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## Characterization of a human $\beta$ V-tubulin antibody and expression of this isotype in normal and malignant human tissue

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### SUMMARY

There are seven distinct  $\beta$ -tubulin isotypes and eight  $\alpha$ -tubulin isotypes in mammals that are hypothesized to have tissue- and cell-specific functions. There is an interest in the use of tubulin isotypes as prognostic markers of malignancy.  $\beta$ V-tubulin, like  $\beta$ III-tubulin, has been implicated in malignant transformation and drug resistance, however little is known about its localization and function. Thus, we generated for the first time, a rabbit polyclonal antibody specific for human  $\beta$ V-tubulin. The antibody did not cross-react with mouse  $\beta$ V-tubulin or other human  $\beta$ -tubulin isotypes and specifically labeled  $\beta$ V-tubulin by immunoblotting, immunofluorescence and immunohistochemistry. Immunohistochemistry of various human normal tissues revealed that  $\beta$ V-tubulin was expressed in endothelial cells, muscle and cells with muscle differentiation, structures with transport and/or secretory function such as renal tubules, pancreatic ducts and bile ducts, and epithelium with secretory function such as prostate.  $\beta$ V-tubulin was also specifically expressed in pancreatic islets and intratubular germ cell neoplasia, where it may have diagnostic utility. In a small number of malignancies, breast, lung and ovarian cancers,  $\beta$ V-tubulin was aberrantly expressed, suggesting that this isoform may be associated with tumorigenesis. Thus,  $\beta$ V-tubulin expression is a potentially promising prognostic marker of malignancy.

### INTRODUCTION

Mammals express seven distinct  $\beta$ -tubulin isotypes, I, II, III, IVa, IVb, V, and VI and eight  $\alpha$ -tubulin isotypes<sup>1-3</sup>. Heterodimers of  $\alpha$ - and  $\beta$ -tubulin assemble head to tail to form protofilaments whose lateral assembly constitutes the microtubule wall. Each of the multiple  $\alpha$ - and  $\beta$ -tubulin isotypes are highly conserved, and are identified primarily by their specific C-terminus sequence<sup>2,4</sup>. Several isotype specific antibodies have been made by designing epitopes to these unique regions<sup>5</sup>.

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Abnormal distribution and expression of  $\alpha$ - and  $\beta$ - tubulin isotypes have been reported in numerous malignancies<sup>6</sup>, thus altered tubulin isotype expression may promote a more aggressive and drug resistant tumor phenotype<sup>7</sup>. For example,  $\beta$ III-tubulin is overexpressed in ovarian, lung, prostate, and breast cancer cell lines<sup>7, 8</sup>, and numerous studies have identified it as a prognosticator of poor survival<sup>9, 10</sup> while others have shown that  $\beta$ III overexpression may be associated with response to microtubule interacting drugs<sup>11, 12</sup>. Furthermore,  $\beta$ III-tubulin overexpression is associated with cell-based models of acquired Taxol resistance<sup>7, 11, 12</sup>, and more recently resistance to DNA-damaging drugs<sup>13</sup>. Most of the evidence that has led to the association between  $\beta$ III-tubulin expression and poor survival were derived from immunohistochemistry using  $\beta$ III-tubulin specific antibodies<sup>9, 12, 14</sup>. Therefore, studies addressing the distribution and expression of the various tubulin isotypes in normal and malignant tissue are limited by availability and specificity of antibodies. For this reason, little is known about the expression of  $\alpha$ -tubulin isotypes or some of the less well-characterized  $\beta$ -tubulin isotypes, such as  $\beta$ V. A mouse BV-antibody has been developed and well characterized<sup>5</sup>, however due to the specificity of the antibody it cannot be used to detect human BV-tubulin.

$\beta$ V-tubulin mRNA has been detected in most human tissue types using qRT-PCR<sup>15</sup> and it has been proposed that it is required for progression through mitosis<sup>16</sup>. It has also been suggested that  $\beta$ V-tubulin overexpression may mediate Taxol-dependence<sup>17</sup>, a characteristic of some Taxol-resistant cells that require small quantities of drug for normal growth in tissue culture<sup>18</sup>. Overexpression of  $\beta$ V-tubulin in Chinese hamster ovary (CHO) cells has been shown to contribute to the dependence of these cells on Taxol for growth<sup>19</sup>. Therefore,  $\beta$ V tubulin expression may be a potentially important marker for defective microtubule stabilization associated with cellular transformation, or drug resistance.

Herein we describe the generation and characterization of a human-specific  $\beta$ V-tubulin antibody and its expression by immunohistochemistry in normal and malignant tissue.

## Materials and methods

### Tubulin peptides and antibodies

The peptides CGEEAFEDDEEEIDG and CYEDDEEESEAQGPK corresponding to human  $\beta$ V- and  $\beta$ III- tubulin C-terminal sequences, respectively, were custom synthesized by the Laboratory for Molecular Analysis at Einstein College. The cysteine residue at the N-terminus of each peptide was introduced for conjugation of peptides to maleimide-activated keyhole limpet hemocyanin (KLH), or maleimide-activated bovine serum albumin (BSA) (Pierce). Rabbits were immunized with  $\beta$ V-tubulin peptide-KLH conjugates by Covance Immunology Services to produce sera containing a rabbit polyclonal  $\beta$ V-specific antibody. Bleeds from naïve and immunized rabbits were analyzed by ELISA using  $\beta$ V- or  $\beta$ III-tubulin peptide-BSA conjugates. Sera from the first bleed were used in all experiments. Other antibodies used were rodent  $\beta$ V-tubulin<sup>5</sup>, (SHM.12G11, a gift from Dr Luduena, UHSC, San Antonio),  $\beta$ III-tubulin (TUJ1 antibody, SDL.3D10, Sigma),  $\beta$ I-tubulin (SAP. 4G5, Sigma),  $\beta$ IV-tubulin (ONS.1A6, Sigma), total  $\beta$ -tubulin (DM1B, Sigma),  $\alpha$ 1-tubulin (4D1, Sigma), actin (AC-40, Sigma), insulin (Dako), glucagon (Dako) and GAPDH (Invitrogen).

### Taxol pelleted microtubules and Immunoblotting

A549 human lung cancer and Hey human ovarian cancer cells from ten 100 mm tissue culture dishes (Corning), at approximately 80–90% confluency, were harvested and Taxol pelleted microtubules were prepared for 2D gel electrophoresis as described previously<sup>3</sup>. Microtubule pellets (containing approximately 100–200  $\mu$ g of protein) were resuspended in

350  $\mu$ L of solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 0.5% ampholyte-containing buffer pH 4.5–5.5, 20 mM DTT, and bromophenol blue), and loaded onto 24 cm IPG strips with a linear gradient of pH 4.5–5.5 (Amersham). The IPG strips were loaded onto Tris-HCl 10% acrylamide criterion gels (Biorad) and run at 200 Volts.

Immunoblots were probed with  $\beta$ V-tubulin rabbit polyclonal antibody at 1:20,000 dilution in 3% milk Tris buffered saline containing 0.05% Tween 20.

### Tissue Culture and Cell Lines

All cell lines were purchased from ATCC and grown in RPMI (Gibco) supplemented with 10% Australian FBS (Gibco) at 37°C, in a 5% humidified CO<sub>2</sub> atmosphere. Mouse embryonic fibroblast cells were generated in our laboratory using standard protocols.

