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Phospho-β-catenin expression in primary and metastatic melanomas and in tumor-free visceral tissues, and associations with expression of PD-L1 and PD-L2.

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Abstract

β-catenin (βcat) is an important downstream effector in the Wnt signaling pathway and plays important roles in the development and progression of many cancers including melanoma. βcat expression is regulated by GSK-3β-mediated phosphorylation at positions 33, 37 and 41. In normal cells, phosphorylation at these sites triggers proteasomal degradation, which prevents accumulation of free cytoplasmic βcat. In cancer cells, stabilized β-catenin translocates into the nucleus, where it associates with TCF/Lef proteins to activate transcription of genes that promote tumorigenesis and metastasis, including PD-L1. It has been suggested that nuclear phospho-βcat (pβcat) staining may be diagnostically useful in differentiating primary from metastatic melanoma. Also, a pβcat peptide (residues 30–39, with only S33 phosphorylated) is naturally presented by melanoma cells as a T-cell target. We evaluated expression of pS33-βcat in primary and metastatic melanomas by immunohistochemistry and found its expression varied widely but

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Competing Interests

RCO and VHE acknowledge conflicting financial interests as shareholders in Agenus, Inc. CLS is an inventor on patents for peptides used in cancer vaccines held by the University of Virginia Licensing and Ventures Group but not for phosphopeptides. CLS also has current or prior consultant roles for Celldex, CureVac and Castle Biosciences, and has institutional support from Celldex, Polynoma, GSK, Merck, 3M, Theraclion and Immatics.

CRediT author statement

Joel Pinczewski: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Software; Validation; Visualization; Writing - original draft; Writing - review & editing; **Rebecca Obeng**: Resources; Roles/Writing - original draft; Writing - review & editing; **Craig Slingluff**: Conceptualization; Project administration; Resources; Supervision; Validation; Writing - review & editing; **Victor Engelhard**: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing

was most commonly cytoplasmic. Nuclear staining was identified in only 18% of metastatic melanomas. Staining with antibodies to pS33-βcat and pS33/37/T41-βcat was most intense in mitotic melanoma cells; however, pS33-βcat intensity was not significantly associated with AJCC stage, tumor location, BRAF mutation status, or immune infiltrates. Yet, PD-L1 and PD-L2 expression by tumor cells were significantly higher in tumors with high pS33-βcat expression. The low rate of nuclear pS33-βcat expression suggests that pS33-βcat may have limited utility for identifying metastatic melanomas. However, high expression in dividing cells and strong associations with PD-L1 and PD-L2 expression may inform future personalized therapies for tumors with high pS33-βcat expression.

Keywords

beta-catenin; immunohistochemistry; human; melanoma; metastasis

Introduction

β-catenin (βcat) forms a downstream portion of the highly conserved canonical Wnt cell signaling pathway. As a member of this pathway, βcat has a pivotal role in embryogenesis and in cell signaling in later life. Mutations in βcat or the proteins that regulate it (e.g., the adenomatous polyposis coli (APC) protein), which lead to accumulation of βcat, are associated with the development and progression of a variety of benign and malignant neoplasms, including familial adenomatous polyposis, Gardner's syndrome, and colorectal cancer[4, 11, 31, 35]. However, mutations resulting in βcat accumulation are also associated with other malignancies including gastric and endometrial adenocarcinomas, hepatocellular cancers, hepatoblastomas, and melanomas[30, 31]. In unstimulated adult cells, βcat is located mainly on the cytoplasmic side of the cell membrane, where it associates with E-cadherin as part of adherens junctions [28]. Free cytoplasmic βcat is maintained at a low level by formation of a destruction complex involving βcat, glycogen synthase kinase-3 β (GSK-3β), casein kinase 1a (CK1a), APC and Axin [3]. This leads to sequential phosphorylation of the complexed βcat at T41, S37, and S33 by GSK-3β, targeting it for destruction by the ubiquitin/proteasome pathway. Signaling through the Wnt pathway results in a different fate for βcat[15]. In this setting, free βcat is protected from phosphorylation and is able to accumulate in the cytoplasm. This build-up in cytoplasmic βcat leads to its eventual translocation to the nucleus where it complexes with T-cell factor (TCF)/ lymphoid enhancer-binding factor (LEF) and other molecules and effects transcription of genes involved in tumorigenesis and metastasis such as c-myc, metalloprotease, and PD-L1 [6, 8, 12, 14, 29]

Zarling et al have identified a βcat peptide spanning amino acids 30–39 and phosphorylated at S33 on melanoma, which is presented by human HLA-A2 molecules as an antigen for cytotoxic T cells [36]. This suggests that N-terminal phosphorylation of βcat occurs in melanoma cells and the resulting peptide may be a target for cancer immunotherapy. Phosphorylation at S33 only occurs after phosphorylation at residues 37 and 41 by the kinase GSK-3β; so, detection of pS33-βcat identifies the tri-phosphorylated form of βcat. pS33-βcat has not been shown to have transcriptional activity, but some authors have

reported that the presence of cytoplasmic and nuclear pS33-βcat staining in malignant cells has diagnostic and prognostic significance. Nakopolou found that cytoplasmic pS33/37/T41-βcat staining was associated with an improved outcome in breast cancer, whereas high levels of nuclear pS33/37/T41-βcat staining were associated with a poor outcome [25]. Kielhorn found that most primary and metastatic melanomas did not stain with an antibody detecting pS33/37/T41-βcat[18]. When present, however, pS33/37/T41-βcat staining was always nuclear and was more commonly seen in metastatic melanoma than in primary melanomas. Also, survival was enhanced for patients whose tumors had lower expression of pβcat; thus, it has been suggested that nuclear pS33/37/T41-βcat staining and its intensity might be useful as a diagnostic and prognostic marker in melanoma.[18]

