Drug Resistant MCF-7 Cells have Altered Expression Levels of ß-Tubulin Isotypes and Mutations in TUBB Gene

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ABSTRACT

Antimicrotubule agents paclitaxel, docetaxel and vincristine are used in treatment of breast cancer. They bind to ß-tubulin subunit of microtubules. Multidrug resistance developed against these drugs remains a serious clinical problem. Different ß-tubulin izotypes possess differential assembly/disassembly dynamics. In this study, expressions of ß-tubulin isotypes, and mutations in TUBB gene were investigated in paclitaxel, docetaxel and vincristine resistant MCF-7 breast carcinoma cell lines. Resistant sublines were developed in the laboratory by continuous drug applications in dose increments and development of resistance were assayed by cytotoxicity analysis. Gene expression levels of ß-tubulin isotypes were investigated by RT-PCR. Exon 4 of the TUBB gene was amplified by PCR and the products were sequenced for determination of mutations. According to expression analysis, mRNA levels of ßII-, ßIII- and ßV-tubulin isotypes were significantly upregulated in paclitaxel and docetaxel resistant cells where they were significantly downregulated in vincristine resistant cells. Sequence analysis of exon 4 of TUBB gene coding for ßI-tubulin revealed that MCF-7/120nMDoc cells carry Gln-327 → His mutation and MCF-7/120nMVinc cells have His-179 → Pro mutation. In conclusion, differential expression levels of ßII-, ßIII- and ßV-tubulin isotypes and mutations in TUBB gene were determined in paclitaxel, docetaxel and vincristine resistant MCF-7 cells. It was shown for the first time that ßV-tubulin expression level may be correlated to resistance to antimicrotubule drugs. Though more functional analysis is required, the data presented here provides an insight into mechanisms of paclitaxel, docetaxel and vincristine resistance through alterations in tubulin/microtubule system.

Keywords: Antimicrotubule drugs, Multidrug resistance, ß-tubulin isotypes, TUBB

ÖZET

İlaç Dirençli MCF-7 Hücrelerinde ß-Tübülin İzotiplerinin Değişen İfadelenme Düzeyleri ve TUBB Gen Mutasyonları

Meme kanserinin tedavisinde kullanılan antimikrotübül ajanlar paklitaksel, dosetaksel ve vinkristin, mikrotübüllerin ß–tübülin alt grubuna bağlanmaktadırlar. Bu ilaçlara karşı gelişen çoklu ilaç dirençliliği tedavinin başarısını kısıtlayan önemli bir problemdir. Bu çalışmada, paklitaksel, dosetaksel ve vinkristine dirençli MCF-7 meme karsinomu hücre hatlarında ß–tübülin izotiplerinin gen ifade düzeyleri ve TUBB gen mutasyonları incelenmiştir. Dirençli hücre hatları artan dozlarda seçici ilaç uygulamaları ile geliştirilmiş ve dirençlilik gelişimi sitotoksisite testleri ile belirlenmiştir. Gen ifade düzeyleri geri transkriptaz-polimeraz zincirleme reaksiyonu (GT-PZR) ile incelenmiştir. TUBB geninin dördüncü egzonu PZR yöntemi ile çoğaltılmış ve DNA dizi analizi yapılmıştır.

Gen ifade düzeyleri incelendiğinde, paklitaksel ve dosetaksele dirençli hatlarda ßII-, ßIII- and ßV-tübülin izotiplerinin ifade düzeylerinde istatistiksel olarak anlamlı artış olduğu, vinkristine dirençli hatta ise bu genlerin ifade düzeylerinde istatistiksel olarak anlamlı azalma olduğu saptanmıştır. DNA dizi analizi sonuçlarına göre, dosetaksele dirençli hücrelerde Gln-327 → His, vinkristine dirençli hücrelerde ise His-179 → Pro mutasyonu bulunduğu belirlenmiştir. Sonuç olarak, bu çalışmada, paklitaksel, dosetaksel ve vinkristine dirençli MCF-7 hücrelerinde farklı mikrotübül dinamiğine sahip ßII-, ßIII- and ßV-tübülin izotip ifade düzeylerinde anlamlı değişiklikler olduğu görülmüştür. Aynı zamanda dosetaksel ve vinkristine dirençli hücrelerde ßI–tübülinini kodlayan TUBB geninde ilaç bağlanması ile ilgili bölgelerde iki farklı mutasyon belirlenmiştir. Dirençli hücrelerde ßV-tübülinin ifade düzeyindeki değişiklik ise ilk defa gösterilmiştir. Bu çalışmadan elde edilen bulgular paklitaksel, dosetaksel ve vinkristine karşı gelişen dirençlilik mekanizmalarında tübülin/mikrotübül dengesindeki değişimlerin önemli olabileceği ile ilgili aydınlatıcı bilgiler içermektedir.

