

# Cytochrome P450 CYP3A4/5 Expression as a Biomarker of Outcome in Osteosarcoma

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**Purpose:** Osteosarcoma, the most common pediatric primary bone tumor, is an aggressive malignancy with a tendency for early pulmonary metastasis. A reliable biomarker to predict clinical outcome at diagnosis has not yet been identified. To date, pathological review of neoadjuvant chemotherapy-induced necrosis is the most useful determinant of which patients are likely to develop metastatic disease. There is a clinical need to identify patients who will benefit from more aggressive preoperative therapy or perhaps require less aggressive chemotherapy. More than 30 human P450 isoenzymes have been characterized, with at least nine possessing some clinical relevance. One of these, CYP3A4/5 is involved in metabolic activation and detoxification of a wide number of carcinogens and chemotherapeutic agents, including many drugs that are useful in the treatment of osteosarcomas.

**Materials and Methods:** Osteosarcoma tissue microarray blocks containing biopsies from 18 primary tumors were used to analyze the expression of P450s 1A1/2,

1B1, 2B6, 2D6, and 3A4/5 by enzyme-linked avidin-biotin complexed immunohistochemistry. The frequencies of expression were 83%, 67%, and 83% for P450s 1A1/2, 1B1, and 3A4/5, respectively. P450s 2B6 and 2D6 were not detectable.

**Results:** These results were extended by developing a fluorescent-based quantitative immunocytochemistry technique to assess the levels of CYP3A4/5 in 18 paraffin-embedded primary biopsy sections. Expression of CYP3A4/5 was found to be significantly higher in primary biopsies of patients who developed distant metastatic disease compared with biopsies from patients with nonmetastatic disease ( $P = 0.0004$ ).

**Conclusion:** High cytochrome P450 CYP3A4/5 expression may predict metastasis and poor prognosis in osteosarcomas. Its use as a biomarker of therapeutic response will have implications for the treatment of these tumors.

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OSTEOSARCOMA (OS) IS the most frequent primary malignant bone tumor, accounting for approximately 30% of all primary bone tumors of the skeleton<sup>1</sup> and occurring mainly in children and adolescents. OS occurs most commonly in the metaphysis of the long bones, the distal and proximal femur, and the proximal humerus. Its natural history is characterized by a rapidly progressive course with early metastases primarily to the lungs, which generally occurs within 1 to 2 years despite amputation. Pulmonary metastasis portends a poor prognosis. Before chemotherapy use became routine, approximately 90% of patients had recurrence within 2 years of diagnosis despite aggressive surgical treatments.<sup>2</sup> The introduction of adjunctive chemotherapy yielded an improvement in long-term survival rate, from 10% to 20% to nearly 50% to 80%.<sup>3</sup> Despite the dramatic improvement, drug resistance and poor clinical outcome are still significant problems for nearly half of the patients.<sup>4</sup>

Several biomarkers have been proposed to predict response of OS, including *HER-2/neu*,<sup>5,6</sup> p53,<sup>7,8</sup> metallothionein,<sup>9-11</sup> glutathione *S*-transferase,<sup>10</sup> P-glycoprotein (multidrug resistance protein [MDR]),<sup>9,12,13</sup> and heat shock proteins.<sup>10</sup> Despite all of these efforts, a reliable marker that predicts therapeutic response at the initiation of chemotherapy has not yet been found.

The cytochrome P450-dependent mixed-function oxidases are heme-containing proteins that play an important role in cell regulation via their involvement in the metabolism of a wide variety of endogenous compounds active in cellular signaling, such as steroids, fatty acids, and eicosanoids.<sup>14</sup> Cytochromes P450 are thought to facilitate tumor invasion and metastasis by

the generation of reactive oxygen species during various metabolic steps and by the activation of protein kinases, cellular proteoglycan changes, and lysosomal enzymes.<sup>15</sup> P450s are involved in the specific activation and detoxification of a large number of anticancer drugs, many of which are used in the treatment of OS (etoposide, ifosfamide, and doxorubicin), with some isoenzymes showing tumor-specific expression.<sup>16</sup> Therefore, P450 enzymes play a role in carcinogenesis and the metabolism of chemotherapeutic agents used in sarcoma treatment.