Activation of the Wnt/βcat pathway in melanoma has been reported to have immunological significance, with reduction in immune cell infiltrates in melanomas with Wnt/βcat activation[33]. Coupled with the finding that a pS33-βcat peptide is an epitope recognized by human $CD8^+$ T cells[36], this heightens interest in understanding the relevance of the S33 phosphorylation site of βcat in melanoma. The relevance of pS33-βcat as a T cell target requires understanding whether the pS33-βcat form of the protein is expressed in normal tissues from vital organs and whether it is selectively expressed by melanoma cells compared to such normal tissues. βcat is expressed in normal tissues including heart and lung, but regulation of βcat phosphorylation is complex enough that expression levels of βcat may not predict expression of pS33-βcat. To our knowledge, the expression of pS33-βcat in human normal tissues has not been assessed. We hypothesized that pS33-βcat would be expressed at higher levels in melanoma metastases than in normal tissues. Thus, in the present study, we performed immunohistochemistry (IHC) studies to determine the breadth of pS33-βcat expression in primary and metastatic melanoma tissues as well as in normal human tissues, and to assess associations with other clinical and prognostic factors. We also compared the staining pattern of the pS33-specific and pS33/37/T41-specific antibodies, the levels and intracellular locations of pS33-βcat staining in primary and metastatic melanoma specimens, and pS33-βcat staining in normal tissues to re-evaluate the potential use of pS33-βcat as a diagnostic and prognostic indicator for melanoma.

Material and Methods

Melanoma tissues for analysis in this study included samples of formalin-fixed and paraffin-embedded (FFPE) specimens from a melanoma tissue microarray (TMA), preparation of which has been described previously [9]. These included 56 metastatic melanomas and two large primary melanomas, as well as samples of normal tissue controls. Staining for p**β**catenin was assessed in the context of clinical outcomes, the pattern of immune cell infiltrates, BRAF V600E mutation status, and expression of PD-L1, and PD-L2, as previously reported [9, 26]. The slides were reviewed by a pathologist (JP) and the staining intensity (on a semiquantitative scale: 0, 1+, 2+, 3+) was recorded in nuclear, cytoplasmic, and membranous regions.

To compare pβcat expression in melanoma metastases and adjacent normal tissues, additional FFPE samples were obtained from the autopsy specimens and from archives of the Department of Anatomic Pathology at the University of Virginia (IRB-HSR 15237

and 10598). These included metastatic melanomas in lung (n=5), heart (n=1) and liver (n=1) with evaluable portions of non-malignant ("normal") tissue from the same organs that had been invaded.

Cells of the human melanoma cell line SLM2 [19] growing in log phase also were pelleted, formalin fixed, paraffin-embedded, and then sectioned for the study. Tissue sections were deparaffinized in xylene and rehydrated by sequential passage through graded alcohol/water solutions. Heat-induced antigen retrieval was performed using Vector's Target Retrieval Solution (Vector Laboratories, Burlingame, CA), pH 6 at 100°C for 20 min.

Working concentrations of antibodies to βcat (1:500, Epitomics, Burlingame, CA # 1247–1) and phospho-βcat (pS33-βcat 1:200, Santa Cruz, Santa Cruz, CA # SC-16743-R; pS33/37/T41-βcat 1:1200, Cell Signaling Technology, Danvers, MA, #9561) were used. In some assays, a 13-mer pS33-βcat blocking phosphopeptide (QSYLDpSGIHSGAT, Genscript, Piscataway, NJ) was added to anti-βcat and anti-pS33-βcat antibodies (1:200), giving a final peptide concentration of 50 μg/ml, and incubated for 30 min prior to being added to slides for blocking. The slides stained for pS33-βcat were incubated overnight at 4 degrees, and slides stained for βcat were incubated for 30 min at room temperature. Expression was detected using Vector's ImmPRESS peroxidase kit and visualized with Vector's ImmPACT™ AEC substrate. Slides were then counterstained with Gill's hematoxylin. Negative control slides were prepared by omitting the primary antibodies. Slides were scanned using an Aperio CS slide scanner (Aperio, Vista, CA) and were analyzed using the Aperio Imagescope "positive pixel count" analytical software algorithm. The resulting data were used to calculate total staining density per unit area.

Results

The specificity of the polyclonal pS33-βcat antibody was tested by staining four normal tissues -- liver, placenta, spleen and kidney (on the TMAs) – with and without pre-incubation with a corresponding pS33-βcat phosphopeptide as a control. As a further control, a commercial anti-βcat antibody was also used. All four normal tissues stained strongly with both the pS33-βcat and βcat specific antibodies (Figure 1). Staining with the pS33-βcat antibody was significantly reduced by pre-incubation with pS33-βcat phosphopeptide, confirming its specificity. As expected, pre-incubation with the blocking phosphopeptide had minimal effect on βcat antibody staining.

To assess the diagnostic and prognostic value of pS33-βcat, we assessed the intensity and staining location of the pS33-βcat antibody in TMA blocks containing 2 primary and 56 metastatic melanomas. Examples are shown in Figure 2A–C. In some cases, the staining was fairly homogeneous, but in others, there was marked heterogeneity, as shown in this example with membranous and cytoplasmic staining (Figure 2A). An example of diffuse cytoplasmic staining (1+ to 2+) is shown in Figure 2B. Some cells in that tumor also have nuclear staining. More consistent nuclear staining is shown in Figure 2C. The two primary melanomas had cytoplasmic staining, but lacked nuclear or membranous staining: one 1+, one 2+ (not shown). Among the 56 metastatic melanomas, 49 (88%) had cytoplasmic staining, ranging from 1+ to 3+. Nuclear staining was observed in 10 cases (18%), of which

8 also had cytoplasmic staining. Membranous staining was observed in 8 (14%), of which 6 also had cytoplasmic staining. A complete lack of staining was observed in 5 tumors (9%). Details are shown in Figure 2D, E.

These 58 patients were categorized based on expression of pβcat, where no expression or only 1+ cytoplasmic expression was considered low pβcat (n = 23). High expression tumors (n = 35) included those with 2+ or 3+ cytoplasmic staining, or any nuclear or membranous staining (all of these were at least 2+). The distributions by sex, age, stage at surgery, status of disease after surgery, tumor location and type, or BRAF V600E mutation status were similar between the two groups (Table 1), and immune cell infiltrates were similar between the two groups (Supplemental Figure). On the other hand, expression of PD-L1 and PD-L2 by melanoma cells was significantly higher in tumors with high pβcat staining ($p = 0.0013$ and $p = 0.0166$, respectively; Table 1). Overall survival was also assessed for tumors with high or low pβcat, where there was a non-significant trend to longer survival for tumors low in pβcat ($p = 0.20$, Figure 3A). When evaluating also by stage, there also was a non-significant trend to longer survival for low pβcat tumors in patients with stage 3 melanoma ($p = 0.38$, Figure 3B).

