Transcriptional and post-transcriptional regulation of βIII-tubulin protein expression in relation with cell cycle-dependent regulation of tumor cells

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Received September 1, 2011;  Accepted October 24, 2011

DOI: 10.3892/ijo.2011.1291

Abstract. The expression of βIII-tubulin (TUBB3) is generally restricted to neurons, but its mRNA is often expressed at low levels in non-neuronal cells. Interestingly, however, a number of non-neural tumors occasionally express high levels of TUBB3 protein, leading to a significant resistance to taxane derivatives. However, the molecular mechanisms controlling TUBB3 expression and its turnover during normal cell growth are largely unknown. Here, we present evidence that TUBB3 expression occurs in a cell cycle-dependent manner, and that its protein levels are controlled by the ubiquitin-proteasome system. Both mRNA and protein of TUBB3 accumulated around the G2/M stage of the cell cycle, and reduction of TUBB3 expression by siRNA resulted in partial inhibition of cell growth. Furthermore, the cell cycle-dependent expression of TUBB3 was mediated by the RE-1-silencing transcription factor REST through its binding to the RE-1 element that is present in the first intron of the TUBB3 gene. These results demonstrate a novel role of TUBB3 in cell cycle progression in non-neuronal cells, and further suggest that dysregulation of the REST-TUBB3 system could be a primary cause of the TUBB3 overexpression.

Introduction

Cell type-specific gene regulation is crucial for the differentiation of tissues and organs, and also for the maintenance of various cellular functions in adult organisms. Dysregulation of such cell type specificity could be a primary cause of tissue malformation during development and also of tissue malfunction in certain diseases, including cancers, in adult life (1). Tumors often show abnormal gene expression, and a number of non-neuronal tumors such as lung, breast, and ovarian cancers reveal aberrant over-expression of βIII-tubulin (TUBB3), which could be a primary reason for their insensitivity to microtubule-targeted anti-cancer drugs such as paclitaxel (2-5). To elucidate the mechanism of this anti-tumor drug resistance, it is important to understand how the expression of TUBB3 is controlled at the transcriptional and post-transcriptional levels, and how its regulation is disrupted in certain types of cancers.

The TUBB3 is a neural-specific tubulin isoform; however, it is occasionally expressed ectopically in certain non-neuronal cancer cells (4,6). While the gene expression of neural-specific TUBB3 is considered to be under the control of the RE-1 silencing transcription factor, REST (also designated NRSF, neuronal restricted silencing factor) (7), there is still no information on its regulatory sequence in the TUBB3 gene. The protein of TUBB3 is often used as a specific differentiation marker for neurons, being immunostained by the antibody Tuj-1 (8). However, it appears that TUBB3 protein expression is not absolutely neuron-specific, there is evidence that TUBB3 is expressed at a low level in normal large intestine, fibroblasts and keratinocytes (9,10). Therefore, it is uncertain whether the afore-mentioned abnormal ectopic expression of TUBB3 protein in certain non-neuronal tumors is dependent on chromatin remodeling or transcriptional de-repression and/or activation.

Tubulins, including TUBB3, are an essential constituent of the cellular cytoskeleton, forming microtubules that play pivotal roles in cell division (as spindle microtubules), cell migration (as cytoplasmic microtubules), and organellar transport (as axonal and dendritic microtubules in neurons) (11-13). Although microtubules are polymers of α- and β-tubulins, forming a large cylindrical structure, the structure is not static, but shows plasticity through the so-called dynamic instability (11). This
microtubule dynamism is controlled by various microtubule-associated proteins (MAPs) and other molecules (14,15), mediating a number of cellular responses including cell division, mitosis, and intracellular traffic. While the specific features of each of the tubulin isoforms may dictate their specialized functions, the roles played by neural-specific TUBB3 in non-neuronal tumor cells remain unknown.

