Published in final edited form as:

Proteomics Clin Appl. 2012 October ; 6(9-10): 502–506. doi:10.1002/prca.201200018.

A Label-Free Mass Spectrometry Method for Relative Quantitation of β -Tubulin Isotype Expression in Human Tumor Tissue

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Abstract

Purpose—Quantitation of β -tubulin isotype expression in taxane resistant human tumor tissue has been difficult to achieve because of the limited availability of validated antibodies. Here we present a label-free mass spectrometry method to quantitate relative expression levels of β -tubulin isotypes.

Experimental Design—Using isotype specific reporter peptides, we determined relative β -tubulin isotype expression levels in human lung tumor tissue.

Results—Four reporter peptides were chosen to quantitate the β I/ β II, β IV, β III and β V tubulin isotypes. These peptides were validated using human cancer cell lines. The label-free method was then used to determine β -tubulin isotype expression in nine human lung tumor samples, which had been described as high or low β III-tubulin expressing using immunohistochemistry. It was found that β I/ β II (accounting for 18.7%–65.7% of total β -tubulin) and β IVa/ β IVb (26.3%–79.1%) were the most abundant isotypes and that the β III (0–8.9%) and β V (1.0–10.4%) were less abundant in the tissue. We also categorized the samples as high or low β III-tubulin expressing.

Conclusions and clinical relevance—With this method we can determine the relative expression levels of β -tubulin isotypes in human tumor tissue. This method will facilitate studies assessing the use of tubulin isotypes as biomarkers of taxane resistance.

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The authors have declared no conflict of interest.

Keywords

β -tubulin isotypes; human lung tumors; label-free quantitation; mass spectrometry. taxane resistance

The microtubule cytoskeleton is the target of one of the most widely used classes of anti-cancer therapeutics, the taxanes, which are microtubule-stabilizing drugs that act by disrupting the inherent microtubule dynamics necessary for mitosis.[1–3] However, resistance to taxanes is a persistent problem, as some tumors are inherently resistant to the drug and others acquire resistance during treatment. There are many possible routes by which cells can become resistant and loss of sensitivity to taxanes is likely due to a combination of factors. One mechanism by which cells develop resistance to antimitotic agents is through the alteration of microtubule dynamics that can be attributed to multiple factors including differences in expressed tubulin isotypes.[4, 5] In humans there is a family of multiple genes for α - and β -tubulin, each of which generates a unique gene product, an isotype.[6, 7] The tubulin isotypes have distinct tissue expression patterns, but anomalous expression has been observed in drug resistant tumors, such as the overexpression of β III-tubulin. The role of β III-tubulin has been extensively examined in lung tumor tissue by immunohistochemistry and it has been found that high expression of β III-tubulin in non small cell lung cancer (NSCLC) is indicative of a poor response to taxanes.[8] Also, it has been found that in NSCLC patients receiving Taxol, those that had low levels of β III-tubulin expression experienced a better response, longer progression-free survival and better overall survival as compared to patients with high levels of β III expression.[9] Although the expression of β -tubulin isotypes has been studied at the RNA level in human tumor and normal tissue and various mass spectrometry methods have been employed to quantitate specific tubulin isotypes in human cancer cell lines, to date, no complete description of expressed tubulin isotypes at the protein level in human tissue has been achieved.[10–13] Here we describe a label-free mass spectrometry-based method that can be used to determine the relative expression ratios of β -tubulin isotypes in human tumor tissue in a single experiment. This method can be used to determine if there are alterations that occur in the tubulin cytoskeleton that could predict treatment response to the taxanes.

The tubulin isotypes have highly similar primary sequences but are characterized by their distinct C-terminal sequences, termed the isotype defining region. Cleavage of tubulin with trypsin results in many identical peptides for both α - and β -tubulin. In addition to the distinctive C-terminal peptides, this digestion also releases unique internal cleavage peptides that can be used to assign the presence of a specific isotype and to determine its relative abundance compared to other isotypes. Our method uses these isotype-specific peptides as reporter ions. The relative abundance of each reporter ion in the mass spectrum serves as a measure of the overall abundance of that isotype in the sample.

