalloproteinase (MMP) have been implicated in physiological as well as pathologic remodeling of the ECM [20]. Human MT1-MMP14 which activates human MMP-2 (Gelatinase A) is high in human tumor tissues [21]. Invasion through ECM and subsequent metastasis is dependent on cooperation between adhesive and proteolytic systems. MMPs hydrolyze ECM components and contribute to the spreading of tumor cells by eliminating the surrounding ECM and BM barriers.

Integrins are major factors underlying decreased cell-substratum adhesion, which permit neoplastic cell motility, metastatic capacity and/or proliferative activity [22]. Integrin α5β1 expressed during tumor invasion, and cell migration selectively recognises fibronectin (FN), an abundant component of the ECM. Cell adhesion to FN results in MMP-9 secretion, in normal and tumor cell systems, leading to increased invasiveness of cells. The FAK/Ras signaling pathway has been implicated in MMP-9 activation in ovarian cancer and melanoma cells [19, 22-24].

Laminins contribute widely to the formation of the ECM, particularly the BM [25] and are associated with cell differentiation, cell shape and movement. They maintain tissue phenotypes, and promote tissue survival [26]. Ln5, is an epithelium-specific subtype that plays an important role in establishing adherence of epithelial cells to the BM and matrix by hemidesmosomes [27, 28]. It is involved in cell anchorage, wound healing, tissue remodeling, and cancer invasion [29].

The cytoplasm of tumor cells at the cancer-stromal interface and at the invasive front expressed Ln5 β3 and γ2 chains in squamous cell carcinoma of the tongue and of colorectal carcinoma [30]. Furthermore, cancer cells invading in a scattered manner showed strong expressions of these two proteins [31-33].

FN provides the substrate for cells to migrate on. It is synthesized in situations in which there is epithielial cell migration and spreading, which can lead to invasion and metastasis [34]. Matrix assembly is initiated when inactive, compactly folded dimeric FN unfolds and polymerizes on binding to integrins α5β1 [4]. Integrins stimulate cell migration by activating GTPases and by anchoring actin filaments to the membrane. The cells thus adhere to integrin α5β1, making new fibril in the process, which is then utilized by the cells to migrate. Cell adhesion to FN stimulates MMP-9 secretion with invasiveness of cells. Integrin α5β1 also contributes to matrix remodeling.

Interaction of Ln5 with α3β1 promotes cell migration-promoting activity in wound healing and tumor invasion [35-37]. Keratinocytes adhere and migrate on deposited unprocessed Ln5 in the provisional BM of the wound [38] via integrins α3β1 and/or α6β4 and on dermal collagen and fibronectin via integrins α2β1 and α5β1, respectively [31, 39].

Production of Ln5 is upregulated upon wounding with deposition of unprocessed arcs or circles of α3 laminin subunit at the leading edge [31]. These interacts with integrin α3β1 to help cell migration over the wound bed. The co-expression of integrins, αvβ3 and αIIbβ3, in human melanoma enhances cell survival and promoted growth in *vivo* [32]. The role of αIIbβ3 and αvβ3 integrin has been outlined in human melanoma growth and survival by Döme B, and his group [33, 40].

In the developing embryo melanocytes, derived from the neural crest migrate from the dorsal midline to distant sites. Migrating neural crest cells (NCCs) adhere to FN in an integrin dependent manner. There is a direct correlation between the concentration of integrin α5β1 and the speed of migrating neural crest cells. Ln5 is a ligand for normal human melanocytes in the BM [41]. The interaction of Ln5 produced in the epidermis, with α3β1 integrin on melanoma cells is involved in cell migration, invasion, and degradation of ECM proteins [42].

Integrin α5β1 functions principally to promote the adhesive events required for cell motility and tension-generated matrix remodeling, and confers strong intercellular cohesion to 3D cellular aggregates [24]. Ln5 is an essential component of epithelial basal laminae that is also expressed in the developing nervous system and it promotes neurite outgrowth [43, 44]. These activities are mediated by the interaction with integrin receptors, α3β1, α5β1, α5β1, and α6β1 [39, 45].