Anahtar kelimeler: Antimikrotübül ilaçlar, Çoklu ilaç dirençliliği, ß-tübülin izotipleri, TUBB

INTRODUCTION

Microtubules are dynamic cytoskeletal polymers. Suppression of microtubule dynamics is important for antimitotic action of antitubulin drugs1. Antimicrotubule agents including paclitaxel, docetaxel (polymerizing) and vicristine, vinorelbine (depolymerizing) are widely used in treatment of variety of tumors including metastatic breast cancer.[2,3] Taxoid drugs, paclitaxel and its semisynthetic derivative docetaxel bind to ß-tubulin of microtubules and stabilize microtubules against depolymerization.[4,5] The vinca alkaloid vincristine also binds to ß-tubulin, however prevents polymerization of microtubules.[6] Distortion of polymerization-depolymerization dynamics results in mitotic arrest and growth inhibition.[7]

There are seven ß-tubulin isotypes in human tissues with differential tissue specific expression pattern.[8] Multiple tubulin isotypes in cells has been correlated to their different physiological properties.[9] Some tubulin isotypes have differing assembly properties.[10,11] Alterations in expression levels of the ß-tubulin isotypes have been correlated to multidrug resistance developed against antimicrotubule agents. Tumor cells resistant to paclitaxel, docetaxel and vinca alkaloids presented altered tubulin composition.[12,13]

Opposing tubulin alterations are responsible for Taxoid and vinca alkaloid resistance. For example, decreased microtubule stability increases taxoid sensitivity where increased microtubule stability increases vincristine sensitivity of cells. ßIII-tubulin has been previously characterized for its low polymerization rate[9] and tumor cells overexpressing this isotype have been shown to overcome stabilizing effects of paclitaxel.[14] Alterations in expression levels of other isotypes have been correlated to paclitaxel, docetaxel[15-18] and vincristine resistance.[19] However, the results demonstrating involvement of other tubulin isotypes in the development of multidrug resistance phenotype are still controversial.

In addition to differential expression patterns, mutations in TUBB gene which encodes the predominant constitutive class I ß-tubulin have also been studied for resistance to antimicrotubule agents.[20-22] Although there has not been any finding that vincristine and taxane resistant breast cancer patients harbour any point mutations on TUBB gene yet, Wiesen et al[23] have previously shown that paclitaxel resistant breast adenocarcinoma cell line MDA-MB-231 harbours a point mutation on the gene. The paclitaxel resistant ovarian cancer cells exhibit impaired paclitaxel driven tubulin polymerization caused by acquired TUBB mutations.[21] These mutations can be either in drug binding site of tubulins and decrease drug binding efficacy of tubulin dimers or along the microtubule and affect polymerization-depolymerization dynamics.

In the present study, paclitaxel, docetaxel and vincristine resistant MCF-7 breast carcinoma cells were selected in our laboratory as previously described[24] and served as in-vitro models for acquired drug resistance. mRNA expression levels of ß-tubulin isotype classes I (TUBB), II (TUBB2B), III (TUBB3), IVa (TUBB4), IVb (TUBB2C) and V (TUBB6) (Table 1), were investigated by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition to expression analysis, mutation studies of TUBB gene in the sublines were also presented.