### Immunohistochemistry of paraffin embedded sections

Asynchronous cells were fixed in 4% methanol-free formaldehyde, pelleted and resuspended in matrigel. The solidified matrigel cassettes were submerged in 10% neutral buffered formalin and paraffin embedded sections were prepared by the Pathology Core at Albert Einstein College of Medicine. Re-hydrated slides were blocked for 1 h at room temperature in 5% donkey serum, 2% BSA. Antibody against  $\beta$ V-tubulin was used at 1:1000 in blocking solution and incubated overnight at 4°C in a humidified chamber. Standard washing and labeling with secondary antibodies were performed. Slides were developed with DAB for 45 seconds, washed for 3 minutes with running tap water and counterstained with hematoxylin and mounted.

Human archived tissue that was formalin fixed and paraffin embedded was processed using the same protocol. These sections were left over from standard of care, and as such were exempt from institutional review board approval. The intensity and the percentage of cells positive for  $\beta$ V-tubulin expression were evaluated using the semi-quantitative H-score, which multiplies the percentage of cells staining positively by staining intensity (scored from 0 to 3).

### Immunofluorescence

HeLa cells were seeded onto Poly-D-lysine coated chamber slides, and after overnight growth, fixed in 3.7% formaldehyde in 1x PBS for 5 min (Polysciences). Cells were permeabilized with 0.1% Triton X-100 in 1x PBS for 3 minutes and slides were blocked in 1% goat serum and 3% BSA in 1x PBS for 30 minutes at room temperature, followed by incubation with the  $\beta$ V-tubulin antibody at a 1:1000 dilution in blocking buffer for 1 h at room temperature. Slides were incubated with goat-anti-mouse alexa 555 and goat-anti-rabbit alexa 488 secondary antibodies (Molecular Probes) for 45 min, and mounted using Prolong Gold anti-fade with DAPI (Molecular Probes).

## RESULTS

### Specificity of a human $\beta$ V-tubulin polyclonal antibody

In previous studies we have demonstrated the ability of narrow-range isoelectric focusing to resolve tubulin isotypes differing in isoelectric point (pI) by as little as 0.01<sup>4</sup>. Subsequent mass spectrometry of excised bands has validated the identity of these isotypes. In addition, SILAC, IEF and MS were combined to quantify relative differences in  $\beta$ III- and  $\beta$ V-tubulin levels of expression in human cancer cell lines<sup>20–22</sup>. These previous studies indicated that  $\beta$ V-tubulin was approximately three to four-fold more abundant in Hey ovarian carcinoma cells compared to human A549 lung cancer cells. To test the specificity of the  $\beta$ V-tubulin

antibody in this study, two-dimensional (2D) immunoblots of Taxol-pelleted microtubules were carried out. Microtubules from Hey and A549 cells were used as sources of high and low  $\beta$ V-tubulin content, respectively<sup>20</sup>. These 2D immunoblots were probed with antibodies specific for  $\text{K}\alpha$ 1-,  $\beta$ I-,  $\beta$ III-tubulins, and the  $\beta$ V-tubulin-specific antibody described in the present study. In order to clearly position  $\beta$ III-tubulin spots, we used microtubules from A549 cells that expressed detectable levels of  $\beta$ III-tubulin. Consistent with high expression in Hey cells, the anti  $\beta$ V-tubulin antibody strongly stained a major specific spot positioned at the expected calculated pI of 4.77 (Fig. 1A)<sup>20</sup>. Minor spots that were more acidic in pI were also observed. Both  $\alpha$ -tubulin and  $\beta$ -tubulins undergo glutamylation and phosphorylation on their C-termini<sup>23</sup> that could account for such additional spots. We previously observed monoglutamylation of  $\text{K}\alpha$ 1 and  $\beta$ I-tubulin in cancer cell lines<sup>24</sup> and more acidic spots corresponding to these isoforms are indicated.

We were able to exclude cross reactivity of the  $\beta$ V-tubulin antibody with  $\beta$ I-tubulin using a high resolution one-dimensional isoelectric focusing gel transferred onto nitrocellulose to analyze the expression of tubulin in microtubule pellets generated from an epothilone-B resistant cell line derived from A549 cells<sup>25</sup>. These cells have a Gln to Glu mutation at residue 292 of  $\beta$ I-tubulin, which causes a loss of 0.02 unit of pI for  $\beta$ I-tubulin. If the  $\beta$ V antibody also cross-reacted with  $\beta$ I-tubulin, two bands with different electrophoretic mobilities would be apparent, similar to the profile shown by the Coomassie blue stain. In fact, the  $\beta$ V antibody reacted with a protein focusing between wild-type (pI= 4.78) and mutant  $\beta$ I-tubulin (pI=4.76) (Fig. 1B). Thus, the  $\beta$ V-tubulin antibody does not cross-react with  $\beta$ I-tubulin. Next, to further test specificity, the  $\beta$ V-tubulin antibody was pre-incubated with  $\beta$ V- or  $\beta$ III-tubulin C-terminal peptides prior to immunoblot analysis for  $\beta$ V-tubulin (Fig. 1C). Since the antibody-defining regions of BIII and BV are the most homologous among the tubulins, we used C-terminal peptides of BIII to confirm BV antibody specificity. As expected, pre-incubation of the  $\beta$ V-tubulin antibody with  $\beta$ V-tubulin C-terminal peptide but not with the  $\beta$ III-tubulin C-terminal peptide prevented the detection of  $\beta$ V-tubulin. Lastly, immunoblots of Hey and A549 carcinoma cell lines were probed with  $\beta$ I-,  $\beta$ III-,  $\beta$ IV-, and  $\beta$ V-tubulin antibodies and respective signals were normalized to those of total  $\beta$ -tubulin and GAPDH (Fig. 1C). The observed differences in expression levels were comparable to those revealed by mass spectrometry<sup>20</sup>.

Human and mouse  $\beta$ V-tubulin have 97.7% overall amino acid identity, while the antigen-defining region has 79% homology (Fig. S1). To test whether the human  $\beta$ V-tubulin antibody that we developed would cross react with mouse  $\beta$ V-tubulin, mouse embryonic fibroblasts (MEFs) were transduced with full length human  $\beta$ V-tubulin, or vector alone. Subsequent immunoblotting indicated that the  $\beta$ V-tubulin antibody was only able to detect human  $\beta$ V-tubulin in the transduced MEFs and not in the endogenous mouse  $\beta$ V-tubulin protein (Fig. 2A). We detected the expression of endogenous mouse  $\beta$ V-tubulin in MEF lysates using a monoclonal antibody specific to rodent  $\beta$ V-tubulin (Fig. 2B). This result indicated that the  $\beta$ V-tubulin antibody we generated is specific for the human isotype. A BLAST protein sequence similarity search was performed with the C-terminus sequence of  $\beta$ V-tubulin. We found that this sequence was similar to an internal sequence (<sub>262</sub>EEDEEEEIDV<sub>271</sub>) of human N-Myc oncoprotein, a protein with a MW similar to tubulin (49 kDa). In order to eliminate the possibility of cross reactivity between the two, particularly for immunohistochemical applications, we performed immunoblot analysis using the  $\beta$ V-tubulin antibody against recombinant full-length N-Myc protein containing a 26 kDa N-terminal GST tag, which decreases its electrophoretic mobility to an approximate MW of 80kDa. Lysates from SH-SY5Y neuroblastoma cells that are known to overexpress N-Myc were used as a control. The  $\beta$ V-tubulin antibody was unable to detect N-Myc by immunoblotting, even with prolonged exposure (Fig. S2). Immunohistochemistry performed on N-myc rich neuroblastoma tissue was negative for expression of  $\beta$ V-tubulin (data not

shown). Therefore no cross-reactivity occurs between  $\beta$ V-tubulin and N-myc. This result and the non cross-reactivity of our anti-human  $\beta$ V-tubulin antibody with the C-terminus of mouse  $\beta$ V-tubulin strongly suggest that the recognized epitope involves the last two amino acid residues and the C-terminal carboxyl.