A theoretical trypsin digest of all human β -tubulin isotypes revealed multiple peptides that could be employed as reporter peptides. In order to have reproducible and robust data, it was necessary to select peptides that were produced consistently from trypsin digestion, generated strong signals in the MALDI-TOF mass spectrometer and had no overlapping peptides in the mass spectrum. Based on these criteria, we selected the peptides shown in Table 1. These peptides have highly homologous sequences, but differ at a few residues, which results in a mass shift but does not greatly affect ionization efficiency. The identity of each of the selected peptides was confirmed by MS/MS (Figure S2). The reporter peptides for the β I and β II isotypes have different sequences, but the peptides have the same mass and thus cannot be distinguished by this method. Also, the β IVa and β IVb reporter peptides have the same sequence and cannot be distinguished from one another, which is not

surprising considering that these two isotypes have 98.4% sequence identity. However, β IVa is known to be predominantly expressed in the brain whereas β IVb is constitutively expressed. Currently there are no antibodies available which can distinguish β IVa from β IVb. The β VI reporter peptide is much shorter than the others and therefore cannot be compared to them. However, β VI is highly specific for the hematopoietic system. Most importantly, β III and β V have unique reporter peptides that can be used to determine their relative abundance compared to the other more commonly observed isotypes. Overall, with this method, we use 4 unique reporter peptides to determine the relative expression levels for the β I & β II (combined), β IV (a & b), β III and β V-tubulin isotypes.

To ascertain if the newly developed method was valid for determining the relative expression levels of tubulin isotypes in human samples, we used human cell lines for which the expression levels of β III had been previously described by alternative methods. We began with the A549 human NSCLC cell line. Our laboratory had determined the relative expression levels of the β -tubulin isotypes based on Coomassie staining intensity of isotype bands after separation by high resolution isoelectric focusing.[13] The β I isotype was the most abundant at 50% of the total β -tubulin with β IVb at 36.5%, β III at 8.0% and β V at 5.5% of the total β -tubulin. With our mass spectrometry method we determined the level of β I/ β II expression to be 50.5 $\pm$ 5.8 %, β IV to be 32.9 $\pm$ 2.4%, β III to be 9.5 $\pm$ 3.6% and β V to be 7.1 $\pm$ 2.7% (Figure S3). These values are in close agreement with what was previously described.

Next we used Hey, a human ovarian carcinoma cell line, for which the relative levels of β -tubulin expression had also been determined by the Coomassie blue staining method and had a different tubulin expression pattern than that of A549[13]. In this case β I/ β II was determined to comprise 39.3% of total β -tubulin with β IV at 39.9%, β V at 20.5% and β III at 3.0% of total β -tubulin. With our mass spectrometry-based method we determined the values to be 35.1 $\pm$ 6.8% for β I/ β II, 30.5 $\pm$ 2.4% for β IV, 31.6 $\pm$ 3.0% for β V and 2.7 $\pm$ 1.9 for β III (Figure S4).

Bovine brain tubulin is a commonly used source for tubulin. The composition of brain tubulin differs from other tissues, with isotypes that are specific to the brain, such as the β IVa-tubulin isotype. Also, the brain has high levels of β III-tubulin. Previous experiments have determined the expression levels of the β -tubulin isotypes to be 61% β I/ β II, 25% β III and 13% β IVa.[14] Our results show similar expression levels in the bovine brain. Using our reporter ions we determined that β I/ β II comprises 55.2 $\pm$ 2.8% of β -tubulin in the brain with β III comprising 23.2 $\pm$ 3.3% and β IV 21.6 $\pm$ 0.7% (Figure S5).

After validating the method with human cell lines and bovine brain tubulin, we applied our method to determine tubulin isotype expression levels in human lung adenocarcinoma tumor tissue. The expression level of the β III-tubulin isotype in each of the tumor tissue samples had been determined by immunohistochemical staining. The β III-tubulin isotype has been extensively studied in samples that are resistant or may become resistant to microtubule-binding drugs. However, not much is known about the expression patterns of the other tubulin isotypes in such samples. Using our mass spectrometry method to analyze the tubulin purified from approximately 50 mg of tumor tissue, we were able to characterize β -tubulin expression patterns in 9 lung tumor samples (Figure 1A). Each sample (except for sample 2) was analyzed three times and an average of the three runs is reported. The average value and the standard deviation for each isotype for each tumor are reported in Table S1. We found tumors 1, 2, 3, 4 and 6 expressed low levels of β III-tubulin, whereas tumors 5, 7, 8 and 9 expressed high levels of β III (Figure 1B). An unpaired t-test was used to validate that the differences between the two groups were significant. Representative data from two different samples are shown in Figure 2, one of which was characterized as high β III

expressing and the other as low β III expressing by immunohistochemical staining. Our results are consistent with what was determined by immunohistochemical staining for all of the tumors except one. Tumor 6 was characterized as expressing high levels of β III by immunohistochemistry, whereas the MS method determined that β III represented 1.1% of the total β -tubulin. This could be due to differences in the sections that were analyzed by immunohistochemistry and mass spectrometry. The mass spectrometrists were blinded to the results of the immunohistochemical staining until completion of the study.