Table 1. ß-tubulin isotypes

ß-tubulin isotype classes	Human gene[d]	Gene Bank accession number	
		mRNA	Protein
ßI[a] (tubulin, beta)[b]	*TUBB*	AF141349	AAD33873
ßII[a] (tubulin, beta-2A/tubulin, beta-2B)[b]	*TUBB2A/TUBB2B*	BC001352	AAH01352
ßIII[a] (tubulin, beta-3)[b]	*TUBB3*	BC000748	AAH00748
ßIV[a,c] (tubulin, beta-4)[b]	*TUBB4*	BC013683	AAH13683
ßIV[a,c] (tubulin, beta-2C)[b]	*TUBB2C*	BC004188	AAH04188
ßV[a] (tubulin, beta-6)[b]	*TUBB6*	NMß032525	NP 115914

[a] ßI, II, III, IVa, IVb,V-tubulin refer for names of different beta tubulin isotype proteins differentiated based on each carboxy terminal region of each protein

[b] New protein names used in NCBI Entrez Protein database

[c] Tubulin, beta-4 for the protein encoded by *TUBB4* gene and tubulin beta-2C for the protein encoded by *TUBB2C* gene are updated and preferred names by NCBI database

[d] Updated and accepted gene names by HUGO Gene Nomenclature Committee

MATERIALS AND METHODS

Chemicals

Paclitaxel (Sigma, St. Louis, MO, USA) and docetaxel (Fluka, St. Gallen, Switzerland) were dissolved in DMSO (Sigma) to prepare stock solutions. Vincristine was obtained from Gülhane Military Medical Academy, Ankara, Turkey. Vincristine and doxorubicin were diluted in deionized water.

Cell Lines

The MCF-7 cell line, a model cell line for human mammary carcinoma, was used as parental cells.[25] These cells exhibit features of differentiated mammary epithelium and was donated by ŞAP Institute, Ankara, Turkey. Paclitaxel, docetaxel and vincristine were applied separately in dose increments to MCF-7 cells for stepwise selection of resistant cells and cells were maintained as previously described.[24,26] In brief, cells were selected in increasing concentrations of drugs and maintained by exposure 400nM paclitaxel, 120nM docetaxel and, 120nM vincristine. DMSO (Sigma) was used as solvent of docetaxel and paclitaxel and it did not exert any antiproliferative efffect when relevant solvent concentrations were tested. Moreover, it was ineffective in development of resistance when applied individually. Cells capable of growing in selection concentrations of drugs became resistant and developed sublines were designated with their respective drug concentrations.

Assay for Cell Proliferation

Antiproliferative effects of paclitaxel, docetaxel and vincristine on parental MCF-7 cells and drug selected sublines were evaluated by means of the Cell Proliferation Kit (Biological Industries, Israel). Assay is a colorimetric test based on the tetrazolium salt, XTT. Assay was performed as previously described[26] and inhibitory concentrations 50 (IC_{50}) values were calculated. Resistance indices (R) for each subline were evaluated by dividing IC_{50} for docetaxel obtained from docetaxel selected cells to IC_{50} for docetaxel obtained from parental MCF-7 cells.

RNA Isolation and RT-PCR

RNA isolation was performed from parental and resistant MCF-7 cells according to guanidium thiocyanide/phenol-chloroform protocol.[27] cDNA synthesis was performed with 5 µg of total RNA, 0.5 µg oligodT or oligonucleotide probes for 5'tagged primers and 40 units of M-MuLV Reverse