Previously published data utilizing a rodent  $\beta$ V-tubulin monoclonal antibody indicated that  $\beta$ V-tubulin is concentrated in the inner ear, localizing specifically to pillar cells and deiter cells in gerbil cochlea<sup>5</sup>. Due to difficulties obtaining equivalent human specimens, we were unable to confirm these results with the human antibody. To test the capability of the  $\beta$ V-tubulin antibody for immunohistochemistry, we prepared formalin-fixed, paraffin-embedded MEF cell pellets sections from MEFs overexpressing  $\beta$ V-tubulin or transfected with the empty vector. The immunohistochemical staining pattern for  $\beta$ V-tubulin antibody was cytoplasmic with a clean background.  $\beta$ V-tubulin was strongly expressed in stably-transfected MEFs and negative in vector-only transfected MEFs (Fig 2C).

### Localization of human $\beta$ V-tubulin in cultured cells

The human  $\beta$ V-tubulin antibody we generated was also evaluated by immunofluorescence in HeLa cells. Localization was contrasted with  $\beta$ III-tubulin. Both  $\beta$ V- and  $\beta$ III-tubulin had diffuse staining that localized throughout the cytoplasm of interphase cells, with intense staining of mitotic cell spindles and of the intercellular bridge in late telophase (Fig. 3). Immunofluorescence using the murine  $\beta$ V-tubulin antibody in NIH3T3 cells also demonstrated that  $\beta$ V-tubulin is concentrated at microtubule spindles and is present in the microtubule network of interphase cells<sup>5</sup>. There may be a subtle difference in the distribution of  $\beta$ III versus  $\beta$ V in the spindles of HeLa cells. Cytoplasmic  $\beta$ V-tubulin immunostaining was also observed in Hey cells, similar to the distribution observed by immunohistochemistry.

### Immunohistochemical analysis of $\beta$ V-tubulin in human tissue

The distribution of  $\beta$ V-tubulin protein in human tissue has never been determined due to the unavailability of a specific antibody. Using our human specific  $\beta$ V tubulin antibody, we determined  $\beta$ V expression in a variety of human normal tissue types (Table 1). As indicated by immunofluorescence,  $\beta$ V-tubulin showed a cytoplasmic pattern of staining by immunohistochemistry, as shown in Figure 4.

In general,  $\beta$ V-tubulin was consistently expressed in the endothelial cells of the blood vessels (Fig 4A), myocytes and cells with muscle differentiation.  $\beta$ V-tubulin was expressed in muscles of all three types with particularly strong expression in smooth muscle. Specialized cells such as myoepithelial cells of the breast and myoid cells of the testis showed strong  $\beta$ V-tubulin staining, consistent with their muscle differentiation. The glomeruli of the kidney have specialized tuft-like vascular structure composed of fenestrated endothelium, podocytes and mesangial cells.  $\beta$ V-tubulin showed an interesting mosaic pattern outlining the glomeruli. This pattern was correlated with the positive expression in endothelial cells and mesangial cells, a specialized cell type with muscle differentiation.  $\beta$ III-tubulin is known to be highly expressed in neuronal cells, and is currently used as a marker of neuronal differentiation<sup>26</sup>.  $\beta$ V-tubulin, unlike  $\beta$ III-tubulin, was not expressed in nerves (Fig. 4B). To confirm our findings by immunohistochemistry, immunoblot analysis of a human microvascular endothelial cell line (HMEC-1) was used (Fig. S2).  $\beta$ V-tubulin, but not  $\beta$ III was expressed minimally in these cells.

Moderate staining was consistently observed in cells with secretory/transport function such as renal tubules, bile ducts of the liver, pancreatic exocrine ducts and ducts of salivary glands (Table 1 and Figure. 4). These cells transport ions and water and have small molecule



exchange/secretion/absorption functions during transport processes, suggesting the possibility that  $\beta$ V-tubulin participates in transport function, and certain ion or small molecule exchange.  $\beta$ V-tubulin was also strongly expressed in sebaceous glands of the skin and variably expressed in prostate glandular epithelium and thyroid follicular cells, which are epithelial tissues with secretory functions. In contrast,  $\beta$ V staining was not detected in the epithelium of the GI tract (stomach, duodenum, gall bladder, small and large intestine), hepatocytes, ovarian surface epithelium and pneumocytes. In normal breast tissue,  $\beta$ V-tubulin was absent in luminal epithelial cells of the ducts/lobules, however, it was strongly expressed in the luminal cells of the breast during lactation (Fig 4H–I). We hypothesize that this specific expression of  $\beta$ V is acquired to meet the demanding secretory needs of the breast during lactation. In addition, macrophages in various tissues showed positive staining for  $\beta$ V-tubulin. Macrophages are inflammatory mediators with secretory function, therefore  $\beta$ V-tubulin expression in macrophages is consistent with the likely secretory-related functions of this tubulin isotype.

### Expression of $\beta$ V-tubulin in pancreatic islets and testis

Interestingly, while  $\beta$ V was largely negative in pancreatic parenchyma (exocrine glands), it was positively expressed in pancreatic islets (Fig. 4E and Fig. 5). Pancreatic islets are endocrine components of the pancreas that are composed of alpha and beta cells that are responsible for the secretion of glucagon and insulin, respectively. We were able to compare the immunostaining pattern of  $\beta$ V-tubulin with that of glucagon and insulin using specific antibodies on serially sectioned pancreatic tissue (Fig. 5).  $\beta$ V-positive cells were localized around the periphery of the islets in a region that qualitatively correlates with the localization of glucagon-stained alpha cells (Fig. 5D). Thus, these data suggest that the glucagon-producing alpha cells may require  $\beta$ V-tubulin for a specific function.

$\beta$ V-tubulin also exhibited an interesting pattern in testis. A testicular biopsy from a child with cryptorchidism showed  $\beta$ V-tubulin positivity highlighting spermatogonia in the seminiferous tubules (Figure 6). In tissue samples of intratubular germ cell neoplasia (ITGCN), surprisingly specific strong expression of  $\beta$ V-tubulin was observed in the neoplastic germ cells, while in contrast, normal seminiferous tubules were negative. Although the number of cases tested was limited (3/3), we believe that  $\beta$ V-tubulin may be a potential diagnostic tool for identifying ITGCN. To date, there is no diagnostic immunohistochemical marker of this entity, and the biology underlying the association of  $\beta$ V-tubulin expression with germ cell neoplasia will require investigation.

### Expression of $\beta$ V-tubulin in human cancer

Since tumorigenesis often causes altered differentiation of affected cells, as is the case with the neuronal-specific  $\beta$ III-tubulin isotype that is expressed in numerous malignancies, we evaluated the expression of  $\beta$ V-tubulin in a small cohort of carcinomas including breast, lung, prostate and ovary. We found that, in contrast to the negative staining in their normal counterparts, lung, breast and ovarian cancers (the latter not shown), had aberrant  $\beta$ V-tubulin expression. Conversely, while normal prostate glands showed variable  $\beta$ V-tubulin staining, expression was completely lost in prostate cancer (Fig. 7).