In the current study, Ln5 and integrin α5β1 are expressed by tumor cells in general areas indicating the potential for adhesiveness and migration on fibronectin matrices (Figure 3). Integrin α5β1 and integrin α5β3 are not expressed in benign melanocytic lesions and early primary melanoma,but emerge in advanced primary melanomas and metastases. Malignant melanoma cells express integrin α5β1 traverse the underlying dermis, gain access to lymphatics and blood vessels to result in metastases [46, 47]. In the general areas, infiltrating margins, outer layers of the TVC, and bordering VM, integrin α5β1 and Ln5 are co-expressed with NFP, Syn and catecholamines.

The tumor cells at the advancing edge produce Ln5 and integrin α5β1 (Figure 4), both of which are involved during transgression of the local ECM to allow the cells to move forth further. Ln5 is frequently expressed at the invading edges of epithelial tumor cells as observed in many immunohistochemical surveys [30, 36, 48, 49]. Epithelial carcinoma cells prefer to adhere onto the Ln5 rich BMs using the specific integrins as receptors.

Tumor angiogenesis is initiated in the pre-existing vasculature accompanied by the growth of endothelial buds towards the tumor margins. The capillary buds cannelise and tumor cells grow and proliferate around these thin walled vessels forming TVCs. The tumor vascular complex was investigated as an in situ model in melanoma. The expression and role of Ln5 and Integrin α5β1 was studied.

In the TVC, Ln5 and integrin α5β1 expression is in the outer layers with maximum positivity in the layers 4 and 5 (Figure 5). Increased integrin α5β1 in the outer layers supports the migration of the tumor cells at the periphery maintaining a compact outline. Integrin α5β1 helps both in construction and destruction of the matrix, leading to rapid tissue remodeling as the cells migrate outward.

TVC shows organized stepwise neural differentiation mimicking embryonic neurogenesis of biogenic aminergic cells. The middle layers show indoleamines with mitotic cells while the outer are positive for catecholamines [8]. The Ln5/integrin α5β1 positivity indicates that the catecholamine cells migrate to the outer layers being motile cells while the indoleamine cells remain in the middle layers. Since integrin α5β1 is also known to mediate strong intercellular cohesion of 3D cellular aggregates [5], contributing to tissue compaction and cohesiveness its expression in the TVC is of particular interest.

In this study, the outer layers of the TVC engage in cell matrix interactions and move forward as a whole, while the cells in the inner layers proliferate to replace the gap created by the migration. Outward movement of cells occurs without disturbing its inner architecture. The leading cells in a collective generate actin and integrin mediated traction as seen in in-vitro studies. A linear cortical actin network extends along cell-cell junctions into deeper regions of the collective, sustaining collective integrity [50]. Collective cell movement is a mechanism for invasion and metastasis in epithelial cancers and melanoma [51, 52].

In local invasion, collective migration result in protruding sheets and strands that maintain contact with the primary site. These characteristics are histologically detectable in invasive epithelial cancer and melanoma. Cell clusters detach from their origin and migrate along interstitial tissue and perineural spaces as seen in melanoma [52]. The large cell collectives produce higher concentrations of promigratory factors and matrix proteases and protect inner cells from immunological assault. Cells of different clonal origin or cells of different biological abilities are likely to function together (mixed clone behaviour), more migratory cells can promote the invasion of less mobile or even immobile cells. Once cancer cell collectives have reached the lymph or blood stream, they might be more efficient in embolising small vessels and survive in even hostile environment to establish metastasis.

Migrating cell collectives are frequently arranged as 2D sheets, like an epithelial layer migrating across substrate or 3D solid strands develop as in neural crest migration [53]. Alternatively, centrally hollow tubular structures represent tubulogenesis, vessel sprouting or branching morphogenesis [54].

In undifferentiated poorly pigmented areas the tumor expands rapidly so that the central zone quickly loses contact with the stroma and the angiogenic vessels. Collections of Ln5, integrin α5β1 and Syn positive cells form loose irregular spaces within the sheets and chords of tumor cells to form the nidus for vasculogenesis (VM) (Figure 6). These enlarge into sinusoids showing haemopoeitic activity and continue into lakes of blood with flattened tumor cells. The spaces extend to form a tubular network which matures into a rich capillary network [15].