To explore the potential roles of TUBB3 in non-neuronal cells as a basis for understanding the malignancy of cancer cells expressing TUBB3, it is important to examine how the expression of TUBB3 is regulated in non-neuronal cells. In examining the expression of both the mRNA and protein of TUBB3, we found unexpectedly that it occurs in a cell cycle-dependent manner, and that the protein level is controlled, at least in part, by protein degradation via the ubiquitin-proteasome system. We also present direct evidence for the involvement of REST/NRSF in the gene expression of TUBB3 via specific interaction with the RE-1 element, confirming that the TUBB3 gene is a direct target of REST/NRSF.

Materials and methods

Cell culture and synchronization. HEK293 and HeLa cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (IDAC), Tohoku University (Sendai, Japan). The cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS). To synchronize the cell cycles, cells were incubated with 2 µg/ml aphidicolin or 2 mM hydroxyurea for 24 h, then released from S-phase arrest by washing them three times with PBS and replacing their culture medium. To obtain M-phase-arrested cells, HeLa cells were incubated with 0.2% Triton X-100 in PBS (-) for 20 min at room temperature. The cells were permeabilized with 4% paraformaldehyde for 20 min at room temperature.

Plasmid construction and transfection. A C-terminal-deleted REST/NRSF (Δ1734 to 3294) expression vector was generated by PCR. The resulting PCR product was cloned into the HindIII and XhoI sites of the pcDNA3.1 (+) expression vector. The pCMV-Myc-ubiquitin vector was a gift from Dr N. Chiba (16). Transfection with plasmids was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). To obtain M-phase-arrested cells, HeLa cells were incubated with 100 µM monastrol or 50 ng/ml nocodazole for 16 h.

Chemicals and antibodies. Aphidicolin (Aphi), hydroxyurea (HU), monastrol, trichostatin A (TSA), and 5-aza-2'-deoxycytidine (5-aza-dC) and MG132 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Polyclonal REST/NRSF (Δ707-579), monoclonal CoREST (Δ707-455), and monoclonal histone deacetylase 1 (HDAC1, Δ50-614) antibodies were purchased from Upstate Biotechnology (Millipore, Billerica, MA, USA). Monoclonal βIII-tubulin (MMS-435P) and monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH, MMS-580S) antibodies were from Covance (Princeton, NJ, USA). Monoclonal Myc (9E10), polyclonal LaminB (M-20) and monoclonal HDAC1 (Δ2394) antibodies were purchased from Upstate Biotechnology (Millipore, Billerica, MA, USA). Monoclonal REST/NRSF (Δ1734 to 3294) expression vector was generated by PCR. The resulting PCR product was cloned into the HindIII and XhoI sites of the pcDNA3.1 (+) expression vector. The pCMV-Myc-ubiquitin vector was a gift from Dr N. Chiba (16). Transfection with plasmids was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). To obtain M-phase-arrested cells, HeLa cells were incubated with 100 µM monastrol or 50 ng/ml nocodazole for 16 h.

Total RNA extraction and cDNA synthesis. Total RNA was extracted using RNeasy Miniprep kit (Qiagen, Valencia, CA, USA), and cDNAs were synthesized using a SuperScriptIII kit (Life Technologies). Total RNA (2 µg) and 50 µM oligo(dT)₁₀ primers were used for cDNA synthesis.

Real-time RT-PCR. Quantification of mRNA levels was performed using real-time RT-PCR. One µl of cDNA was amplified with TaqMan Universal PCR master mix (Life Technologies) using specific primers and hybridization probes, and assessed using an ABI 7500 real-time PCR system (Life Technologies). Relative expression levels of mRNA were calculated by the ΔΔCT method. The forward and reverse primers for TUBB3 were 5'-GGGCCCAAATCTCGGGAAGTC-3' and 5'-AGT CGCCCACGTGTTGGC-3', respectively. The hybridization probe was 5'-FAM-TGATGAGCATCGGAGT CGACCCAG-TAMRA-3'. The forward and reverse primers for GAPDH were 5'-GAATGGAAGTCGGAGTCC-3' and 5'-GATGATGTGATGGGATTTC-3', respectively. The hybridization probe was 5'-FAM-CAGCTTCCCCTTCTAGCC-TAMRA-3'. Values were normalized relative to GAPDH mRNA.