Immunoblotting for  $\beta$ V-tubulin was also performed in a series of lung (NSCLC), ovarian, and breast cancer cell lines to determine if there was a histological association with its expression (Figure S3).  $\beta$ V-tubulin was expressed in all but one cell line, although at highly variable levels, supporting previous studies suggesting that it is ubiquitously expressed in cancer cell lines<sup>27</sup>. Thus these data are consistent with the immunohistochemical data obtained for lung, ovarian and breast cancer.

We conducted a study of  $\beta$ V-tubulin expression in 32 cases of breast cancers with various differentiation states.  $\beta$ V-tubulin was aberrantly expressed in ten cases (30%), of which nine were poorly differentiated, suggesting that expression is associated with malignant transformation and high tumor grade. Thus, our preliminary data indicate that  $\beta$ V-tubulin is aberrantly expressed in lung, ovarian and breast cancer and collectively imply that like  $\beta$ III-tubulin,  $\beta$ V-tubulin has irregular expression in transformed cells, though additional studies to confirm and extend these observations are needed.

## Discussion

Several studies have established that mammalian microtubules will incorporate all available tubulin isotypes as evidenced by immunofluorescence using isotype specific antibodies and secondly, by transfection of non-mammalian isotypes into mammalian cells<sup>28-30</sup>.  $\beta$ V-tubulin is a minor tubulin isotype, composing less than 10% of the total tubulin in most tissues in the chicken,<sup>31</sup> less than 20% in mouse embryonic fibroblasts, and less than approximately 7% of total tubulin in Chinese hamster ovary cells<sup>28, 30, 31</sup>.

Numerous studies have documented overexpression of  $\beta$ III-tubulin in different malignancies, and its utility as a marker of poor survival and, in some cases response to therapy<sup>7,9</sup>. This has prompted widespread interest in characterizing the expression of other tubulin isoforms in human malignancies. In the absence of a commercially available specific  $\beta$ V-tubulin antibody, many of the studies to date have employed overexpression or RNA-based knockdown techniques to modulate  $\beta$ V in numerous cell lines, without a real appreciation of the tissue-specific expression of this isotype in normal tissue. A previous qRT-PCR study reported ubiquitous expression of  $\beta$ V-tubulin in human tissue<sup>15</sup>, however we did not find this by immunohistochemistry. It is possible that the levels of  $\beta$ V-tubulin protein in the RT-PCR positive tissues are extremely low, or that the ubiquitous expression was an artefact due to contamination with a  $\beta$ V-tubulin positive cell type, such as endothelial cells or macrophages, since laser-capture microdissection was not performed.

To circumvent this inherent problem with mRNA expression profiling, we sought to generate a human-specific  $\beta$ V-tubulin antibody that could be used to delineate the repertoire of expression in normal and malignant tissue, and as such provide evidence about the potential function of this specific tubulin isoform.

Unlike  $\beta$ III tubulin that is normally expressed only in neuronal tissue and cells,  $\beta$ V was expressed in a wide range of human non-neuronal cell and tissue types. The observation that  $\beta$ V-tubulin was preferentially expressed in cells with a secretory function, such as pancreatic ductal cells, was unexpected. One of the functions of microtubules is to move secreted proteins within cells. Colchicine, a microtubule destabilizing agent that interacts with the tubulin dimer, has been shown to inhibit the secretion of several proteins from secretory tissues. Specifically, colchicine inhibits the secretion of thyroid hormones from thyroid epithelial cells, lipoproteins from liver cells, insulin from pancreatic islet cells,  $\text{HCO}_3^-$  from pancreatic ducts, and apolipoprotein E from macrophages<sup>32-35</sup>. Interestingly, cell types that constitute the pancreatic islet, salivary ducts and bile duct showed positive staining for  $\beta$ V-tubulin, reinforcing the view that there are isotype-specific functions for tubulins. Future studies to modulate the intrinsic expression of  $\beta$ V tubulin in these cell types will be required to fully determine whether this isotype actually participates in the transport of protein cargo as part of the secretory process, or is an authentic marker of a secretory cell type.

Although not a major focus of this study, the data from tumor tissue indicate potentially interesting alterations in the expression of  $\beta$ V in normal, versus malignant breast, lung, ovary, and prostate tissue. Specifically,  $\beta$ V was undetectable in epithelial cells, yet was

aberrantly expressed in approximately one-third of invasive breast carcinomas analyzed. Similarly,  $\beta$ V-tubulin was not expressed in normal lung pneumocytes or ovarian epithelium, however, some lung and ovarian cancers were positive. Conversely, while variably expressed in normal prostate glands,  $\beta$ V-tubulin expression was lost in prostate cancer.

Therefore, similar to the expression of  $\beta$ III that occurs in malignant cells versus normal cells, there also appears to be a trend toward reversal of  $\beta$ V-tubulin expression during malignancy. Thus, it is plausible that the altered expression in malignant cells reflects an inherent change in the differentiation status of the normal tissue, such that  $\beta$ V-tubulin transcripts that are normally not expressed in a given cell type become expressed; while in tissues that normally expresses  $\beta$ V-tubulin, transcripts are silenced.

Overexpression of mouse  $\beta$ V-tubulin, like  $\beta$ III, has been demonstrated to confer resistance to Taxol in cells,<sup>11, 16, 19</sup> although  $\beta$ V-tubulin was thought to confer more deleterious effects on cell division<sup>36</sup>. In contrast, the overexpression of  $\beta$ I-,  $\beta$ II-,  $\beta$ IV-, or  $\beta$ VI-tubulin had no effect on microtubule assembly or drug resistance when overexpressed in cells<sup>16, 19</sup>. Such studies make it tempting to speculate that  $\beta$ V-tubulin, like  $\beta$ III, may be implicated in poor prognosis for some malignancies and possibly poor response to therapy. However we caution against overstating the significance of cell based studies until more extensive immunohistochemical analyses have been performed. Furthermore, human  $\beta$ V-tubulin has a different sequence in the Taxol binding site relative to mouse  $\beta$ V-tubulin<sup>20</sup>, thus conclusions about the role  $\beta$ V-tubulin obtained from mouse cell models may not be applicable to human  $\beta$ V-tubulin. The availability of this human-specific  $\beta$ V-tubulin antibody will enable mechanistic studies to shed light on the biological function of this tubulin isoform, and its potential involvement in drug resistance.

