Fluorescence-based DNA sequencing has been used to decipher the functional importance of DNA polymorphisms and mutations. One gene that has received recent attention is the class I β-tubulin isotype M40. Mutations have been identified in M40, which accounts for more than 85% of total β-tubulin RNA in cell lines used in the National Cancer Institute anti-cancer drug screen and in 12 human tumor xenografts (1). Using four overlapping sets of primers (GenBank accession number J00314), two mutations—a Phe270Val and an Ala364Thr—were identified in M40 from paclitaxel-resistant human ovarian cancer cells (1A9PTX10 and 1A9PTX22, respectively) (2). Recently, when sequencing complementary DNA (cDNA) with similar primers, a heterozygous Leu240Ile
mutation was identified in vincristine-resistant CCRF-CEM human lymphoblastic T-cell leukemia cells (3). Furthermore, cDNA from Chinese hamster lung tumor cells was sequenced for several α- and β-tubulin isotypes, and multiple nucleotide changes located outside the open reading frame were identified (4).

Using the one-dye labeled primer method for the ALFExpress sequencer (thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP, Amersham Pharmacia, Uppsala, Sweden), we analyzed genomic DNA from 49 non-small-cell lung cancer (NSCLC) patients. We detected numerous heterozygous single nucleotide substitutions in the phosphate, ribose, and base regions of the β-tubulin exon 4 GTP-binding site [see Fig. 1 and Table 2 of our original article (5)]. Since 1996, we have used multiple alignments of the related genomic sequences (www.ncbi.nlm.nih.gov) and used DNA analysis software (Sequencher; Gene Codes Corp., Ann Arbor, MI), confirming the presence of these mutations and ruling out the possibility that the mutations might correspond to pseudogenes. We agree that Kelley et al. (6) correctly point out in their Table 1 that some missense changes found in the ribose region (codon 180) overlap several pseudogenes (shown here in Table 1). However, except for these changes, none of the mutations from our original study (5) were pseudogenes. In a later study (7), we used a primer pair (forward primer 5'-AAGAGATAGATCCGAGGGAATTAT-3' from intronic position 2901 and a reverse primer 5'-GGCTGTTGAGTAAGCGGCTAA-3' from exonic position 4117) (GenBank accession number AC006165) to obtain a polymerase chain reaction (PCR) product of 1216 base pairs, which encompasses the entire exon 4, a region much larger than that described by Kelley et al. By fluorescence-based sequencing of PCR products, we detected multiple heterozygous single nucleotide substitutions in genomic serum DNA from 131 patients with NSCLC (7). However, on checking the sequences in BLAST (www.ncbi.nlm.nih.gov/blast), we found that a number of these substitutions corresponded to pseudogenes, including a Val-Ile change detected in four patients.

We concede that the originally identified sequence for M40 may contain some errors. In fact, basing our work on GenBank J00314, where the codon 11 sequence is CAA, we had identified a CAA (Gln) to CAG (Gln) polymorphism at amino acid 11 of M40 in all of 131 serum DNA samples (7). However, the new database (GenBank AC006165, cDNA NM_001069) identifies this supposed variant (CAG) as the normal sequence.

Because the presence of pseudogenes could not be completely ruled out at the time of our original study, the relationship between mutations and survival remains unclear. On the basis of the findings by Kelley et al. (6), we cannot rule out the possibility that some of the mutations represent rare nonpathogenic variants. Conflicting data on β-tubulin research should be elucidated by sequencing cDNA, obviating the cumbersome worst-case scenario of pseudogenes.

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Recently, Kelley et al. (1) reported that there were no amino-acid-altering mutations in the class I β-tubulin (also known as M40 or TUBB) among 25 lung cancer cell lines and 20 primary non-small-cell lung cancers (NSCLCs). This result contrasts with a report by Monzo et al. (2) that 33% of 49 NSCLC samples harbored mutations in the β-tubulin gene and were “a strong predictor of the response to the antitubulin drug paclitaxel.”

We have recently reported strong conservation of the class I β-tubulin gene among human populations and an absence of mutations of this gene in ovarian and lung cancers (3). DNA was isolated from 93 control individuals representing a wide variety of ethnicities and from 79 cancer specimens or human tumor cell lines. There were 62 specimens derived from ovarian carcinomas, including 32 without prior exposure to paclitaxel, and 30 known paclitaxel-resistant specimens. We also included 17 nontreated NSCLC specimens. We screened for sequence variations in exons 1, 2, 3, the first half of exon 4, and flanking noncoding regions of human class I β-tubulin by using denaturing high pressure liquid chromatography (DHPLC). The second half of exon 4 was analyzed by direct sequence analysis because the 3‘-UTR region contains a run of G’s that cause slippage and, consequently, formation of heteroduplexes that may affect the ability of DHPLC to detect mutations.