Table 2. Primer sets for RT-PCR and PCR

Primer set	Forward/ Reverse	Denaturation	Annealing	Extension	Number of cycles	Amplicon size (bp)
Class I	F: 5'CCCCATACATACCTTGAGGCGA3', TUBB exon 1					
ß-tubulin[a]	R: 5' GCCAAAAGGACCTGAGCGAA 3', TUBB exon 3	94°C, 45 s	51°C, 45 s	72°C, 45 s	33	289
Class II	F: 5' CATCTCCGAGCAGTTCACGG3', TUBB2B exon 4					
ß-tubulin[b,c]	R: 5' AGACCGTGTGGGTCGCCCTC 3', TUBB2B exon 4	94°C, 1 min	62°C, 45 s	72°C, 45 s	31	200
Class III	F: 5'ATGCGGGAGATCGTGCACAT3', TUBB3 exon 1					
ß-tubulin[a]	R: 5'CCCCTGAGCGGACACTGT3', TUBB3 exon 3	94°C, 45 s	52°C, 45 s	72°C, 45 s	37	238
Class IVa	F: 5'TCTCCGCCGCATCTTCCA3', TUBB4 exon 1					
ß-tubulin[a]	R: 5'GCTCTGGGGACATAATTTCCTCCT3', TUBB4 exon 3	94°C, 45 s	53°C, 45 s	72°C, 45 s	35	272
Class IV[b]	F: 5'GCTGTTTGTCTACTTCCTCCTGCT3', TUBB2C exon 1					
ß-tubulin[a]	R: 5'CAGTTGTTCCCAGCACCACTCT3', TUBB2C exon 4	94°C, 45 s	54°C, 45 s	72°C, 45 s	31	344
Class V	F: 5'CGGGGAGGAAGCTTTTGAGG3', TUBB6 exon 4					
ß-tubulin[b,d]	R: 5' AGACCGTGTGGGCTGGGTAG 3', TUBB6 exon 4	94°C, 1 min	62°C, 45 s	72°C, 45 s	33	256
B-2m	F: 5'TCTCTCTTTCTGGCCTGGAG3', Exon 1					
	R: 5'GGATGGATGAAACCCAGACA3', Exon2	94°C, 30 s	55°C, 30 s	72°C, 30 s	30	122
TUBB[e,f]	F: 5' TGTATTGGAGTGCTAATACAG 3', 3409-3429					
	R: 5' CTCCCTTGAAGCTGAGATGG 3', 5199-5218	98°C, 20 s	58°C, 30 s	72°C, 45 s	35	1810
4-1[e]	F: 5' CATGTATCTTCCATACCCTG 3', 3577-3596					
	R: 5' CTGAAGGTATTCATGATGCG 3', 3835-3854	98°C, 20 s	59°C, 30 s	72°C, 25 s	30	278
4-2[e]	F: 5' GAATGGGCACTCTCCTTATC 3', Exon 4, 3788-3807					
	R: 5' GGACCATGTTGACTGCCAAC 3', Exon 4, 4107-4126	98°C, 20 s	59°C, 30 s	72°C, 25 s	30	339
4-3[e]	F: 5'ATGAGTGGTGTCACCACCTG 3', Exon 4, 4048-4067					
	R: 5' GACTGCCATCTTGAGGCCAC 3', Exon 4, 4427-4446	98°C, 20 s	59°C, 30 s	72°C, 25 s	30	399
4-4[e]	F: 5'CCCAACAATGTCAAGACAGC 3', Exon 4, 4387-4406					
	R: 5'CAAGATAGAGGCAGCAAACAC 3', Exon 4, 4767-4787	98°C, 20 s	59°C, 30 s	72°C, 25 s	30	401

[a] 43; [b] Reverse primers were designed according to 5'tagged cDNA sysnthesis[28,29] and forward primers were previously described[18,43].
[c] Accession number: BC001352; [d] Accession number: NMß032525
[e] 22; [f] Accession number: NMß178014

Transcriptase according to the manufacturer's instructions (MBI Fermentas, Lithuania). cDNA synthesis of ßII- and ßV-tubulin were performed using oligonucleotide probes with high Tm and 5'tagged primers according to previously described protocols.[28,29] This method provides unique sites on cDNA which are not present on genomic DNA thus preventing amplification of any contaminating DNA. Beta-2 microglobulin (ß-2m) was used as internal standard. The oligonucleotide probes for cDNA synthesis of class II and V ß-tubulin are: 5'AGACCGTGTGGGTCGCCCTCCTCCTCCTCGA3' (Tm: 75°C) and 5'AGACCGTGTGGGCTGGGTAGAACCCGCAATTCTCT3' (Tm: 74°C), respectively. Primer sets and amplicon sizes are listed in Table 2. PCR products were examined by native agarose (2% w/v) gel electrophoresis and visualized with ethidium bromide staining. Densitometry measurements of band intensities were performed using Scion Image Software (Scion Corporation, Maryland, USA).