Moreover, the unique profile of  $\beta$ V-tubulin expression that we have characterized in several malignancies lends support to our hypothesis that altered expression of this tubulin isoform may be associated with tumorigenesis. These initial studies in breast and lung cancers will form the basis of extended analyses that will probe the relationship between  $\beta$ V-tubulin, differentiation, stage, survival and response to therapy. Immunohistochemical-based analyses of  $\beta$ V-tubulin expression may be a useful diagnostic tool and a potential prognostic marker.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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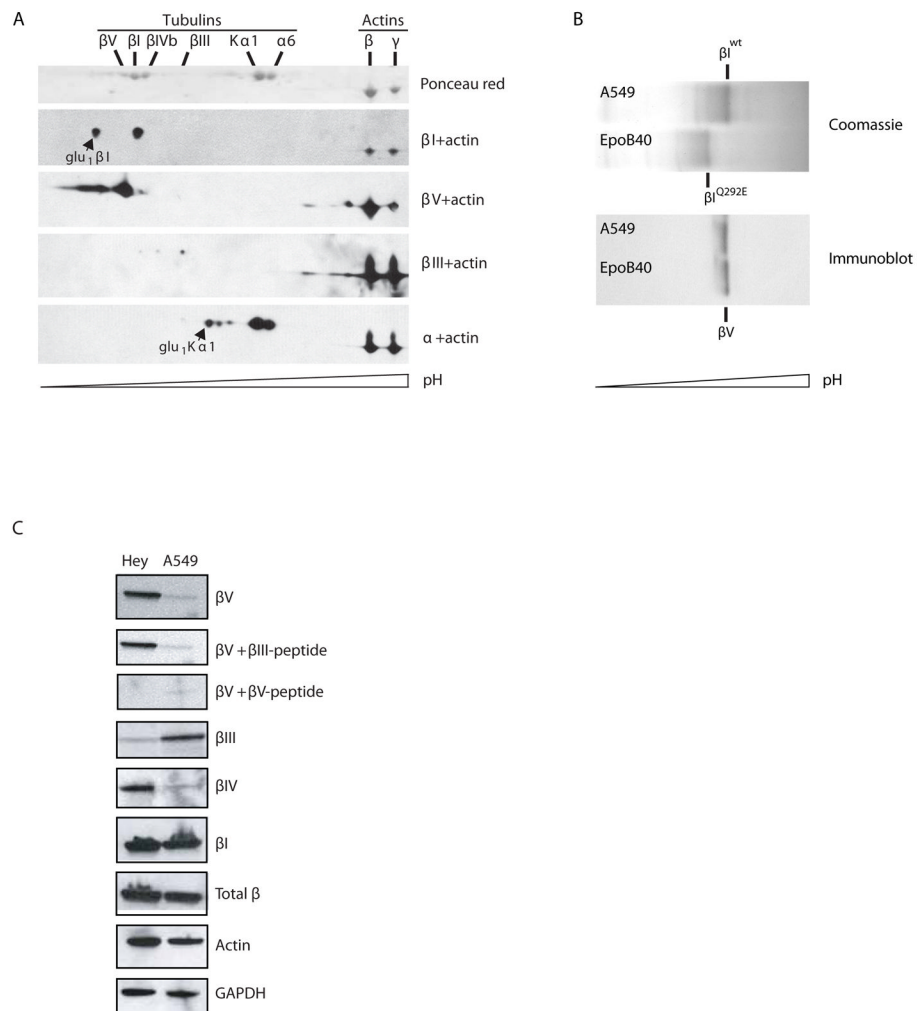
## References

1. Joshi HC, Yen TJ, Cleveland DW. In vivo coassembly of a divergent beta-tubulin subunit (c beta 6) into microtubules of different function. *J Cell Biol.* 1987; 105:2179–2190. [PubMed: 3316249]
2. Luduena RF. Multiple forms of tubulin: different gene products and covalent modifications. *Int Rev Cytol.* 1998; 178:207–275. [PubMed: 9348671]
3. Miller LM, Xiao H, Burd B, Horwitz SB, Angeletti RH, Verdier-Pinard P. Methods in tubulin proteomics. *Methods Cell Biol.* 2010; 95:105–126. [PubMed: 20466132]
4. Verdier-Pinard P, Pasquier E, Xiao H, Burd B, Villard C, Lafitte D, Miller LM, Angeletti RH, Horwitz SB, Braguer D. Tubulin proteomics: towards breaking the code. *Anal Biochem.* 2009; 384:197–206. [PubMed: 18840397]