To investigate the potential role of P450s in OS, we examined the expression of five major cytochrome P450 isoenzymes in a cohort of OS primary biopsies by enzyme-linked avidin-biotin complexed immunohistochemistry (IHC). We subsequently used a quantitative immunofluorescence (QIF) technique to assess the levels of CYP3A4/5 in OS tissue sections, and correlated the expression with clinical outcome.

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## MATERIALS AND METHODS

### Patients and Tumors

Formalin-fixed, paraffin-embedded blocks originally derived from primary bone tumors were obtained from the files of the Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI. The diagnosis was confirmed, and institutional review board approval was obtained.

### Immunohistochemical Staining for Cytochromes P450

Initially, tissue microarray blocks that contained 18 biopsies and their corresponding resections were assembled as described.<sup>17</sup> These were sectioned, deparaffinized, and stained as follows. Five-micrometer sections were microwave-preheated in citric acid buffer to retrieve antigenicity. Sections were incubated with blocking solution for 60 minutes at room temperature before being exposed to the primary antibody of P450s CYP1A1/2 (1:500, goat polyclonal antibody, cat# 299124; Gentest, Woburn, MA), CYP1B1 (1:500, rabbit polyclonal antibody, cat# A211; Gentest), CYP2B6 (1:500, rabbit polyclonal antibody, cat# A226; Gentest), CYP2D6 (1:500, mouse monoclonal antibody, cat# A246; Gentest), and CYP3A4/5 (1:500, mouse monoclonal antibody, cat# A254; Gentest) for 30 minutes at room temperature. According to the antibody manufacturer's package enclosure, each antibody was specific for the respective cytochrome P450 subspecies by immunoblot analysis. The immunocomplex was visualized by the immunoglobulin enzyme bridge technique, using Vector ABC-peroxidase kit (Vector Laboratories, Burlingame, CA). The enzyme substrate, 3,3' diaminobenzidine tetrachloride was used, resulting in a brown reactant. Sections were then weakly counterstained with 0.1% hematoxylin. Concurrent sections were stained with antibodies to vimentin to assess antigen preservation. Appropriate negative (no primary antibody) and positive (kidney for CYP1A1/2 and CYP1B1; liver for CYP2B6, CYP2D6, and CYP3A4/5) control samples were stained in parallel with each set of OS blocks. The immunoreactivity of the malignant cells were scored by a four-tier (negative, low-positive, medium-positive, and high-positive) modification of the grading scheme previously described by Wang et al<sup>18</sup> for *HER-2/neu* staining. Weak staining was defined as a brown blush compared with the negative control.