None of the 172 tumor samples, cell lines, xenografts, or controls had non-synonymous (i.e., amino acid altering) mutations or polymorphisms in the coding region. We detected two silent polymorphisms in exon 4—Leu217Leu (CTG/CTA) and Gly409Gly (GGC/GGT)—with minor allele frequencies of 17% and 0.5%, respectively. The amino acid sequence of class I β-tubulin was completely conserved among chimpanzee, orangutan, gorilla, and humans. Nucleotide diversity analysis revealed that human class I β-tubulin is one of the most conserved genes studied so far, with 4.4-fold less sequence diversity than that found in 106 other human genes (4).

What are the reasons for the discrepancies between the study by Monzo et al. (2) and the reports by Kelley et al. (1) and our group (3)? Specificity of the primers is one of the crucial issues in the analysis of single nucleotide polymorphisms and mutations in any gene, particularly those with more than one iso-type or with pseudogenes. Sequence variations in β-tubulin are especially difficult to study because there are six β-tubulin isoforms and more than 20 β-tubulin pseudogenes, all of which are highly homologous.

In analyzing class I β-tubulin, Monzo et al. (2) designed primers TP4 and STP4, which have 100% identity not only for class I β-tubulin but also for four β-tubulin pseudogenes (GenBank accession numbers V00598, K00841, J00317, and M24191). Moreover, another primer for exon 4, STB4, does not have a 100% match with any gene, including class I β-tubulin. The closest match is located on chromosome 16 and is not specific for the whole primer sequence. Lack of specificity for the class I β-tubulin iso-type could have resulted in a mixture of polymerase chain reaction (PCR) products and a false-positive detection of mutations. Kelley et al. (1) provide experimental evidence supporting this hypothesis. We used the class I β-tubulin sequence retrievable from GenBank (accession number J00314) to design five pairs of primers encompassing all exons, adjacent intrinsic sequences, and 5‘- and 3‘-UTRs and to test amplicon specificity (Fig. 1).

The paper of Monzo et al. (2) is also problematic in its discussion of the functional domains of the β-tubulin protein designating the domain encoded by exon 1 as the paclitaxel binding region. Electron crystallographic analysis has shown that the paclitaxel binding site is located in the intermediate region of the β-tubulin protein encoded by exon 4 (5).

Although Kelley et al. (1) and our group used different primers and sequencing methods, we obtained similar results, thus providing convincing evidence that sequence variations in class I β-tubulin in NSCLC and ovarian cancer are uncommon. The extremely high conservation of amino acid sequence in control individuals and the absence of mutations in NSCLC and ovarian carcinomas make it unlikely that variations in class I β-tubulin isotype are a clinically relevant cause of resistance to taxanes in these diseases. We agree with Kelley et al. (1) that it may be wise to postpone any ongoing clinical trials in which the treatment for NSCLC is based on the β-tubulin “mutation” status of the patients. The example of class I β-tubulin highlights the need to verify and confirm reports of mutations in genes that are found to be associated with various clinical parameters, such as drug response or survival.

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In their letter to the Journal, Monzo et al. mistakenly state that we did not include the base-binding region in our analysis of TUBB. We amplified and sequenced the entire coding region and adjacent splice sites from all four exons in tumor cell lines and from the entire exon 4 (including the base-binding region) in tumor samples. We replicated their analysis of the ribose- and phosphate-binding regions and provided evidence for nonspecific amplification resulting in the detection of artifactual sequence variants in these regions. We could not replicate their analysis for the base-binding region of exon 4 because of an error in the sequence of an oligonucleotide in their original publication. The absence of TUBB mutations in tumors and cell lines calls into question the finding of mutations in serum DNA, as reported by Rosell et al. (1).

Sale et al. (2) summarize their published analysis of the TUBB gene in human tumor samples, cell lines, and normal samples from the great apes. Using primers independently designed to avoid amplification of non-TUBB sequences and denaturing high-pressure liquid chromatography (dHPLC), no sequence variants were found that resulted in an altered amino acid sequence. Tsurutani et al. (3) have also been unable to detect any mutations of TUBB in 42 lung cancer samples by sequence analysis of cDNA, a technique that also avoids amplification of non-TUBB sequences. Taken together with results from my laboratory (4), it is clear that mutation of TUBB in human lung cancer is a rare occurrence or does not occur at all.

Oncologists have long sought the ability to predict the variable clinical tumor responses observed after chemotherapy treatment. Unfortunately, our current understanding of the complex and multifactorial mechanisms of drug resistance is insufficient to direct selection of drugs for patients with lung cancer. As increasing numbers of candidate markers of drug resistance and other potential predictors of clinical outcome emerge from the tools of the biotechnology revolution, we will be obligated to select only those markers with compelling and confirmatory preclinical studies for further analysis in clinical trials.

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References


Notes

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