DNA Isolation and Sequence Analysis of TUBB Gene

DNA samples were isolated from each MCF-7, MCF-7/400nMPac, MCF-7/120nMDoc and MCF-7/120nMVinc sublines with AbsoGene genomic DNA isolation kit (RTA, Turkey) according to manufacturer's instructions. Genomic DNA samples isolated from each subline were used as template for PCR and the primers were selected so as to prevent amplification from pseudogenes according to previous report.[22] The 4th exon of the TUBB gene was amplified by PCR and subsequently, the PCR product was amplified by four nested PCR as four overlapping fragments as previously described.[22] All PCRs were performed with error free high fidelity DNA polymerase enzyme, Phusion (Finnzymes, Finland). Primer sets and amplicon sizes are given in Table 2. The PCR products were examined by native agarose (2% w/v) gel electrophoresis. UltraClean™ GelSpin DNA Purification Kit (MO BIO Laboratories, Inc., CA, USA) was used to elute PCR products from agarose gel. Eluted samples were sequenced automatically bidirectional by a company (Iontek, Istanbul-Turkey).

Statistical Analysis

The results of XTT cytotoxicity assay and band intensity values of RT-PCR were subjected to two-tailed t-test by using SPSS Software (SPSS Inc., Illinois, USA) to determine significant difference between means of groups ($\alpha = 0.05$) .

RESULTS

Resistance Profiles

According to cell proliferation assays, drug selected sublines gained varying degrees of resistance to their selective agents. The paclitaxel (MCF-7/400nMPac), docetaxel (MCF-7/120nMDoc) and vincristine (MCF-7/120nMVinc) resistant sublines are 150-, 47- and 30-fold resistant to these drugs, respectively, when compared to the parental MCF-7 cells.[26]

mRNA Expression Levels of β-tubulin Isotypes in Sensitive and Resistant Cells

According to fold change values obtained from RT-PCR results (Table 3), expression of class II ß-tubulin was upregulated in docetaxel resistant sublines and in MCF-7/120nMDoc cells it was 1.68-fold higher than that of sensitive cells. The increased expression was also observed in paclitaxel resistant sublines where 0.6-fold downregulation was observed 120nM vincristine resistant cells. Class III ß-tubulin expression was significantly upregulated in 120nM docetaxel selected cells (1.90-fold). There was also a 1.53-fold increase in paclitaxel resistant sublines. On the contrary, its expression level in MCF-7/120nMVinc cells significantly decreased (0.28-fold). Class Vß-tubulin level was upregulated in paclitaxel and docetaxel resistant sublines (significant 1.78-fold and 1.90-fold increase, respectively). However, the expression level was significantly downregulated in MCF-7/120nMVinc cells (0.53-fold). Results demonstrated that class I, IVa and Vb ß-tubulin expression levels did not significantly change in drug resistant sublines (data not shown).

Mutation Analysis

In order to identify possible mutations in TUBB gene, DNA was isolated from MCF-7/400nMPac,

Table 3. Changes in expression levels of ß-tubulin isotype classes

ß-tubulin	Cells	ß/b2m 1[a]	ß/b2m 2[a]	Mean ß/b2m ± SEM[b*]	FC 1[c]	FC 2[c]	Mean FC ± SEM[b]
ßII	MCF-7	1.13	1.09	1.11±0.02	-	-	-
	400nM Pac	1.54	1.77	1.65±0.12	1.36	1.62	1.49±0.13
	120nM Doc	1.84	1.90	1.87±0.03	1.63	1.74	1.68±0.06
	120nM Vinc	0.77	0.57	0.67±0.10	0.68	0.52	0.60±0.08
ßIII	MCF-7	1.03	1.06	1.05±0.02	-	-	-
	400nM Pac	1.48	1.72	1.60±0.12	1.44	1.62	1.53±0.09
	120nM Doc	2.04	1.93	1.98±0.06	1.98	1.82	1.90±0.08
	120nM Vinc	0.28	0.32	0.30±0.02	0.27	0.30	0.28±0.02
ßV	MCF-7	1.13	1.18	1.16±0.03	-	-	-
	400nM Pac	2.07	2.03	2.05±0.02	1.83	1.72	1.78±0.06
	120nM Doc	2.02	2.37	2.20±0.17	1.79	2.01	1.90±0.11
	120nM Vinc	0.55	0.67	0.46±0.03	0.49	0.57	0.53±0.04