### QIF Staining for CYP3A4/5

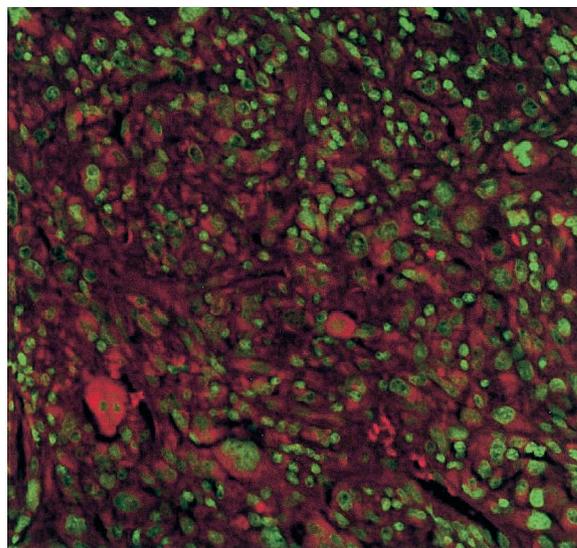
Deparaffinized sections were microwave preheated in citric acid buffer to retrieve antigenicity. Then they were permeabilized with 0.1% saponin for 10 minutes at room temperature and treated with three washes of 20 mmol/L of glycine and 0.1% paraphenyldiamine in tri-buffered saline-(TBS-) Tween 20 to inactivate free formaldehyde molecules that might otherwise cause autofluorescence. Sections were blocked with blocking solution (5% goat normal serum and 1% fetal bovine serum in TBS-Tween 20) for 30 minutes at room temperature before being exposed to the CYP3A4/5 primary antibody (1:300) overnight at 4°C. Sections were then exposed to the labeled secondary antibody (Alexa Fluor 568 goat antimouse immunoglobulin G, cat# A-1104, 1:100; Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Cell nuclei were stained with Syto 16 (cat# S-7578; Molecular Probes, 1:4,000) for 30 minutes at room temperature. The sections were mounted in an antifading medium and kept in the dark at 4°C until examined.

### Fluorescent Microscopy and Digital Imaging

Labeled sections were initially excited at  $\lambda = 488$  nm and the fluorescing nuclei images were acquired at a 20 $\times$  magnification by digital micrograph and UltraView imaging software (Perkin-Elmer, Boston, MA). Consequently, sections were excited at  $\lambda = 568$  nm to acquire the fluorescing CYP3A4/5 enzyme image. Computer-generated composite images of nuclei and enzyme allowed visualization of cellular distribution (Figs 1 and 2). All images were acquired under exactly the same conditions.

### Statistical Analysis

All images were partitioned into equally sized square areas small enough to provide more homogeneous regions, and pixel intensities were



**Fig 1.** A composite image of fluorescing nuclei (stained by Syto 16) in green and P450 3A4/5 in red (probed by Alexa Fluor 568 goat antimouse) expressed in a primary osteosarcoma biopsy from patient 16. Magnification  $\times 200$ .

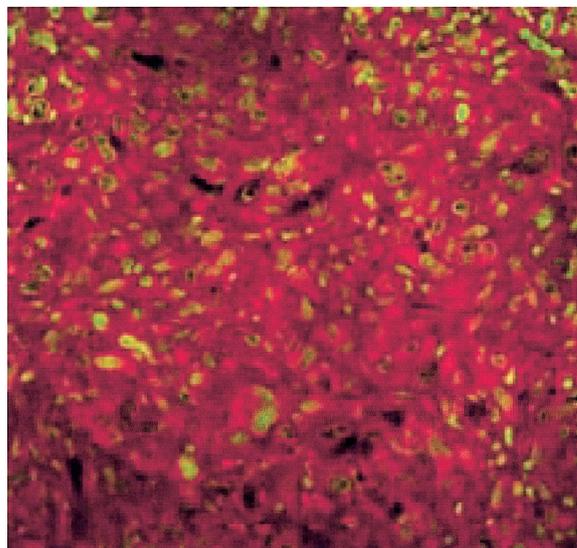
acquired from each of these areas. Nuclear-density-weighted average enzyme pixel intensity ( $F$ ) was then computed for each sample according to the following formula:

$$\sum W_i J_i$$

where  $J_i$  is the enzyme pixel intensity at the  $i^{\text{th}}$  area and  $W_i$  is the weight of the  $i^{\text{th}}$  area whose value is given by the following formula:

$$F = I_i / \sum I_i$$

where  $I_i$  is the nuclei pixel intensity of  $i^{\text{th}}$  area with the sum of  $W_i$  normalized such as  $\sum W_i = 1$ .<sup>19</sup>



**Fig 2.** A composite image of fluorescing nuclei (stained by Syto 16) in green and P450 3A4/5 in red (probed by Alexa Fluor 568 goat antimouse) expressed in a primary osteosarcoma biopsy from patient 7. Magnification  $\times 200$ .

