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IMMUNOHISTOCHEMICAL EVALUATION OF CYTOCHROME P450 (CYP) AND P53 IN BREAST CANCER

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1. ABSTRACT

In breast cancer, cytochrome P450 (CYP) metabolizes both endogenous substrates (i.e. estradiol) and exogenous substrates (i.e. anticancer drugs), which is associated not only with tumor development and progression but also with efficacy of cancer treatment. Therefore, we examined the expression of CYPs (CYP2A6, CYP1B1 and CYP3A) and p53 in specimens from 34 Japanese patients with breast cancer by immunohistochemistry. The expression of CYP3A was not detected in the 34 cases. CYP2A6 was detected only in one specimen (2.9%). Twenty-eight specimens (82.4%) showed positive signals for CYP1B1 expression. Eight of 34 (23.5%) were positive for p53 expression. Positive rate of CYP1B1 in stage I disease (100%) was statistically higher than that in stage II – IV disease (70.0%). Positive rate of p53 was 21.4% (6/28) in CYP1B1-positive cases and 33.3% (2/6) in CYP1B1-negative cases. There was no significant relationship between CYP1B1 expression and p53 expression. In conclusion, the expression of CYP3A in breast cancer may be less frequent in Japanese population although the expression of CYP3A has been reported in 20% of breast cancer in Caucasian, suggesting that the CYP3A expression in breast cancer may be dependent on ethnic groups. Since CYP3A is involved in the conversion of tamoxifen to its metabolites, the variation of the CYP3A expression in breast cancer tissues among ethnic groups might cause differences in the efficacy of tamoxifen.

2. INTRODUCTION

In the body, xenobiotics are metabolized by the phase I enzymes including the microsomal P450 (CYP) families and epoxide hydrolases. The metabolites are then modified by the phase II enzymes that include UDP-glucuronosyltransferases (UGTs), sulfotransferases, glutathione S-transferases, and N-acetyltransferases. These reactions generally result in pharmacological inactivation or detoxification of xenobiotics. Conjugated metabolites of xenobiotics are further subjected to specific transport machineries, thus facilitating elimination of the metabolites from the body. These metabolisms occur mostly in the liver. However, the phase I and phase II enzymes are also expressed in a variety of tissues. CYPs incidentally convert xenobiotics to more active and/or toxic compounds that may form DNA-adducts resulting in the initiation and promotion of tumor (1-4). Therefore, CYP expression has been studied in a variety of human tumors, such as breast cancer, colon cancer, and lung cancer (5).

In addition to CYP enzymes, a nuclear phosphoprotein p53, which transcriptionally activates the genes downstream of its binding site (6), is also frequently involved in the development of cancer via the incidence of its mutation. The mutations in this tumor suppressor gene that have been found in 37% of all human malignancies (6) are involved in carcinogenesis

and in the prognosis of the majority of human malignancies (7). Missense mutations of p53 frequently found in the central exons 5 through 8 are associated with prolonged protein half-life, leading to a nuclear accumulation of p53 protein that can easily be detected by immunohistochemical analysis (8) while the wild type p53 are hardly detected because of its low abundance in most tissues. Therefore, p53 proteins detectable by immunohistochemical analysis can be considered as a marker for *p53* gene mutations. Since the carcinogenic metabolites derived from the CYP system may contribute the mutation of p53, the immunohistochemical analysis of both CYPs and p53 is important for better understanding of cancer initiation and progression.

In humans, CYP19 (aromatase) that converts androgens to estrogens is expressed both in normal and malignant breast tissues. The aberrant expression of CYP19 in breast cancer excessively produces estrogens, resulting in the enhancement of breast tumor growth and development. In addition to CYP19, the expression of CYP1A1, CYP1B1, CYP2C, CYP2E1, and CYP3A4 has been reported in breast cancer tissues. Of them, CYP1A1 and CYP1B1 catalyze the conversion of estradiol to 4-hydroxyestradiol, a potent carcinogen that may form DNA-adducts. CYP1A1 and CYP3A4 convert estradiol to its less active metabolite 2-hydroxyestradiol. Nitrosamine compounds related to tobacco smoke are also activated by several CYPs including CYP2A6. Nitrosamine metabolites mediated by CYP2A6 may form DNA-adducts and cause specific mutations in *p53* gene.

Tamoxifen is currently the most widely used chemotherapeutic agent for the adjuvant treatment of breast cancer. It likely acts as a prodrug by conversion to the active metabolites (N-desmethyl-tamoxifen, *trans*-4-hydroxytamoxifen, and 4-hydroxy-N-desmethyl-tamoxifen) that bind the estrogen receptor with similar or enhanced binding affinity compared with the parent compound (9-11). In breast cancer tissues, CYP3A4 may contribute the conversion of tamoxifen to N-desmethyl-tamoxifen and *trans*-4-hydroxytamoxifen, while CYP1B1 may catalyze the isomerization of *trans*-4-hydroxytamoxifen to the weakly estrogenic *cis*-4-hydroxytamoxifen. Therefore, the analysis of CYP expression in breast cancer is important not only for better understanding of initiation and development of breast cancer but also for the evaluation of efficacy of chemotherapeutic treatment.