[a] Expression levels of ß-tubulin isotypes normalized to b2-m expression levels. Values 1 and 2 were obtained from two independent experiments.
[b] SEM: Standard error of the means obtained from mean values of two independent experiments
* Mean expression levels were statistically significant between sensitive and resistant cells with $p < 0.05$
[c] FC (fold changes) = ß/b2m resistant / ß/b2m sensitive (FC1 and FC2 values were obtained by dividing expression levels of independent experiments 1 and 2, respectively)

MCF-7/120nMDoc, MCF-7/120nMVinc, and sensitive cells. Site encoding the drug binding site of class I ß-tubulin (TUBB gene; exon 4) was amplified with overlapping primer sets and sequenced. Sequence differences between sensitive and resistant cells were evaluated. DNA of MCF-7/120nMDoc and MCF-7/120nMVinc cells had nucleotide sequence differences compared with sensitive cells. There was a CAG → CAT mutation at codon 327 in MCF-7/120nMDoc cells and CAT → CCT mutation at codon 179 in MCF-7/120 nMVinc leading to Gln to His and His to Pro changes, respectively. There were not any nucleotide sequence differences in MCF-7/400nMPac cells when compared with sensitive cells.

DISCUSSION

Resistance to chemotherapy is a multifactor phenomenon such that chemoresistance developed against a drug may depend on alterations more than one cellular pathway.[30] Recent studies represent that changes in the tubulin/microtubule system may play an important role in resistance to antimicrotubule agents targeting this system.[31,32] These include altered expression levels of the genes encoding b-tubulin isotypes and microtubule-associated proteins, and mutations in drug binding regions.[22] These changes can lead to altered microtubule dynamics or stability in the cell and decreased sensitivity to antimicrotubule agents.[12]

Mammals have different tubulin proteins having differential assembly properties.[14] In vitro studies with purified tubulins[33,34] revealed that, relative abundance of these proteins determine tubulin polymerization kinetics since capacity of ß-tubulins to form heterodimers with ß-tubulins differs and leads to changes in microtubule assembly. According to gene expression analysis, all the isotypes were intrinsically expressed in sensitive MCF-7 cells. Class II ß-tubulin mRNA expression level significantly increased in MCF-7/400nMPac and MCF-7/120nMDoc cells (1.53 and 1.68-fold, respectively) with respect to parental MCF-7. It was previously reported that[35] increased mRNA expression

levels of class II ß-tubulin seemed to be a promising predictive marker of docetaxel response in breast cancer patients. Expression level of class III ß-tubulin also significantly increased in paclitaxel and docetaxel resistant sublines (1.53- and 1.90-fold, respectively) where it significantly decreased in MCF-7/120nMVinc cells (0.28-fold). Decreased mRNA expression level of class III ß-tubulin have been correlated with vincristine and vinblastine resistance in human leukemia cells[20] since class III ß-tubulin has relatively higher microtubule-destabilizing potency.[9,36,37] In addition, increased expression levels were also correlated with taxol resistance in previous studies.[14,16,17] Similarly, antisense reduction of class III beta tubulin mRNA and protein expression in Taxol resistant lung cancer cells corresponded to an increase in sensitivity.[37] Class V ß-tubulin was significantly overexpressed in MCF-7/400 nMPac cells (1.78-fold) and MCF-7/120nMDoc (1.90-fold) cells. On the other hand, it was significantly decreased in MCF-7/120nMVinc cells (0.53-fold). Interestingly, it was previously reported that similar to class III ß-tubulin, mouse class V ß-tubulin had also destabilizing properties11. Furthermore, Horwitz et al.[38] demonstrated a possible regulated balance between calss III and V ß-tubulin expressions in cells based on their similar topology that has been correlated to microtubule-destabilizing properties. However, alterations in expression of class V ß-tubulin have not been correlated with antimicrotubule resistance, previously. Conclusively, in agreement with the previous findings, changes in mRNA expression levels of class II, III and V ß-isotypes seem to be important alterations in drug resistant cells. It is also interesting that different alteration patterns were observed in the expression levels of ß-tubulin isotypes in response to effect of microtubule stabilizing and destabilizing agents.