To explore whether there were differences in the level of pS33-βcat in tumors and normal tissues that might have relevance in the diagnosis and treatment strategies for melanoma patients, we compared staining in melanoma metastases versus adjacent lung, liver, and heart tissue that did not contain melanoma. Whole sections of lung, liver and heart tissue, containing both metastatic melanoma and adjacent uninvolved tissue, were stained with the pS33-βcat antibody. There was intense staining in uninvolved lung tissue and in respiratory epithelia (Figure 4A, C, E, G, I). Metastatic melanoma in the lung tissue (Figure 4) mainly had cytoplasmic staining but also included cases with minimal staining (Figure 4F, J) and others with more significant staining (Figure 4B, D). Variations in melanoma staining for pS33-βcat were also noted in some specimens: an example image in Figure 4H shows a region with no evident staining adjacent to another with moderate staining plus isolated mitotic tumor cells with intense staining. As shown in Figure 5A, cardiac myocytes had higher staining intensities than the metastatic melanoma in heart tissue, with the most intense staining occurring in the intercalated discs (Figure 5B). In liver tissue, there was only very weak staining for both metastatic melanoma and adjacent uninvolved hepatocytes (Figure 5C).

Phosphorylation of pS33 requires prior phosphorylation of S37 and T41 by GSK-3β[16]. We therefore hypothesized that an antibody to pS33-βcat would produce comparable staining to an antibody to pS33/37/T41-βcat. We stained an SLM-2 melanoma cell line with both antibodies (Figure 6). Indeed, the two antibodies produced similar staining. For both antibodies, staining was largely cytoplasmic, with a minority of cells having combined nuclear/cytoplasmic staining. Nuclear staining was somewhat more evident with the pS33/37/T41-βcat antibody than the pS33-βcat antibody, and cytoplasmic staining may be slightly more evident with the pS33-βcat antibody. One particularly striking finding was that mitotic cells showed much more intense staining with both pβcat antibodies than melanoma cells without mitotic figures.

Discussion

We have found that melanoma metastases express pS33-βcat at varied levels and that the most common staining pattern in melanoma cells was cytoplasmic. In contrast to expectation, there also was significant expression of pS33-βcat in multiple normal tissues, including liver, placenta, spleen, kidney, lung, and heart. The validity of the antibody stain was confirmed by blocking with a specific phospho-β-catenin peptide. When evaluating lung and heart tissue containing melanoma metastases, the expression of pS33-βcat was commonly higher in the surrounding normal tissue than in melanoma cells of the same specimen, though heterogeneity of staining was noted in some normal and malignant tissues. A striking finding, however, was that the intensity of pS33-βcat staining in melanoma cells was greatest in actively dividing cells (Figure 4H and Figure 6.).

βcat serves an important function in embryogenesis and tissue development and is also an important downstream mediator of the development and progression of cancer. The N-terminus is of particular interest since this region contains phosphorylation sites that are critical for regulation of cellular levels of βcat that in turn affect the protein's transcriptional activity. It has been suggested that pβcat expression may have diagnostic and/or prognostic value and specifically that nuclear staining of melanoma by an antibody to pS33/37/T41-βcat might be useful as a prognostic marker and also a diagnostic marker to differentiate metastatic melanoma from primary melanoma. However, we found that cytoplasmic pS33-βcat staining was the most common staining pattern. In fact, exclusively nuclear staining only occurred in a single melanoma, and a complete lack of staining was also uncommon. Our finding of widespread cytoplasmic pS33-βcat staining in melanoma is much more in keeping with what might be expected if GSK-3β activity is maintained in the majority of melanomas. Kielhorn had reported that nuclear staining with the pS33/37/T41-βcat antibody was associated with metastatic melanoma[18]. The antibody used in that prior work detects the triply phosphorylated peptide (S33/37/T41) whereas our antibody was generated using a pS33 peptide. In our comparison of the two antibodies, nuclear staining was somewhat more evident with the pS33/37/T41-βcat antibody than the pS33-βcat antibody. Thus, the discrepancy in the localization of pβcat between our results and previous observations may be related in part to the pβcat antibodies used. However, only a minority of metastatic melanomas had nuclear staining for pβcat in both studies (18% in this study and 41% in the Kielhorn study); so, staining for pβcat is likely to be of limited sensitivity for identifying metastatic melanomas. Kielhorn acknowledged that their finding was paradoxical, and suggested that it might be due to a defect in the proteasomal breakdown machinery in melanoma cells [18]. However, while this would explain why pβcat might build up in the cells, it does not explain why the protein would be exclusively found in the nucleus. Our findings using both pS33-βcat antibodies are consistent with expectation that the most common location of pβcat is the cytoplasm.

To evaluate whether pS33-βcat expression differed between melanomas and normal tissues in vital organs, we selected tissue blocks containing metastatic melanoma with attached uninvolved tissue from the invaded organ to exclude tissue handling as a source of variability. We found that cytoplasmic pS33-βcat staining was less intense in the metastatic melanomas than in the uninvolved tissues in most cases. This could be due to a decrease in

βcat phosphorylation in the metastatic cells; however, it seems unlikely since mutations in the N-terminus of βcat or in the components of the destruction complex such as APC are rare in melanoma [30, 31]. Alternatively, these findings may also reflect an overall decrease in the level of βcat protein in melanoma, as previous studies have shown [16, 21, 32]. This may be due to enhanced degradation of the protein as opposed to decreased gene expression since increased levels of mRNA have been found in metastatic melanoma tissues compared to primary melanoma [7]. It is also possible that because melanoma cells are actively in cycle, pβcat is degraded more rapidly in melanoma cells than in normal tissues. βcat has been shown to accumulate in the cytoplasm during the cell cycle, with peak levels evident in the G2/M phase [27]. Degradation of βcat, by phosphorylation, is needed for the cells to progress from the G2 phase through the M-phase[27].