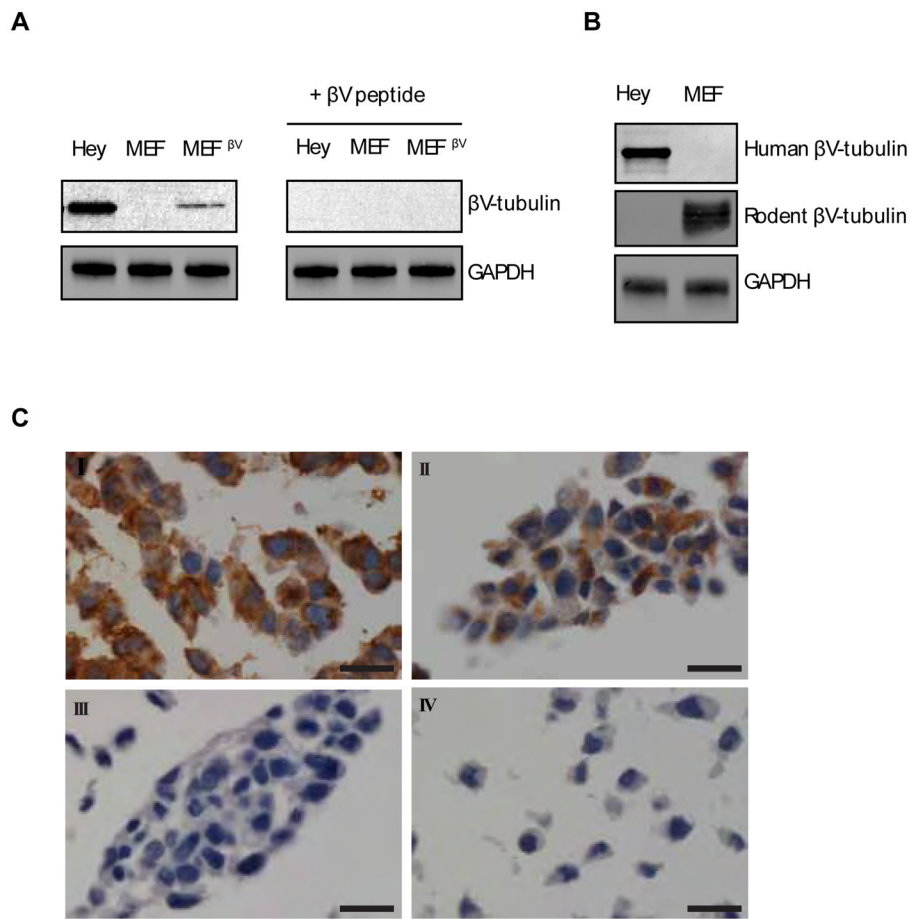
5. Banerjee A, Jensen-Smith H, Lazzell A, Prasad V, Elguezabal G, Hallworth R, Luduena RF. Localization of beta tubulin in the cochlea and cultured cells with a novel monoclonal antibody. *Cell Motil Cytoskeleton*. 2008; 65:505–514. [PubMed: 18412253]
6. Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of Taxol resistance related to microtubules. *Oncogene*. 2003; 22:7280–7295. [PubMed: 14576838]
7. Kavallaris M. Microtubules and resistance to tubulin-binding agents. *Nat Rev Cancer*. 2010; 10:194–204. [PubMed: 20147901]
8. Seve P, Dumontet C. Class III beta tubulin expression in nonsmall cell lung cancer. *Rev Mal Respir*. 2010; 27:383–386. [PubMed: 20403547]
9. Ferrandina G, Zannoni GF, Martinelli E, Paglia A, Gallotta V, Mozzetti S, Scambia G, Ferlini C. Class III beta-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. *Clin Cancer Res*. 2006; 12:2774–2779. [PubMed: 16675570]
10. Seve P, Dumontet C. Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents? *Lancet Oncol*. 2008; 9:168–175. [PubMed: 18237851]
11. Kavallaris M, Kuo DY, Burkhart CA, Regl DL, Norris MD, Haber M, Horwitz SB. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isoforms. *J Clin Invest*. 1997; 100:1282–1293. [PubMed: 9276747]
12. Seve P, Reiman T, Lai R, Hanson J, Santos C, Johnson L, Dabbagh L, Sawyer M, Dumontet C, Mackey JR. Class III beta-tubulin is a marker of paclitaxel resistance in carcinomas of unknown primary site. *Cancer Chemother Pharmacol*. 2007; 60:27–34. [PubMed: 17021819]
13. McCarroll JA, Gan PP, Liu M, Kavallaris M. betaIII-tubulin is a multifunctional protein involved in drug sensitivity and tumorigenesis in non-small cell lung cancer. *Cancer Res*. 2010; 70:4995–5003. [PubMed: 20501838]
14. Lee KM, Cao D, Itami A, Pour PM, Hruban RH, Maitra A, Ouellette MM. Class III beta-tubulin, a marker of resistance to paclitaxel, is overexpressed in pancreatic ductal adenocarcinoma and intraepithelial neoplasia. *Histopathology*. 2007; 51:539–546. [PubMed: 17714470]
15. Leandro-Garcia LJ, Leskela S, Landa I, Montero-Conde C, Lopez-Jimenez E, Leton R, Cascon A, Robledo M, Rodriguez-Antona C. Tumoral and tissue-specific expression of the major human beta-tubulin isoforms. *Cytoskeleton (Hoboken)*. 2010; 67:214–223. [PubMed: 20191564]
16. Bhattacharya R, Cabral F. Molecular basis for class V beta-tubulin effects on microtubule assembly and paclitaxel resistance. *J Biol Chem*. 2009; 284:13023–13032. [PubMed: 19282281]
17. Ganguly A, Yang H, Cabral F. Paclitaxel-dependent cell lines reveal a novel drug activity. *Mol Cancer Ther*. 2010; 9:2914–2923. [PubMed: 20978163]
18. Martello LA, Verdier-Pinard P, Shen HJ, He L, Torres K, Orr GA, Horwitz SB. Elevated levels of microtubule destabilizing factors in a Taxol-resistant/dependent A549 cell line with an alpha-tubulin mutation. *Cancer Res*. 2003; 63:1207–1213. [PubMed: 12649178]
19. Bhattacharya R, Cabral F. A ubiquitous beta-tubulin disrupts microtubule assembly and inhibits cell proliferation. *Mol Biol Cell*. 2004; 15:3123–3131. [PubMed: 15121885]
20. Verdier-Pinard P, Shahabi S, Wang F, Burd B, Xiao H, Goldberg GL, Orr GA, Horwitz SB. Detection of human betaV-tubulin expression in epithelial cancer cell lines by tubulin proteomics. *Biochemistry*. 2005; 44:15858–15870. [PubMed: 16313188]
21. Verdier-Pinard P, Wang F, Martello L, Burd B, Orr GA, Horwitz SB. Analysis of tubulin isoforms and mutations from taxol-resistant cells by combined isoelectrofocusing and mass spectrometry. *Biochemistry*. 2003; 42:5349–5357. [PubMed: 12731876]
22. Miller LM, Menthen A, Chatterjee C, Verdier-Pinard P, Novikoff PM, Horwitz SB, Angeletti RH. Increased levels of a unique post-translationally modified betaIVb-tubulin isoform in liver cancer. *Biochemistry*. 2008; 47:7572–7582. [PubMed: 18570381]
23. Verhey KJ, Gaertig J. The tubulin code. *Cell Cycle*. 2007; 6:2152–2160. [PubMed: 17786050]
24. Verdier-Pinard P, Wang F, Burd B, Angeletti RH, Horwitz SB, Orr GA. Direct analysis of tubulin expression in cancer cell lines by electrospray ionization mass spectrometry. *Biochemistry*. 2003; 42:12019–12027. [PubMed: 14556633]
25. Yang CP, Verdier-Pinard P, Wang F, Lippaine-Horvath E, He L, Li D, Hofle G, Ojima I, Orr GA, Horwitz SB. A highly epothilone B-resistant A549 cell line with mutations in tubulin that confer drug dependence. *Mol Cancer Ther*. 2005; 4:987–995. [PubMed: 15956256]

26. Lee MK, Tuttle JB, Rebhun LI, Cleveland DW, Frankfurter A. The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. *Cell Motil Cytoskeleton*. 1990; 17:118–132. [PubMed: 2257630]
27. Bhattacharya R, Frankfurter A, Cabral F. A minor beta-tubulin essential for mammalian cell proliferation. *Cell Motil Cytoskeleton*. 2008; 65:708–720. [PubMed: 18553364]
28. Lopata MA, Cleveland DW. In vivo microtubules are copolymers of available beta-tubulin isotypes: localization of each of six vertebrate beta-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. *J Cell Biol*. 1987; 105:1707–1720. [PubMed: 3312237]
29. Lewis SA, Gu W, Cowan NJ. Free intermingling of mammalian beta-tubulin isotypes among functionally distinct microtubules. *Cell*. 1987; 49:539–548. [PubMed: 3552250]
30. Sawada T, Cabral F. Expression and function of beta-tubulin isotypes in Chinese hamster ovary cells. *J Biol Chem*. 1989; 264:3013–3020. [PubMed: 2644275]
31. Sullivan KF, Havercroft JC, Machlin PS, Cleveland DW. Sequence and expression of the chicken beta 5- and beta 4-tubulin genes define a pair of divergent beta-tubulins with complementary patterns of expression. *Mol Cell Biol*. 1986; 6:4409–4418. [PubMed: 3025656]
32. Kockx M, Guo DL, Huby T, Lesnik P, Kay J, Sabaretnam T, Jary E, Hill M, Gaus K, Chapman J, Stow JL, Jessup W, Kritharides L. Secretion of apolipoprotein E from macrophages occurs via a protein kinase A and calcium-dependent pathway along the microtubule network. *Circ Res*. 2007; 101:607–616. [PubMed: 17660382]
33. Williams JA, Wolff J. Colchicine-binding protein and the secretion of thyroid hormone. *J Cell Biol*. 1972; 54:157–165. [PubMed: 4338961]
34. Montague W, Howell SL, Green IC. Insulin release and the microtubular system of the islets of Langerhans. Identification and characterization of tubulin-like protein. *Biochem J*. 1975; 148:237–243. [PubMed: 1098658]
35. Stein O, Sanger L, Stein Y. Colchicine-induced inhibition of lipoprotein and protein secretion into the serum and lack of interference with secretion of biliary phospholipids and cholesterol by rat liver in vivo. *J Cell Biol*. 1974; 62:90–103. [PubMed: 4366104]
36. Bhattacharya R, Yang H, Cabral F. Class V beta-tubulin alters dynamic instability and stimulates microtubule detachment from centrosomes. *Mol Biol Cell*. 2011; 22:1025–1034. [PubMed: 21289088]