Table 1. CYP3A4/5 Levels in 18 Primary Osteosarcoma Biopsies by QIF

Subject	Date of Biopsy	Level of CYP3A4/5 Described as Pixel Intensity	% Necrosis	Lung Metastasis	Date of Lung Metastasis Surgery	Date of Last CXR
1	3/8/89	1436	—	Y	3/20/89	—
2	9/4/98	1495	50	Y	11/10/99	—
3	5/17/00	1438	15	Y	5/25/00	—
4	4/13/89	1052	—	Y	7/26/89	—
5	5/23/89	1214	100	Y	10/16/92	—
6	11/25/97	1579	99	Y	9/11/01	—
7	6/27/96	1037	95	Y	11/3/00	—
8	7/11/86	1470	98	Y	4/5/89	—
9	10/13/94	1290	98	Y	7/23/98	—
10	12/9/96	807	40	Y	12/8/98	—
11	4/6/01	689	15	Y	12/18/01	—
12	5/2/89	604	100	N	—	8/30/00
13	12/10/93	531	—	N	—	6/22/00
14	10/19/94	999	75	N	—	6/9/00
15	4/22/97	532	100	N	—	7/31/02
16	9/6/01	438	99	N	—	10/11/02
17	2/4/94	177	90	N	—	4/29/01
18	11/4/99	466	15	N	—	10/23/02

Abbreviations: QIF, quantitative immunofluorescence; CXR, chest radiograph; Y, yes; N, no.

The null hypothesis of no difference of weighted average enzyme intensity between biopsies from patients who ultimately developed distant metastasis and biopsies from patients who remained disease-free was tested using an exact permutation test at a significance level of  $P = .05$ .

## RESULTS

### Clinicopathologic Data

The OS patients ( $n = 18$ ) had an age range of 6 to 29 years, with a mean age of 14.6 years and a male-female ratio of 10:8. All 18 patients had pretreatment primary biopsies with 11 tumors that ultimately metastasized to the lung and seven tumors from patients with nonmetastatic disease. The primary site in nine patients was the femur; eight tumors were found in the tibia, and one was found in the humerus.

### Expression of P450s by Standard Immunohistochemistry

Immunohistochemical analysis of OS tissue microarray blocks representing all 18 biopsies using anti-P450s 1A1/2, 1B1, 2B6, 2D6, and 3A4 antibodies was performed. Both CYP1A1/2 and CYP3A4/5 immunopositivity were detected in 15 (83%) of 18, and CYP1B1 immunopositivity was detected in 12 (67%) of 18. CYP2B6 and CYP2D6 were absent in these osteosarcomas. CYP1A1/2 demonstrated moderate immunoreactivity with little variation among the different tumors. CYP1B1 also showed moderate immunoreactivity but with some greater variation. CYP3A4 showed strong immunoreactivity with noticeable variation among the different tumors. Biopsies, posttherapy, metastatic, and recurrent lesions were assessed for the presence of CYP3A4/5 by immunohistochemistry using the previously described peroxidase technique. The results demonstrated that there was increased staining quality in the recurrent and metastatic lesions compared with the biopsy specimens. All positive and negative immunohistochemical controls were appropriate.

### Expression of CYP3A4/5 by QIF

Eighteen primary OS (11 metastatic and seven nonmetastatic) were stained and scored in duplicate and averaged, as described previously. Resections and metastatic lesions were not assayed for the presence of CYP3A4/5 by this method. Samples were then placed in two groups on the basis of occurrence of distant metastasis as shown in Table 1. Table 1 also shows the date of biopsy as well as the date when metastasis was proved or date of last contact. Results showed higher CYP3A4/5 levels in primary biopsies of patients who developed distant metastatic disease with a weighted average mean intensity of 1,228 (95% CI = 1,030 to 1,428) compared with lower levels in patients with nonmetastatic disease, with a weighted average mean intensity of 536 (95% CI = 307 to 763; Fig 3). An exact permutation test comparing the average pixel intensities of metastatic primary bone tumors and nonmetastatic tumors showed a  $P$  value of .0004. Both groups were significantly different at an alpha level of .004 or greater.