This correlates with our finding that mitotic figures in melanoma tissues show increased pS33-βcat staining intensity compared to cells not in the M phase of the cell cycle. Thus, the increased frequency of cell division in melanoma may account for an overall low level of pβcat while the increased levels observed in normal tissues may reflect steady state levels and a decreased rate of degradation owing to decreased frequency in cell division.

To our knowledge, the expression of pβcat in normal tissues has not been reported elsewhere. We found that some normal heart and lung tissues have high levels of pS33-βcat, with the most intense staining in the intercalated discs of myocytes and respiratory epithelial cells, respectively. βcat forms part of the adherens junction in intercalated discs. βcat has previously been reported to accumulate in the intercalated discs of myocytes in patients with hypertrophic cardiomyopathy[23]. Furthermore, Maher et al have reported that pβcat and components of the destruction complex (axin, APC2, and GSK-3β) can localize to areas of cell-cell contact that are distinct from cadherin-catenin complexes at the cell membrane and facilitate phosphorylation of βcat, thereby leading to speculation about the possible role of βcat in cell fate during development and tissue morphogenesis [22]. Thus, the high-level expression of pS33-βcat at the intercalated discs of the myocytes may be due to accumulation and phosphorylation of βcat at that site in normal tissues.

One further interesting finding from our work was that pβcat staining was sharply increased in mitotic melanoma cells with antibodies both to pS33-βcat and to pS33/37/T41-βcat. This may be due to an increase in βcat during mitosis. Indeed it has been shown that βcat and APC, a key component of the destruction complex, accumulates in cells during the cell cycle, with the highest level of βcat being detected at the G2/M phase followed by a sharp decrease in βcat levels when cells enter the G1 phase [27]. The expression of pS33-βcat in mitotic cells may also be related to the role of βcat in centrosome disjunction and splitting [1, 2, 17]. Phosphorylation of S33/37/T41 and S45 can also be mediated by the NIMA-related kinase 2 whose kinase activity is highest during mitosis [2, 10, 24]. pβcat has been shown to accumulate in centrosomes and may assist in proper bipolar spindle formation[5, 24]. Defects in cell polarity and microtubule organization have been observed in neuronal progenitor cells expressing a stable form of βcat that has alanine substitutions for the S33/37/T41 and S45 phosphorylation sites [1, 2, 5]. Thus, pβcat may be necessary for microtubule organization and proper cell division. Eventual degradation of βcat is necessary for cells to progress through the M phase. Thus, the strong pS33-βcat

staining we observed in the mitotic melanoma cells is likely indicative of the imminent degradation of βcat in melanoma cells in this phase of the cell cycle. This finding may be useful in assessing whether pβcat could be targeted for cancer immunotherapy.

The most effective immunotherapy for melanoma is checkpoint blockade with antibodies to PD1 or to PD-L1, and its effectiveness is associated with expression of PD-L1. PD-L1 is upregulated on tumor cells in the presence of IFNγ[34] and functions to limit immune-mediated destruction of melanoma cells in the tumor microenvironment. Thus, it mediates immune dysfunction, but is commonly induced in the presence of functioning CD8 T cells [34]. However, in this study, we find that pβcat expression is associated with PD-L1 and PD-L2 expression, but not with increased infiltrates of CD8 cells or other immune cells. Thus, this finding may reflect an intrinsic pathway for increasing PD-L1 and PD-L2 expression independent of T cell infiltrates, and possibly related to pβcat expression. Activation of the Wnt/βcat pathway has been reported to upregulate PD-L1 expression in fibroblasts [14]. In glioblastoma cells, the β-catenin/TCF/LEF complex induces PD-L1 expression by binding its promoter, and AKT plays a role in that induction[8]. These data support combinatorial blockade of AKT and PD-L1 for GBM [8, 13]. Further, β-catenin has been downregulated by a histone demethylase inhibitor (5-carboxy-8-hydroxyquinoline (IOX1)), with resultant downregulation PD-L1 [20]. Also, inhibition of Wnt and/or βcat has enhanced tumor response to PD-L1 blockade in a murine lung cancer model. Our findings raise interesting questions of whether the role of PD-L1 as a biomarker for response to PD-1 blockade may be enhanced by distinguishing whether the PD-L1 expression is interferon-γ-dependent or due to a different intrinsic pathway associated with βcat activation. It is possible that precision therapy approaches may benefit from using the antibody to pS33-βcat to identify those in whom constitutive PD-L1 expression may be IFNγ-independent and/or to consider combination therapies that concurrently block Wnt/βcat and PD-L1.

Conclusions

Overall, our results indicate that the pS33-βcat and pS33/37/T41-βcat antibodies produce similar staining patterns in melanoma. Cytoplasmic pβcat is most predominant in the tumor cells in both primary and metastatic melanomas. The pS33/37/T41-βcat antibody may stain nuclei somewhat more readily and the cytoplasm less readily than the pS33 antibody. Our data do not support use of the pS33-βcat antibody for diagnosis of metastatic melanoma. However, there was a weak trend toward improved survival with low pβcat expression, which may or may not be meaningful in a much larger dataset. Interestingly, we observed strong pβcat staining in mitotic melanoma cells. Other studies have demonstrated that degradation of βcat is necessary for progression through the M phase. Thus, targeting the pS33-βcat peptide might be a way to target proliferating melanoma cells. Finally, pS33-βcat expression was significantly associated with PD-L1 and PD-L2 staining on tumor cells, despite no association with T cell infiltration. These findings suggest that PD-L1 expression may be interferon-γ-dependent or due to a different intrinsic pathway. It is possible that precision therapy approaches may benefit from using the antibody to pS33-βcat to identify patients who may benefit from combinations of PD1 antibody and an agent targeting Wnt/βcat.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

APC	adenomatous polyposis coli protein
βcat	beta-catenin
pβcat	phospho-beta-catenin
CK1a	casein kinase 1a
FFPE	formalin-fixed paraffin embedded
GSK-3β	glycogen synthase kinase-3 β
IHC	immunohistochemistry
LEF	lymphoid enhancer-binding factor
PD-L1	programmed death ligand1 a
PD-L2	programmed death ligand 2
TCF	T cell factor
TMA	melanoma tissue microarray