In mixed tumors showing both TVC and VM, sheets of tumor cells surrounding angiogenic vessels show collections of Ln5 and integrin α5β1 positive tumor cells lining slit like spaces which connect with the surrounding angiogenic capillary network (Figure 7). The LN5/integrin α5β1 positivity and the presence of mitosis suggest that these are cells which have switched to an aggressive invasive phase in the bordering cells. These findings resemble the VM looping patterns described in the 3D studies [55-57] where they are identifiable by the laminin positivity of the bordering cells [58-61]. Thus

there is a switch over to the rapid fire vascularisation to facilitate rapid growth, mimicking embryonic conditions as the melanoma becomes poorly differentiated. Ln5 and integrin α5β1 positivity in the tumor melanocytes heralds this switch.

Ln5 and integrin α5β1 are important for the invasion of the stroma. The tumor cells generate acellular microcirculatory channels composed of ECM and lined externally by tumor cells [62] in aggressive primary and metastatic melanomas,. Vasculogenic mimicry describes the generation of non-endothelial cell-lined channels delimited by ECM in aggressive tumors. The molecular profile of aggressive cutaneous and uveal melanoma cells reveals multiple phenotypes simulating a pluripotent, embryonic-like stem cell [55-57].

Remodeling of the extracellular matrix is an integral part of normal tissue growth and differentiation. MMPs generally function to degrade proteoglycans and matrix glycoproteins. Important to cancer progression, loss of basement membrane integrity may correlate with an increased probability of distant metastasis and poor prognosis [67]. Therefore, overexpression of MMPs may be one part of the multistep process by which the neoplastic cell can proliferate and metastasize. Proteolytic degradation of the ECM aids tumour invasion by clearing a pathway for the invading tumour cells. In addition, the proteolytic activity probably releases several factors from the ECM that promote cell proliferation and angiogenesis.

Integrin α5β1 upregulates MMP expression. Highly invasive melanoma cells express relatively high levels of α5β1 and low levels of α5β3. The switch in integrin expression leads to even higher MMP-2 production, which correlates with higher invasion capacity of these cells [40, 68]. Thus high integrin expression at the invasive front leads to higher proteolysis related activities, which in turn leads to the alteration of Ln5 towards a motility inducing structure.

Increased FN matrix assembly in this context can promote cell aggregation, spheroid formation, and cohesion supporting tissue compaction and remodeling. The net effect of integrin-FN interactions in 3D is force generation and cellular rearrangement into a close-packed, spherical conformation [69]. Thus the presence of higher α5β1 expression in the outer layers of the TVC is crucial for maintaining the spherical closely packed structure.

Integrin α5β1 promotes in vitro and in vivo survival of cells in metastatic melanoma [68, 70] and correlates with migratory behavior of malignant cells as well as the normal human melanocytes to adhere, spread and migrate in skin wounds. In tumor cells, it is of advantage to have more integrin α5β1 and less integrin αvβ3 to facilitate rapid binding to blood clots and other matrix components. Thus a shift from αvβ3 to α5β1 signals enhanced invasiveness increased motility [19, 46, 71, 72].

In conclusion, Ln5/ integrin α5β1 expression along the infiltrating margins at the tumor-stroma inter-phase show that the matrix proteins and the matrix-degrading proteases become focalized to substrate contacts for the advancing cell mass. The tumor cells move forward over Ln5 matrix aided by the motive force generated by integrin.

The TVC behaves like a single unit and the cells migrate outward collectively with integrin α5β1 providing attachment and dynamic force generation to maintain the architecture. Cells proliferating in the middle layers of the lobule replace the gap created by the outward migration. Organized neural differentiation with co-expression of NFP/Syn and LN5/integrin indicate the migratory nature of the catecholaminergic melanotic/ neuronal cells.

Ln5/integrin α5β1 positivity at sites of vasculogenesis correlates with aggressive primary and metastatic melanomas. Acellular microcirculatory channels composed of ECM and lined externally by tumor cells are generated by Ln5/integrin α5β1 positive tumor cells [63]. In vasculogenic mimicry aggressive tumor cells generate non-endothelial tumor cell-lined channels delimited by ECM.

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Conflicting interests

The author declares that there are no conflicting interests.

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