Flow cytometry. Flow cytometry analysis was carried out using a CycleTEST™ Plus DNA Reagent kit (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed using a FACScalibur (Becton-Dickinson).

Small-interfering RNA (siRNA). Control siRNA duplexes (negative universal control HI #2) were purchased from Life Technologies. Cells were transfected with 40 nM of siRNA duplexes using Lipofectamine™ RNAi MAX (Life Technologies). The sequence of siRNA-1 sense oligo was 5'-GCUGCGACACCCCUCUUGUdTdT-3', corresponding to nt 1432 to 1450, which is the 3'-non-coding region. The sequence of siRNA-2 sense oligo was 5'-CCGCCCUCUGUCGCUUdTdT-3', corresponding to nt 1475 to 1493, which is the 3'-non-coding region.

Whole-cell extracts, and cytosolic and nuclear protein preparation. For preparation of whole-cell lysates, cells were lysed with RIPA buffer. Preparation of cytosolic and nuclear proteins was performed essentially as described by Kim et al (17).

Immunoprecipitation. HEK293 cells in 6-cm dishes were transfected with 2 µg of empty vector or pCMV-myc-ubiquitin plasmid and cultured for 48 h, then lysed with immunoprecipitation buffer [10 mM HEPES (pH 7.6), 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM PMSF, 1x Complete™ , 50 µM MG132]. After brief centrifugation, the antibody was added to each supernatant, followed by incubation at 4°C overnight with gentle rotation. Samples were then mixed with protein G-Sepharose and incubated at 4°C for 2 h. The beads were then washed, and the samples were subjected to electrophoresis in 10% SDS-PAGE.

Immunohistochemistry. HeLa cells were cultured on 25 x 25-mm cover slips, then rinsed with PBS (-), and fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in PBS (-) for
15 min, then blocked with 5% FBS in PBS (-) for 30 min at RT. Diluted antibodies were dropped onto the cover glasses and incubated overnight at 4˚C in a humidified chamber. The cells were then incubated with diluted Alexa488- or 594-conjugated second antibodies (Life Technologies) for 2 h at RT in a dark, humidified chamber. Nuclear staining was carried out by incubation with 4,6-diamidino-2-phenylindole (DAPI) for 5 min.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was carried out essentially by the method of Luo et al (18). HeLa cells were grown to about 60% confluency in a 10-cm dish, and the culture medium was replaced just before cross-linking treatment. The forward and reverse primers for the TUBB3 RE1-site were 5'-GCACCAAGGACAGCGC-3' and 5'-GCAACAGAGCCAAACTCCATC-3', respectively. The hybridization probe was 5'-FAM-CTGGCCTCGAACGCCGGGTT-TAMRA-3'.

Results

Cell cycle-dependent control of TUBB3 expression. To address whether TUBB3 expression is cell cycle-dependent, we analyzed synchronously growing HeLa cells released from S-phase arrest imposed by treatment with aphidicolin (Aphi) or hydroxyurea (HU). Cell cycle progression into G2/M phase was monitored by cyclin B1, which is expressed in this phase (19). TUBB3 mRNA was increased after release from S-phase arrest (Fig. 1A). Protein expression also increased 8 h after the release and peaked at 16 h (Fig. 1B), but the other isotype of β-tubulin, Class I β-tubulin, showed no change in its expression level throughout the cell cycle. These results suggest that TUBB3 is induced during cell cycle progression.

Next, we examined the effect of another reagent, monastrol, to address whether TUBB3 is involved in mitosis. Monastrol is a potent, cell-permeant inhibitor of mitosis, arresting cells at M phase by disrupting kinesin Eg5, the mitotic molecular motor protein (20). Monastrol-arrested cells, characterized by a monopolar spindle, were positive for TUBB3 (Fig. 1C), which was also detected by Western blotting (Fig. 1D). Moreover, TUBB3 expression was detected in the cyclin B1-positive population (Fig. 2), among asynchronized HEK293 or HeLa cells. These results suggest that βIII-tubulin is involved in cell division in non-neuronal cells.