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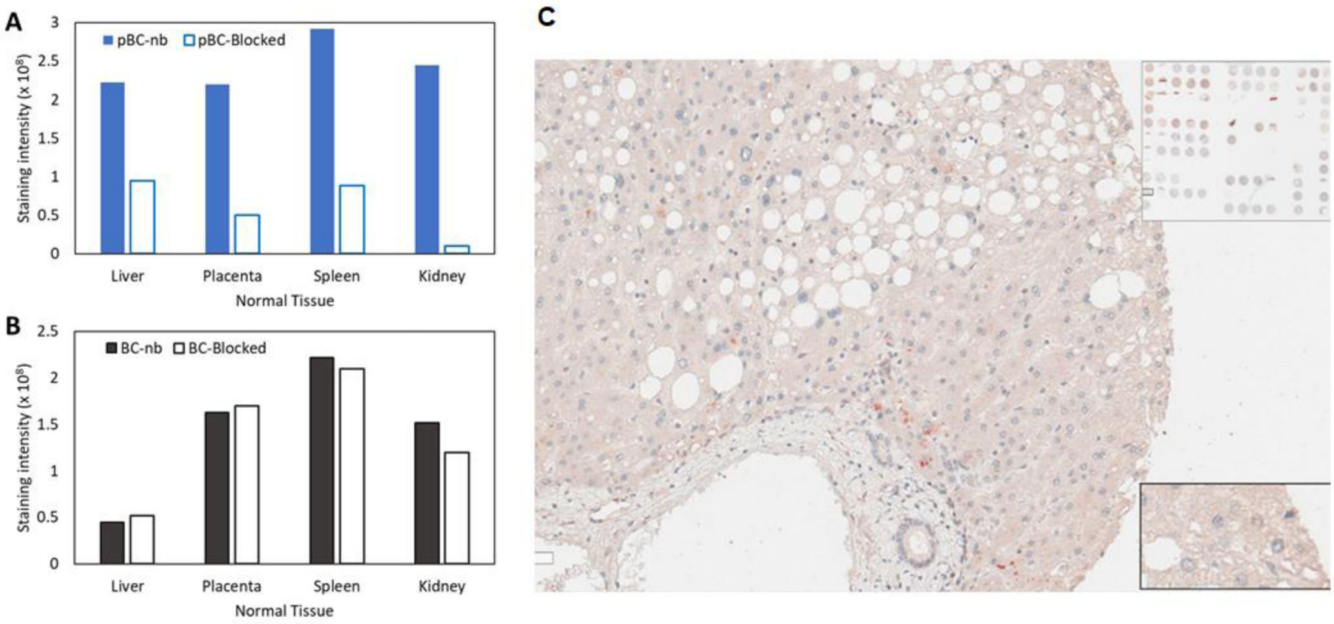

Figure 1.

Phospho-β catenin in normal human tissues. Total staining density per unit area for core samples of normal liver, lung, spleen and placenta, as normal controls from the TMA blocks, stained with antibodies to (**A**) pS33-βcat and (**B**) βcat, with pre-incubation with pS33-βcat blocking phosphopeptide (pβcat-Block and βcat-Block) or without blocking (pβcat-nb and βcat-nb). An example of weak cytoplasmic pβcat staining of non-malignant liver control is shown in (**C**).

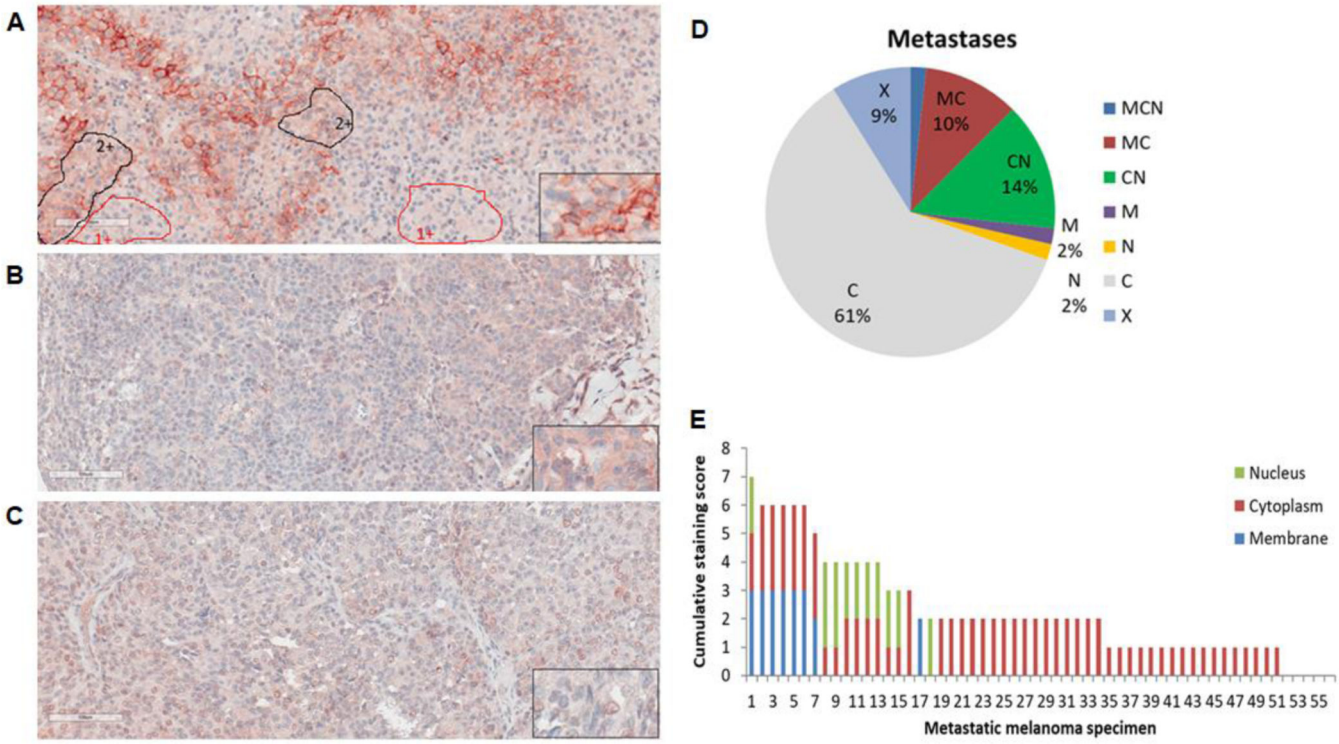

Figure 2.