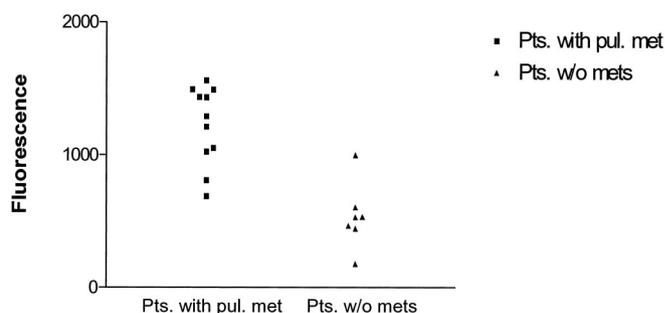


Fig 3. QIF CYP3A4/5 Osteosarcoma Bx. P450 3A4/5 levels described as weighted average pixel intensity in the total osteosarcoma population ( $N = 18$ ). Pts, patients; met/mets, metastasis/metastasis.

## DISCUSSION

In this study, primary OS biopsies using tissue microarray blocks were analyzed for the expression of five major cytochrome P450 isoenzymes using standard immunohistochemical methods. Individual subfamilies of the cytochromes P450 have been reported to be present in several varieties of sarcomas and carcinomas.<sup>16,20-24</sup> Our results showed expression of CYP1A1/2, CYP1B1, and CYP3A4/5, whereas P450s 2B6 and 2D6 were not detectable. A number of studies have found CYP1A1/2 to be highly expressed in various types of cancer, including sarcomas, with frequencies ranging from 50% to 70%.<sup>16,23,24</sup> Furthermore, genotypic and phenotypic polymorphisms of CYP1A1 have been associated with an increased risk of smoking-related cancers.<sup>24</sup> Although our results show CYP1A1/2 to be expressed in OS at higher frequencies than in most cancers investigated (80%), they do not reveal any variation in immunoreactivity among different samples with regard to this particular isoenzyme. Some CYP1B1 genetic variants have been suggested as susceptibility factors for ovarian, prostate, breast, and lung cancers.<sup>25-27</sup> CYP1B1 is involved in the activation of a large number of procarcinogens,<sup>28</sup> and some studies suggest that it has an endogenous role in tumors.<sup>28</sup> Our results show a high expression frequency for CYP1B1, consistent with frequencies found in other types of cancers,<sup>29</sup> but a weak immunoreactivity variation among the different OS samples.

CYP3A4/5 involved in the metabolism of various anticancer drugs<sup>30,31</sup> is considered to be the most clinically relevant P450 in view of its broad specificity and that it is the dominant P450 found in the liver accounting for 30% to 60% of total liver cytochromes P450. Our results show a high expression frequency for CYP3A4/5 (80%), consistent with results from other studies investigating P450 3A subfamily expression in soft tissue sarcomas.<sup>23</sup> CYP3A4/5 showed an interesting immunoreactivity variation among the different OSs investigated.