Treatment with TUBB3 siRNA decreases cell growth. Next, we assessed the effects of a siRNA specific for TUBB3 mRNA in HEK293 and HeLa cells. The siRNA decreased the expression of endogenous TUBB3 mRNA by about 80-90%
throughout the experimental period (Fig. 3A). The numbers of cells at the indicated time points after transfection indicated that knockdown of endogenous TUBB3 mRNA decreased the cell growth rate relative to the control in both cell lines (Fig. 3B). These results indicate that TUBB3 is required for normal cell growth.

Control of TUBB3 levels by the ubiquitin-proteasome system in normally growing cells. We next explored how TUBB3 protein expression is controlled. The ubiquitin-proteasome-dependent degradation pathway is important for maintenance of cellular homeostasis through irreversible destruction of proteins (21). To test the possible involvement of the ubiquitin-proteasomal degradation pathway, we first examined the effect of MG132, a proteasome inhibitor, on TUBB3 protein turnover. As shown in Fig. 4A, Western blot experiments revealed 3.5- to 4-fold accumulation of TUBB3 protein after MG132 treatment in HEK293 and HeLa cells. Immunohistochemistry confirmed that this TUBB3 protein accumulation occurred after MG132 treatment in a time-dependent manner in HeLa cells (Fig. 5). As the effect of MG132 on TUBB3 levels suggested that the protein is ubiquitinated, we further examined whether TUBB3 is poly-ubiquitinated in normally growing cells. We transfected HEK293 cells with a myc-tagged ubiquitin expression vector, and then carried out immunoprecipitation with TUBB3 antibody, then ubiquitinated proteins were detected by myc antibody.
tion with a specific antibody against TUBB3, followed by immunoblotting with an anti-myc antibody. As shown in Fig. 4B, TUBB3 was detected as high-molecular-weight smears, suggesting that TUBB3 is polyubiquitinated in vivo in normally growing cells. These results demonstrate that the level of TUBB3 protein is physiologically controlled, at least in part, by the ubiquitin-proteosome system in normally growing non-neuronal cells.

Epigenetic regulation for TUBB3 expression. TUBB3 expression is regulated at the chromatin level and affected by HDAC inhibitor. We next investigated whether the TUBB3 gene might be regulated at the chromatin level. We determined a RE-1 sequence, which bind transcription regulator, REST/NRSF, on TUBB3 gene. These sequences were also found in mouse and rat TUBB3 genomic DNA (Fig. 6A). The expression of REST/NRSF and its related proteins was observed predominantly in the nucleus in both cell lines (Fig. 7A). To confirm the involvement of epigenetic regulation of TUBB3 in non-neuronal cells, we examined the effects of the DNA demethylation reagent, 5-Aza-dC, and the histone deacetylase inhibitor, TSA, on normally growing HeLa and HEK293 cells. Increased expression of TUBB3 protein was detected upon treatment with TSA in both cell lines (Fig. 7B, lanes 5-7), whereas no such increase was observed with 5-Aza-dC (lanes 2-4). This result suggests that TUBB3 expression in non-neuronal cells is regulated mainly by histone acetylation status. To investigate whether TUBB3 gene is directly regulated by REST/NRSF, we transiently transfected C-terminal-deleted REST/NRSF into normally growing HeK293 cells. As REST/NRSF forms complexes with silencing factors (HDAC, mSin3a, MeCP2) through the C-terminal CoREST binding motif and is impor-
Another cell cycle synchronizing reagent, nocodazole, which arrests the cells in M-phase, showed distinct pattern. Occupancy was minimum at 0 h and peaked at 16 h after release (Fig. 6B), the time point at which majority of cell population were in M- and S-phase, respectively (Fig. 6C). These results suggest that REST/NRSF dissociates from the TUBB3 NRSE/RE-1 sequence in G2/M phase and recovers in G1 phase, and that the switching mechanism of REST/NRSF is involved in cell cycle-dependent expression of the TUBB3 gene.