Expression and localization of phospho-β-catenin in metastatic melanomas and associations with survival. Representative sections of pS33-βcat stained tissue from 3 TMA cores, with (**A)** 3+ membranous staining and a mixture of 1+ and 2+ cytoplasmic staining (some areas containing each type of staining are indicated on the image), (**B**) 1+ cytoplasmic staining on the left and 2+ on the right., and (**C**) nuclear staining. (**D)** The proportion of melanoma metastases with nuclear (N), cytoplasmic (C), membranous (M) staining, combinations (MCN, MC, CN), or no staining (X). (**E**) The extent of staining in each region is shown per metastatic tumor based on scoring of 0 to 3 in each cellular area (N, C, and M). The intensity of staining was graded from 0 (no staining) to 3+ (heavy staining).

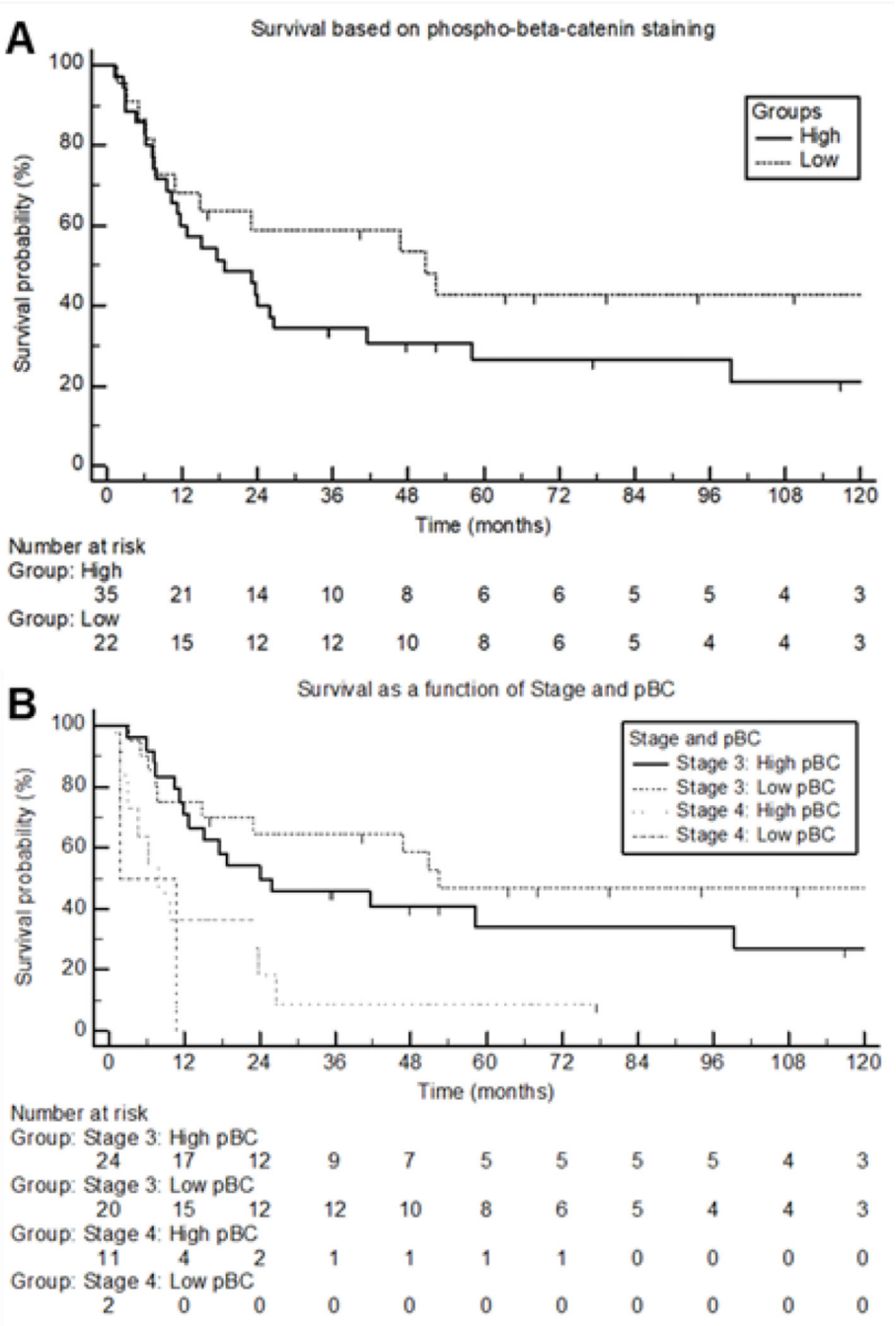

Figure 3.

Overall survival by phospho-β catenin expression. (**A**) Kaplan-Meier estimates of patient survival for those with high or low expression of pS33-βcat (not significant, p = 0.20). (**B**) Kaplan-Meier estimates of patient survival by stage for those with high or low expression of pS33-βcat (not significant, p = 0.38).