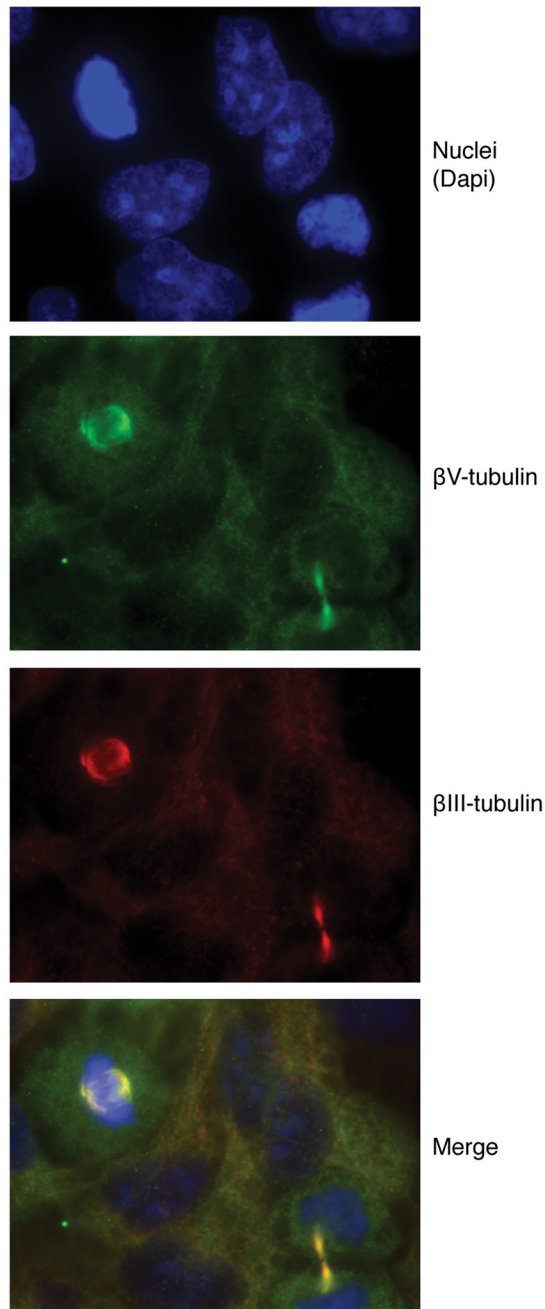
**Figure 1. Isotype-specific immunoreactivity of a human  $\beta V$ -tubulin antibody**

A) Taxol-pelleted microtubules from Hey cells were resolved by isoelectric focusing using IPG strips at pH 4.5–5.5, followed by SDS-PAGE, total protein staining and immunoblotting with isotype specific antibodies as indicated. B) Isoelectric focusing of wild type and mutant  $\beta I$ -tubulin in A549 and A549.EpoB40, respectively. IEF gels were either stained with Coomassie blue (top), or electrotransferred onto nitrocellulose for immunoblotting with anti human  $\beta V$ -tubulin antibody (bottom). C) Pre-incubation of antibodies with  $\beta III$ - or  $\beta V$ -tubulin C-terminal peptides and subsequent immunoblotting using the  $\beta V$ -tubulin antibody on Hey and A549 cell lysates. Also shown are immunoblots with the indicated isotype specific antibodies for  $\beta V$ -,  $\beta III$ -,  $\beta I$ -, and total  $\beta$ -tubulin.



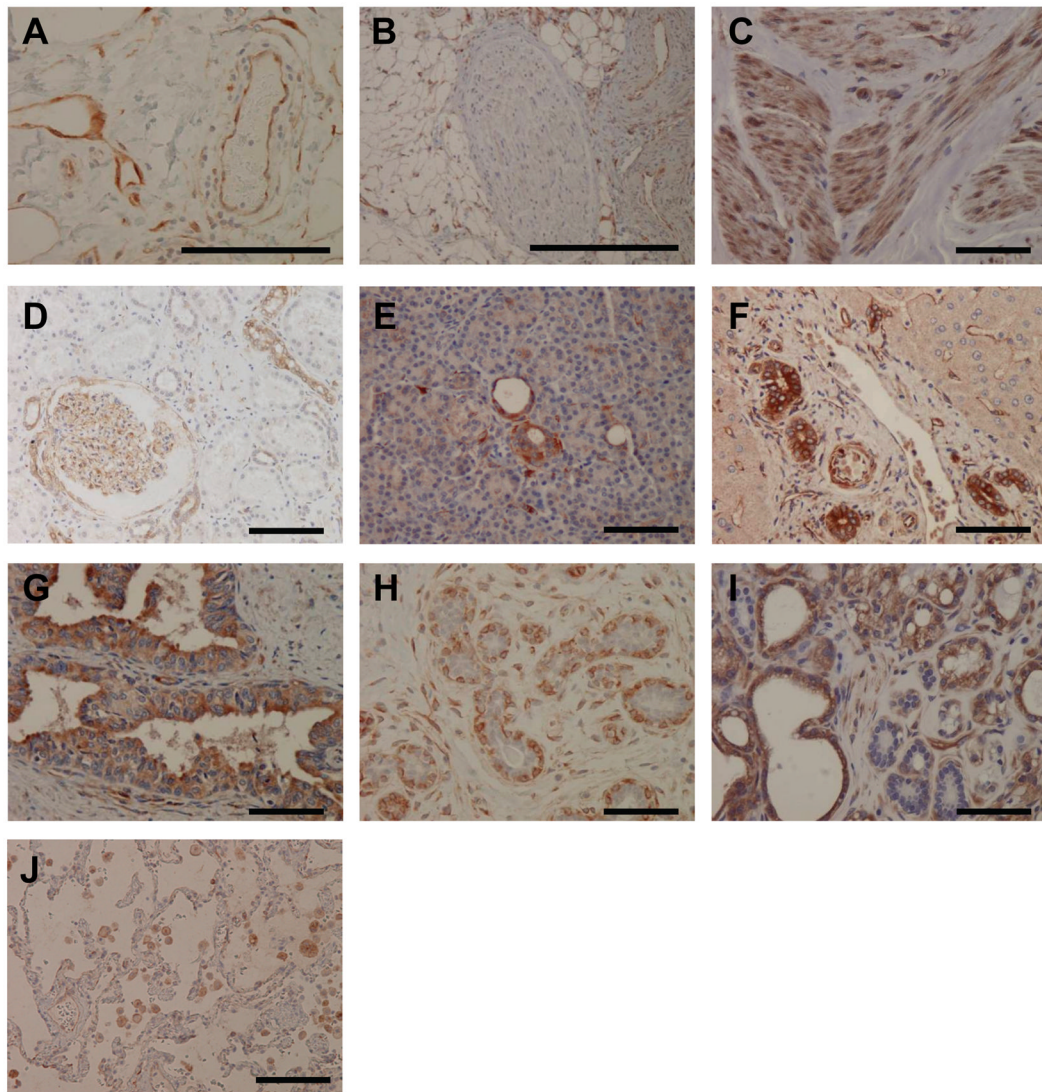
**Figure 2. Specificity of human  $\beta$ V-tubulin immunoreactivity in mouse embryonic fibroblasts (MEFs) expressing exogenous human  $\beta$ V-tubulin**

A) Mouse embryonic fibroblasts transfected with empty vector or full length, human  $\beta$ V-tubulin (MEF <sup>$\beta$ V</sup>) were immunoblotted with the  $\beta$ V-tubulin antibody in the absence and presence of  $\beta$ V-tubulin peptides. B) Immunoblot analysis of MEF and Hey cells with a rodent specific  $\beta$ V-tubulin monoclonal antibody. C) Immunohistochemical analysis of  $\beta$ V-tubulin expression in formalin-fixed paraffin-embedded cells (I) Hey cells, positive control for high expression of endogenous  $\beta$ V-tubulin, (II) MEFs expressing exogenous human  $\beta$ V-tubulin, (III) MEFs transfected with empty vector, and (IV) Hey cells incubated with secondary antibody only. All slides were counterstained with hematoxylin. Scale bars, 20 $\mu$ m.