The results obtained with class I, IVa and IVb ß-tubulin mRNA expression level in drug resistant sublines were not statistically significant. However, in previous studies published, increased class I and IVa ß-tubulin mRNA levels in paclitaxel resistant cells have been reported.[19,22] Such variations in expression patterns may be correlated to differences in tissue types, resistance indices and selection doses. In addition, direct functional relevance of these isotypes with microtubule stabilizing and/or destabilizing agents still remains unclear.

There is evidence that mutations in class I b-tubulin may play an important role in resistance to drugs that target the tubulin/microtubule system in resistant cell lines.[12] In this study mutational analysis revealed single base substitutions in MCF-7/120 nMDoc and MCF-7/120nMVinc cells resulting in missense mutations at amino acid level. MCF-7/120 nMDoc cells acquired a Gln-327 → His-327 mutation in comparison with parental cells and gene sequence form NCBI database. Previously, this site was found to be close to common binding sites of taxanes in taxol selected human ovarian cancer cell line.[21] Codon 327 may also be close to taxane binding site in MCF-7 and amino acid mutation at this site may inhibit binding of docetaxel to microtubules as previously proposed by Giannakakou et al.[21] Amino acid change from neutral amino acid glutamine to positively charged amino acid histidine having cyclic side chain may result in impaired binding ability of docetaxel to microtubules. MCF-7/120 nMVinc cells have His-179 → Pro-179 mutation in class I b-tubulin. This mutation is near GTP binding site (ß173) and possibly alters microtubule stability.[20] Since GTP binding is an essential regulatory mechanism of microtubule stability, changes in that site may seriously influence microtubule stability.[39] The cells may have developed this mechanism to rearrange microtubule stability and compensate for the effect of presence of microtubule disassembly agent vincristine. This amino acid alteration may be one of the mechanisms of drug resistance in MCF-7/120nMVinc cells although a more comprehensive research at protein level is required to support the finding. According to previous reports, important ß-tubulin amino acids for paclitaxel binding were identified by electron crystallography and these were located between 1-31 and 217-233[40,41] amino acids. In this study, MCF-7/400nMPac cell line did not have any sequence difference in TUBB gene. Although there are several cases that represent base substitutions in TUBB gene in paclitaxel resistant cell lines[21,39], it has been also reported that alterations in this gene sequence have not been correlated to paclitaxel resistance in patients.[42] Furthermore, acquired ß-tubulin mutations may differ among cell and tissue types. In addition to mutations that directly affect drug binding, mutations at promoter regions of the genes encoding ß-tubulin isotypes may also cause alterations

in expression levels of these genes. For instance mutations at the core promoter that change any consensus sequence required for the initiation may possibly cause major changes in initiation resulting in a decrease of the production transcripts.

In this study, for the first time changes in class V ß-tubulin mRNA expression in antimicrotubule resistant breast carcinoma cells were demonstrated. Although changes in mRNA expression levels provide a promise insight to subject, supportive research on determination of corresponding protein level should be conducted with attempts to knockdown the TUBB6 gene in order restore sensitivity and correlate expression levels with drug resistance. Similarly, the differential expressions of class II and III ß-tubulin and missense mutations in TUBB gene may be one of the mechanisms of acquired resistance to paclitaxel, docetaxel and vincristine in MCF-7 cells.

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