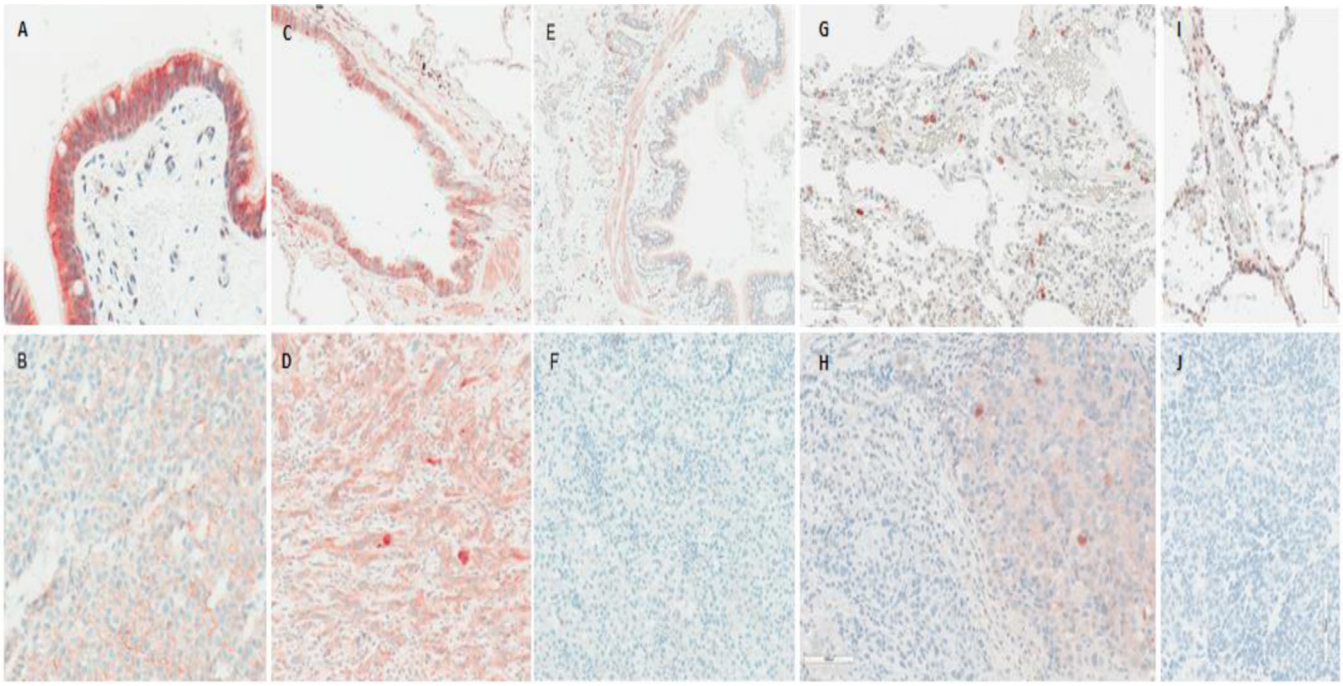

Figure 4.

pβcat staining of lung tissue and melanoma metastasis from the 5 patients. Representative sections of pS33-βcat stained tissue from five cases of metastatic melanoma to lung. (**A, C, E, G, I)** staining of uninvolved respiratory epithelium from 5 patients. (**B, D, F, H, J**) the corresponding metastatic melanomas from these cases.

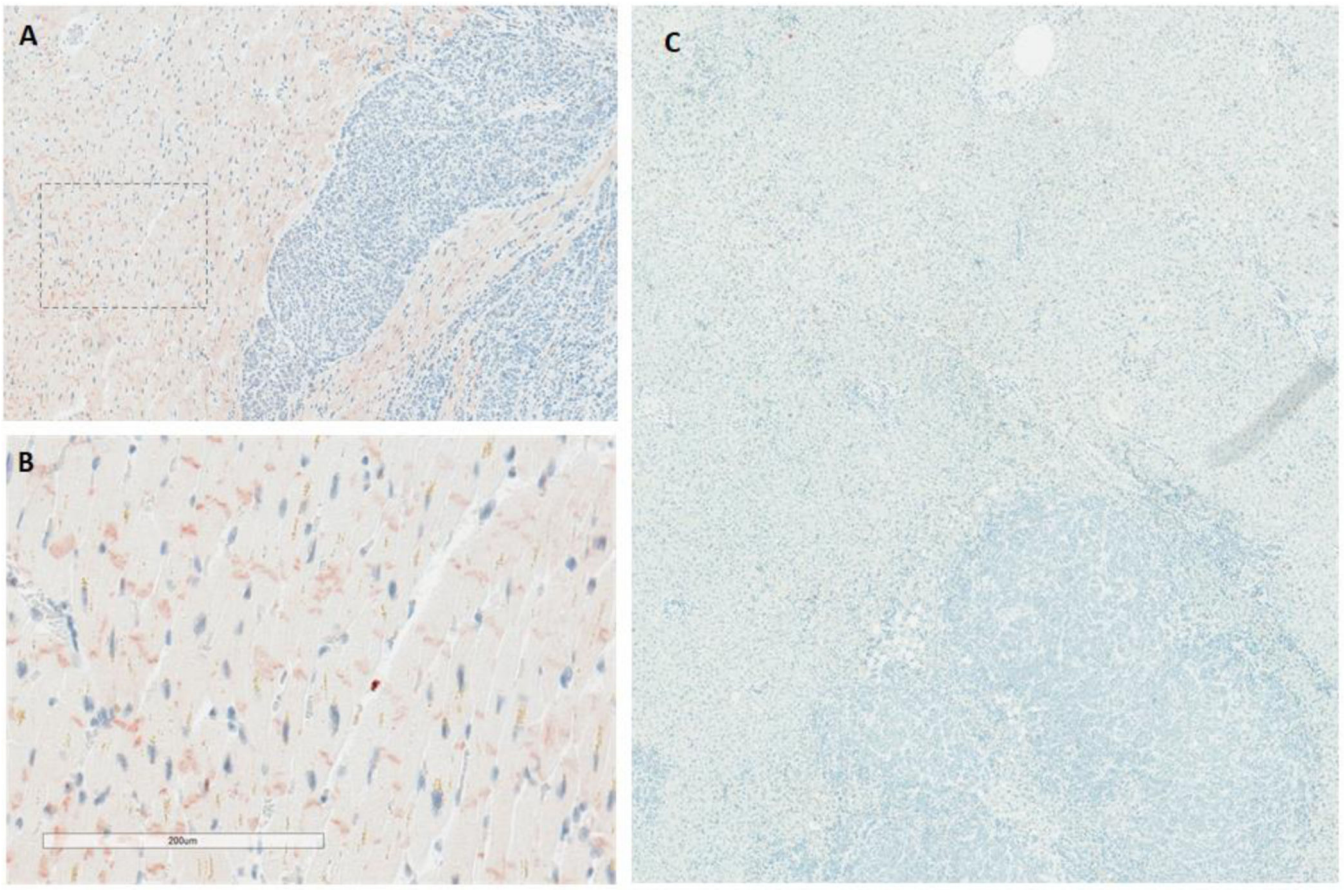

Figure 5.

pβcat staining in heart tissue and liver with melanoma metastasis. Representative section of pS33-βcat stained heart tissue from a patient with metastatic melanoma to heart at a 30x magnification. The metastatic melanoma is on the right side of the figure and has no significant staining. The uninvolved heart tissue is on the left side of the figure and has weak cytoplasmic staining (A). The region in (A) marked with a rectangle is shown at higher magnification in (B), with intense staining of the intercalated discs. Representative section of pS33-βcat stained liver (20x magnification) containing a melanoma metastasis. Neither the uninvolved liver (upper aspect of figure) nor the melanoma (lower aspect of figure) had significant staining for pS33-βcat.