**Discussion**

Several non-neuronal tumors such as ovarian, breast and lung cancers show aberrant expression of TUBB3 and significant resistance to taxane derivatives (4,23-27). However, details of the mechanisms underlying TUBB3 overexpression in non-neuronal cancer cells are largely unknown. In this study, we demonstrated that i) cell cycle-dependent expression of TUBB3 is involved in cell growth in non-neuronal cells and ii) that the timed expression of TUBB3 is directly regulated by REST/NRSF during the cell cycle. Dysregulation of the REST/NRSF-TUBB3 system may be a primary cause of malignancy in taxane-resistant tumors.

Microtubules are involved in various cellular events, their role as a mitotic spindle component being particularly critical for normal cell growth (dividing cells) and for neuronal plasticity (non-dividing cells) (11-13,28). TUBB3 is highly expressed in mature neurons and generally used as a reliable marker of neuronal differentiation (8). With regards to the physiological involvement of TUBB3 in non-neuronal cells, it is expressed in the male germ line and essential for viability and fertility in *Drosophila melanogaster* (29). However, little is known about its involvement in mammalian cells except in mature neurons.

Although microtubules have a variety of functions in cellular events, their role as a mitotic spindle component is most crucial for normal cell growth. Our results demonstrated that TUBB3 is expressed in HEK293 and HeLa cells, albeit at a low level, but is strictly regulated in a cell cycle-dependent manner. We therefore assume that TUBB3 is directly involved in mitotic spindle control in normal cells. As removal of TUBB3 from unfractionated tubulin increases the rate and extent of tubulin assembly (30), and the tubulin isotype composition regulates microtubule dynamics (31), it is possible that cell cycle-dependently expressed TUBB3 contributes to mitotic spindle dynamism by controlling microtubule instability in mitotic cells.

In glioblastoma cells, TUBB3 forms complexes with γ-tubulin (32), which is important for the nucleation and polar orientation of microtubules (12). Furthermore, TUBB3 expression has been demonstrated by immunohistochemistry in the large intestine, fibroblasts and keratinocytes under physiological conditions (9,10). These lines of evidence support our finding that TUBB3 is an important component in mitosis.

It has been shown that REST/NRSF plays a critical role as a key transcriptional repressor of neuron-specific gene regulation in non-neuronal cells (33-35). TUBB3 is one of the target genes regulated by REST/NRSF during neurogenesis (7). However, no detailed analysis of its REST/NRSF binding site has yet been carried out. In this context, we...
showed that a highly conserved RE-1 sequence is located in the first intron of the TUBB3 gene. Surprisingly, REST/NRSF occupancy in the RE-1 sequence of the TUBB3 gene was altered in a cell cycle-dependent manner, being minimal in the G2/M phase. This switching mechanism is involved in the intrinsic expression of TUBB3 in non-neuronal cells. One recently discovered aspect of this underlying mechanism is regulation of phosphorylated REST/NRSF by β-transducing repeat-containing protein (β-TrCP) during the cell cycle (36). Furthermore, β-TrCP overexpression causes oncogenic transformation of human mammary epithelial cells through REST/NRSF degradation (37). As several types of cancer show aberrant expression of β-TrCP (38), it would be interesting to determine whether TUBB3 expression involves activation of the β-TrCP-REST/NRSF axis.

There is increasing evidence to support the role of REST/NRSF as a tumor suppressor gene, and linkage of its dysregulation or abnormal expression to tumorigenesis (37,39-42). Thus, it is possible that dysfunction of REST/NRSF is involved in TUBB3 overexpression in non-neuronal cancer cells and is a primary cause of taxol resistance. To confirm this, detailed analysis of REST/NRSF in other cancer cells, such as ovarian cancer cells expressing high levels of TUBB3, is required. Our results suggest possible mechanism underlying TUBB3 overexpression in non-neuronal cancer cell lines.

In conclusion, this study is the first to indicate the physiological involvement of TUBB3 in mitosis in normally growing non-neuronal cells. Taken together, our present findings suggest that disruption of the novel mechanisms that underlie TUBB3 regulation is responsible for the constitutively high expression of TUBB3 in various non-neuronal cancer cells.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research for Young Scientists No. 19790267 for M.S., and the ‘Open Research Center’ Project at Iwate Medical University from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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