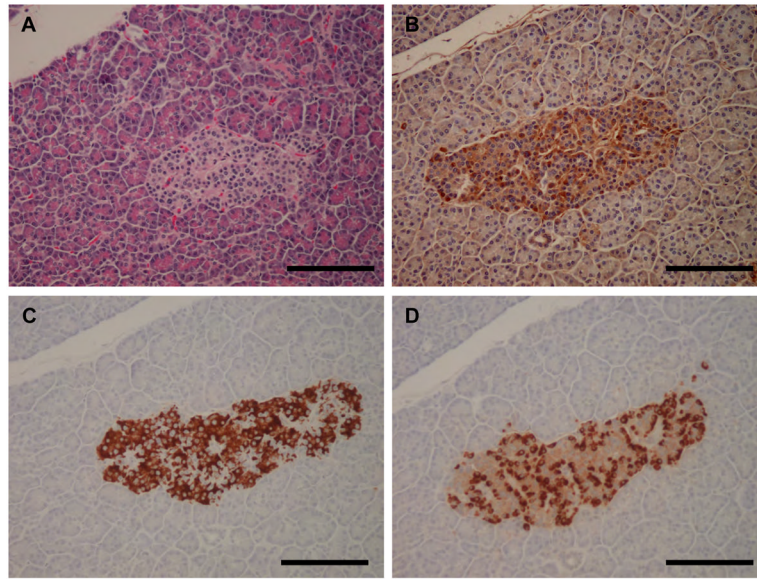
**Figure 3. Immunofluorescence of endogenous  $\beta$ V- and  $\beta$ III-tubulin in HeLa cells**  
Field includes interphase and mitotic cells. From top: dapi staining of nuclei, alexa 488 labeling of  $\beta$ V-tubulin (green), alexa 555 labeling of  $\beta$ III-tubulin (red), and merged image.





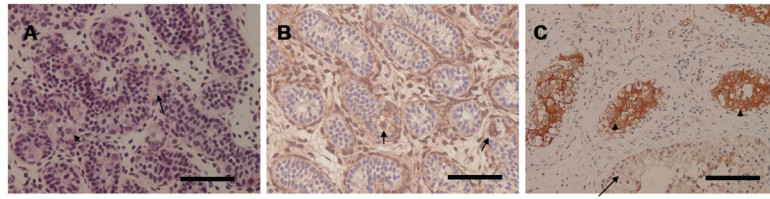
**Figure 4. Immunohistochemistry of  $\beta$ V-tubulin in normal human tissue**

A) endothelial cells of vessels and capillaries, B) nerve fiber, C) smooth muscle, D) glomerulus and renal tubules, E) pancreatic ducts, F) bile ducts of liver; G) prostate glands, H) normal breast ducts/lobules; I) lactating breast, J) normal lung. Scale bars, 100  $\mu$ m.



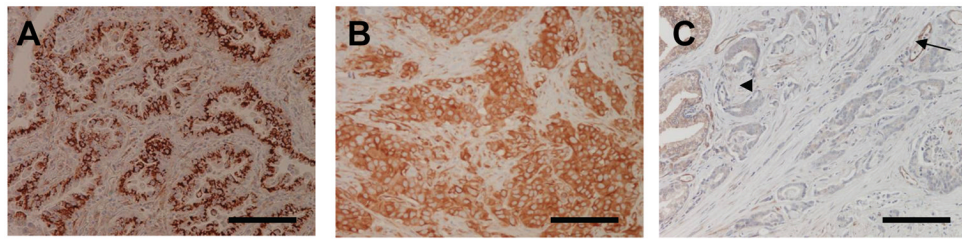
**Figure 5. Immunohistochemistry of  $\beta$ V-tubulin in pancreatic islets**

A) Hematoxylin and eosin, B)  $\beta$ V-tubulin, C) insulin, D) glucagon. Scale bars, 100  $\mu$ m.



**Figure 6. Immunohistochemistry  $\beta$ V-tubulin in the germ cells of immature testis**

A) Hematoxylin and eosin staining of a tissue section from a one year old child with testis cryptorchidism, B)  $\beta$ V-tubulin was expressed in the germ cells- spermatogonia in the seminiferous tubules (arrows). Sertoli cells were negative. The surrounding outer myoid cells were positive staining for  $\beta$ V-tubulin due to their muscle differentiation C) An adult testis with intratubular germ cells neoplasia (ITGCN).  $\beta$ V-tubulin was strongly expressed in ITGCN (arrowheads). Normal seminiferous tubules in the background were negative for  $\beta$ V-tubulin (arrow). Scale bars, 100  $\mu$ m.



**Figure 7. Immunohistochemistry of  $\beta$ V-tubulin in human malignancy**

$\beta$ V-tubulin was strongly expressed in A) lung adenocarcinoma and B) invasive breast cancer. C) loss of  $\beta$ V-tubulin in infiltrating prostate adenocarcinoma and perineural invasion of the adenocarcinoma. A nerve fiber that is negative for  $\beta$ V-tubulin is indicated by the arrowhead. Variable expression of  $\beta$ V-tubulin in normal prostate glands is present in the upper left. Endothelial cells lining blood vessels positive for  $\beta$ V-tubulin are indicated (arrow), Scale bars, 100  $\mu$ m.

**Table 1**Distribution of  $\beta$ V-tubulin in Normal Tissues by Immunohistochemistry

<b>Tissue</b>		<b>Intensity</b>
Skin	Sebaceous glands	+++
	Squamous epithelium	+ (variable)
	Basal cells	+
Muscle	Smooth muscle	+++
	Skeletal muscle	++
	Cardiac Muscle	++
Blood vessel	Endothelial cells	+++
	smooth muscle	++
GI Tract <sup>¶</sup>	Epithelium	-
Liver	Hepatocytes	-
	Bile ducts	++
Pancreas	Ducts	+
	Islets	++
	Acini	-
Kidney	Renal tubules	- to ++ (variable)
	glomeruli	++
Testis	Seminiferous tubules	-
	Mature germ cells	-
	Immature germ cells	++
Prostate	Sertoli cells	-
	Glandular cells	++ (variable)
Mammary gland	Basal cells	-
	Luminal cells	-
	Lactating secretory cells	++
Ovary	Myoepithelial cells	+++
	Surface epithelium	-
Fallopian tube	Stromal cells	+
	Epithelium	-
Lung	Pneumocytes	-
	Respiratory epithelium	-
Thyroid	Follicular cells	- to ++ (variable)
Adrenal gland	Cortex and medulla	-
Salivary Gland	Acini	+ (variable)
	Ducts	++
Nerve	Neuronal Cells	-

Abbreviations: (-) no staining, (+) weak staining, (++) moderate staining, (+++) strong staining

<sup>¶</sup>(Stomach, Small Intestine, Appendix, Colon, Gall bladder)