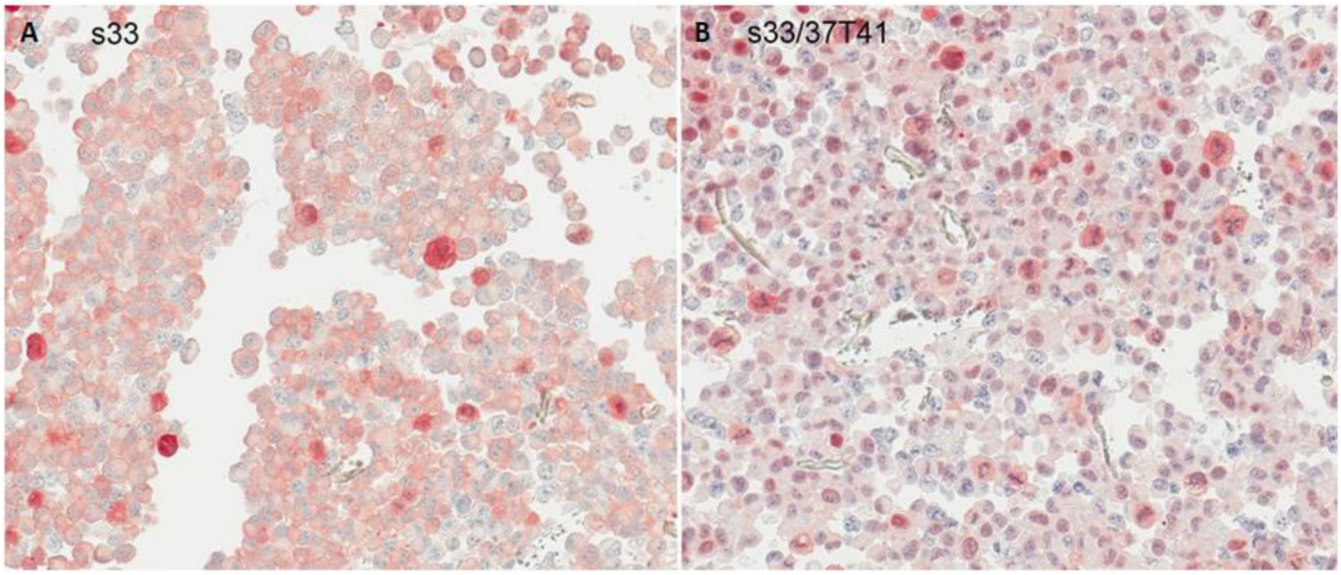

Figure 6.

pβcat staining of melanoma cell block with two antibodies. A cell block of the SLM2 melanoma cell line was stained with antibodies to pS33-βcat (left panel) and pS33/37/T41-βcat (right panel). Staining for both antibodies was predominantly cytoplasmic but also included some nuclear staining with a slight predilection for nuclear staining with the pS33/37/T41-βcat antibody. Mitotic cells stained much more intensely than adjacent non-dividing cells.

Table 1.

Clinical features of patients with melanomas evaluated for pβcatenin expression specimens

Variable	Subsets	N	pBC-High*	pBC-Low**	P value pBC High vs Low ****
Total	**All**	**58**	**35**	**23**	**--**
Sex	Male	37 (64%)	23 (66%)	14 (61%)	0.70
	Female	21 (36%)	12 (34%)	9 (39%)	
Age	Median (range) years	56 (19–89)	58 (19–89)	53 (27–82)	NS
Stage at tumor excision (AJCC version 7)	Stage IIIA	2 (3%)	1 (3%)	1 (4%)	0.276
	Stage IIIB	17 (29%)	7 (20%)	10 (43%)	
	Stage IIIC	25 (43%)	16 (46%)	9 (39%)	
	Stage IV	14 (24%)	11 (31%)	3 (13%)	
Tumor type	Primary melanoma	2	1	1	N/A
	Melanoma metastasis	56	34	22	
Disease status after surgery	No evidence of disease	47 (81%)	26 (74%)	21 (91%)	0.11
	Advanced melanoma	11 (19%)	9 (26%)	2 (9%)	
Tissue site	Metastasis: lymph node	40 (69%)	23 (66%)	17 (74%)	0.138
	Metastasis: skin/subcutis	15 (26%)	10 (29%)	5 (22%)	
	Metastasis: small bowel	1 (2%)	1 (3%)	0 (0%)	
	Large primary	2 (3%)	1 (3%)	1 (4%)	
BRAF V600E status	V600E mutated	29 (50%)	19 (54%)	10 (44%)	0.46
	V600E non-mutated	29 (50%)	16 (46%)	13 (56%)	
PD-L1 expression	>1% of tumor cells***	22 (39%)	19 (56%)	3 (14%)	**0.0013**
PD-L2 expression	>1% of tumor cells	15 (26%)	13 (37%)	2 (9%)	**0.0166**
Immunotype	A	15 (26%)	11 (31%)	4 (17%)	0.243
	B	37 (64%)	19 (54%)	18 (78%)	
	C	6 (10%)	5 (14%)	1 (4%)	

* pBC-High includes those with at least 2+ staining (nuclear, membranous, and/or cytoplasmic)

** pBC-Low includes those with no pBC staining or 1+ cytoplasmic staining only

*** one tumor not evaluable for PD-L1 expression.

**** chi-square tests (MedCalc software). P values < 0.05 shown in bold.