In all of the lung tumor tissue samples, β I/ β II and β IVa/ β IVb were the most abundant isotypes, with values ranging from 18.7%–65.7% and 26.3%–79.1% of total β -tubulin, respectively. The β III & β V isotypes had relatively low or no detectable expression, with levels for β III ranging from 0–8.9% and β V from 1.0–10.4% of total β -tubulin. In the samples characterized as expressing low levels of β III by immunohistochemistry, β III expression on average was 1.4% of the total β -tubulin content, whereas β V was 2.5% of total β -tubulin. In tumor sample #3, no β III expression was observed at all by our method. In the samples characterized as high β III expressing, β III expression on average was 6.2% and β V 5.4% of the total β -tubulin content, with both isotypes detected in all 5 samples.

We present a pilot study of a novel method for the relative quantitation of tubulin isotypes in human tumor tissue. This method can be readily used by any laboratory with access to a MALDI-TOF mass spectrometer, and does not require internal standards labeled with heavy isotopes (*e.g.*, ^{15}N , ^{13}C) or labeling of the sample directly with heavy isotope tags. Our label-free method directly determines the relative expression levels of tubulin isotypes without the need for isotype-specific antibodies. This method can be applied to the examination of tubulin isotypes in any tissue sample and will be useful both in the clinical setting and by researchers further investigating the roles of the β -tubulin isotypes in drug resistance and tumor aggressiveness. This method allows for determination of the relative expression levels of β -tubulin isotypes in human tumor tissue and will permit determination of the validity of using specific tubulin isotypes as biomarkers to identify patients who would benefit from taxane treatment and those that would likely benefit from alternative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Berta Burd for her assistance in preparing samples for analysis by mass spectrometry. This work was supported by National Cancer Institute Grant CA 124898, The National Foundation for Cancer Research (to S.B.H.) and NIDA5P20 DA026149 (to R.H.A.).

Abbreviations

NSCLC non small cell lung cancer

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Statement of Clinical Relevance

Microtubules have proven to be an effective target for anticancer drugs, such as the taxanes, a class of microtubule-stabilizing agents which are routinely used in the treatment of human cancers. However, there is a recurring problem of resistance to the taxanes and the process by which tumors develop resistance is complex and not thoroughly understood. While there are most likely many factors contributing to resistance, one area of interest is the β -tubulin isotype composition of the microtubules. A change in β -tubulin isotype expression is thought to alter the stability and dynamics of the microtubules which could lead to reduced efficacy of the taxanes. We present a label-free mass spectrometry method for relative quantitation of β -tubulin isotypes and demonstrate the validity of the method with human lung tumors which were previously characterized by immunohistochemistry as high or low β III-tubulin expressors.

Figure 1. β -tubulin expression levels in human lung adenocarcinoma tissue

(A) The average relative expression levels of each β -tubulin isotype for 9 tumor tissue samples, determined by the MS-based method. (B) The levels of β III expression in each sample determined by immunohistochemical staining. Samples 1, 2, 3, 4, & 6 expressed low amounts of β III whereas samples 5, 7, 8 & 9 expressed high levels of β III. Samples were categorized as high (black) or low β III expressing (gray).

Figure 2. Mass spectrometry method using reporter peptides to determine relative expression levels of β -tubulin isotypes in human lung adenocarcinoma tissue

(A) Immunohistochemical staining of human lung adenocarcinoma tissue for β III. Tumor 1 (top) expressed low levels of β III whereas tumor 9 (bottom) expressed high levels of β III.

(B) Relative expression patterns of all β -tubulin isotypes in tumors 1 & 7. (C) Mass spectra showing the relative abundance levels for each of the reporter peptides for tumors 1 & 7.

This data was used to determine the overall relative abundance of each isotype.

Table 1
Sequence alignment and mass of β -tubulin trypsin reporter peptides

Residues that differ from the β I sequence are shown in bold type.

Tubulin Isotype	Gene	Accession Number	Mass (Da)	Reporter Peptide	Trypsin Reporter Peptide Sequence
βI	TUBB	NP_821133	3101.40		²⁰ FWEVISDEHGIDPTGTYHGDSDLQLDR ₄₆
βII	TUBB2B	NP_821080	3101.40		²⁰ FWEVISDEHGIDPTGSYHGDSDLQLER ₄₆
βIII	TUBB3	NP_006077	3076.41		²⁰ FWEVISDEHGIDPSGNYVGDSDLQLER ₄₆
βIVa	TUBB4	NP_006078	3115.42		²⁰ FWEVISDEHGIDPTGTYHGDSDLQLER ₄₆
βIVb	TUBB2C	NP_006079	3115.42		²⁰ FWEVISDEHGIDPTGTYHGDSDLQLER ₄₆
βV	TUBB6	NP_115914	2959.40		²⁰ FWEVISDEHGIDPAGGYVGDSALQLER ₄₆
βVI	TUBB1	NP_110400	2060.93		²⁰ FWEMIGEEHGIDLAGSDR ₃₇