CYP3A4 is of particular interest because it has been shown to be involved in the oxidation of ifosfamide, vinblastine, etoposide, and doxorubicin,<sup>32</sup> all four types of compounds used as chemotherapeutic agents for the treatment of OS. On this basis, we decided that it would be useful to quantitate this enzyme in formalin-fixed, paraffin-embedded bone tumors. We developed a fluorescent-based quantitative immunohistochemistry technique that allows us to measure the levels of this enzyme in archival tumor sections. An uneven distribution of tumor cells and bony areas might lead to problems in estimating the levels of the enzyme. Moreover, a nonweighted average of enzyme intensity leads to biased results because of the heterogeneity of the tumor's cellular distribution. Therefore, each image was partitioned into equally sized square areas, and nuclear density as well as enzyme intensity was estimated within each square area. The size and number of square areas were chosen in a way to render each subimage more homogeneous with respect to nuclear density. A weight average of enzyme intensity was then computed for each tissue section with weights given by the normalized nucleic intensity, assuming that it represents nuclear density. The weighted average intensity method allows targeting of richly cellular areas in the tumor by giving them a larger

weight and at the same time gives an appropriately smaller weight for less cellular—more bony—areas, thus allowing a more reliable reading of the protein or enzyme in question.

Because of the small sample size, an exact permutation test was used instead of a two-tailed *t* test to test the null hypothesis of no difference between the two groups of tumors. A permutation test is a nonparametric statistical analysis that is more appropriate for small sample size populations.<sup>33</sup> An exact permutation test of the null hypothesis of no difference in the average CYP3A4/5 intensity between biopsies that eventually metastasized compared with ones that did not resulted in a *P* value of 0.0004, a highly statistically significant difference illustrated in Fig 3. This suggests that CYP3A4/5 may be a prognostic factor for metastasis in OS and may even be playing a role in the resistance to chemotherapy.

Thus far, several proteins expressed in OS and present in serum levels have been proposed as prognostic factors, including p53 and *HER-2/neu*. Expression of p53 and its mutants has been reported in many human malignancies and was reported as a prognostic marker in some types of cancer,<sup>34,35</sup> but several studies did not find any correlation between its expression and prognosis or response to chemotherapy, suggesting that p53 is not a marker for OS.<sup>7,8</sup> Several recent studies that examined large cohorts of OS patients have defined several independent prognostic factors.<sup>36,37</sup> Although some of these factors, such as tumor site and size, are assessable at diagnosis, a reliable prediction of prognosis is not possible until later in the course of the disease, when information on tumor response and quality of surgical remission becomes available.

Thus, in the absence of a reliable biomarker in OS, CYP3A4/5 higher expression in primary tumors that metastasize presents an interesting finding. CYP3A4/5 overexpression in malignancies with metastatic potential may be a cellular protective mechanism conferring survival advantage by providing these tumors with the metabolic mechanisms for the inactivation of chemotherapeutic agents. It is also possible that 3A4/5 may be involved in growth regulation through metabolism of endogenous growth regulatory factors. Although it is tempting to speculate that 3A4/5 may play a role in metastasis and in the progression of OS, this possibility warrants further study. Our results support the notion that a quantitative analysis of P450 3A4/5 expression may make it possible to identify high-risk patients more precisely and may help in the selection of an appropriate postoperative follow-up schedule. Our developed quantitative technique may be a useful tool in identifying new prognostic factors not only in OS but also in other types of malignancies.

In summary, this is the first report to identify variable expression of the 3A subfamily of cytochrome P450s in OS. We have developed a novel fluorescent-based quantitative immunohistochemistry technique to analyze for the levels of expression of CYP3A4/5. Although our results represent a small sample population, they demonstrate that CYP3A4/5 could be a reliable marker of OS metastasis and poor prognosis. These results require further validation but are consistent with the findings of another group, which demonstrated decreased response to docetaxel in breast cancer patients whose tumors possessed high CYP3A4 mRNA expression levels.<sup>38</sup>

Aside from its clinicopathologic and prognostic significance, the overexpression of CYP3A4/5 in primary biopsies that metastasize may have implications for both the development and

the treatment of these tumors; it might form the basis of a new therapeutic strategy to provide a molecular target for chemotherapeutic agents that can be activated by P450 3A4/5.<sup>39</sup>

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The acknowledgment is included in the full text version of this article only, available on-line at [www.jco.org](http://www.jco.org). It is not included in the PDF version.

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