In this study, we investigate the immunohistochemical analyses of CYPs (CYP2A6, CYP1B1 and CYP3A) and p53 in 34 breast cancer tissues (invasive ductal carcinoma) from Japanese patients. The results from immunohistochemical analyses were not correlated to clinical features of the patients, except that the early stage of breast cancer may express CYP1B1 more frequently. Further, the CYP3A expression in breast cancer was not detected in this study, suggesting that CYP3A may be expressed less frequently in Japanese population than Caucasian.

3. MATERIALS AND METHODS

3.1. Surgical specimens

We examined 34 of 54 (63%) consecutive Japanese patients with breast cancer who underwent surgical resection at the Department of Surgery II, School of Medicine, University of Occupational and Environmental Health in Kitakyushu, Japan between 1996 and 1998. The criteria for inclusion into the study were based on the availability of immunohistochemical inspection. There were 34 women ranging in age from 27 to 78 years (mean, 55.6 years). Fourteen patients had stage I disease (T: tumor 2 cm or less, N: no lymph node metastasis) and 20 stage II - IV disease, according to the TNM staging (12). Histological typing of the tumors was performed according to the WHO classification (13). Resected specimens were fixed in 20 % formalin for 3 days and were embedded in paraffin. For histological study, sections were stained with hematoxylin and eosin. CYP (CYP2A6, CYP3A and CYP1B1) and p53 staining were examined 34 breast cancer.

3.2. Immunochemistry

Immunohistochemical staining was performed on 3 μm paraffin-sections. Anti-CYP2A6 (Cat. No. 458106), anti-CYP3A (Cat. No. 458254), recognizing 3A4, 3A5, and 3A7, and anti-CYP1B1 (Cat. No. 458211) were purchase from Gentest Corp., Woburn, MA. Anti-human p53 (the mouse monoclonal antibody DO-1) was from Oncogene Science Inc., Cambridge, MA. This anti-p53 recognizes the codon regions 37 - 45 of the p53 protein using a standard immunohistochemical method as described (7). We defined as CYP and p53 positive cases when more than 10% of tumor cell were stained (CYP and p53 negative cases; 10% > positivity, CYP and p53 positive cases; 10% ≤ positivity) (7).

3.3. Statistical analysis

The relationship between the positivity of immuno-staining and various clinical or pathological parameters was evaluated by the t-test. Students' t-test was also used in characterizing the relationship of positivity of immuno-staining between CYP and p53.

4. RESULTS AND DISCUSSION

4.1. Expression of CYP1B1 in breast cancer

Because of the conversion of 17β-estradiol to the carcinogenic 4-hydroxyestradiol and *trans*-4-hydroxytamoxifen to *cis*-4-hydroxytamoxifen, the increased expression of CYP1B1 in normal and malignant breast tissues may be associated not only with the increased risk but also with the management of breast cancer. By immunohistochemical analysis, twenty-eight (82.4%) of 34 specimens were stained positive by anti-CYP1B1. Figure 1A showed a representative image of the specimen that CYP1B1 and CYP2A6 were strongly expressed in the cytoplasm of the same invasive ductal carcinoma with a predominant intraductal component while CYP3A was not detected. In this study, CYP1B1 positive rate of stage I disease (100%) was likely to be higher than that of stage II – IV disease (70.0%). CYP1B1 tended to be expressed

Table 1. Relationship between the immuno-reactivity (CYP2A6, CYP1B1 and p53) and various clinical or pathological parameters in patients with invasive ductal carcinoma

		CYP2A6			CYP1B1			p53		
	N	+	-	P	+	-	P	+	-	P
Age										
<55	17	1 (5.9)	16		15 (88.2)	2		3 (17.6)	14	
≥55	17	0 (0.0)	17	1.0	13 (76.5)	4	0.65	5 (29.4)	12	0.69
Pathological stage										
I	14	0 (0.0)	14		14 (100)	0		4 (28.6)	10	
II-IV	20	1 (5.0)	19	1.0	14 (70.0)	6	0.07	4 (20.0)	16	0.87
Estrogen receptor										
Positive	26	1 (3.8)	25		23 (88.5)	3		5 (19.2)	21	
Negative	8	0 (0.0)	8	1.0	5 (62.5)	3	0.23	3 (37.5)	5	0.57
Progesterone receptor										
Positive	22	1 (4.5)	21		20 (90.9)	2		3 (13.6)	19	
Negative	12	0 (0.0)	12	1.0	8 (66.7)	4	0.19	5 (41.7)	7	0.16
Total	34	1 (2.9)	33		28 (82.4)	6		8 (23.5)	26	

N:Number, P: *p* Value

Table 2. The CYP expression in breast cancer and corresponding normal tissues

Breast Cancer	Tumor tissue		Normal tissue	
	Protein	RNA	Protein	RNA
CYP1A	IHC (40%)[21, 22]			
CYP1A1	IHC[3, 20, 27]		IHC[20, 27]	
CYP1B1	IHC (82%)*, IB[15, 16]	RT-PCR[14]		
CYP2A6	IHC*			
CYP2C	IHC (33%)[33], IB[34]		IB[34]	
CYP2E1	IHC[20, 35]		IHC[20, 35]	
CYP3A	IHC (22%)[21, 22]			
CYP3A4	IHC[20]		IHC[20]	

IHC: Immunohistochemistry, **IB**: Immunoblotting, **RT-PCR**: Reverse transcriptase-polymerase chain reaction, * In this study, % positive rate in the specimens are included in parentheses. Superscript: reference number

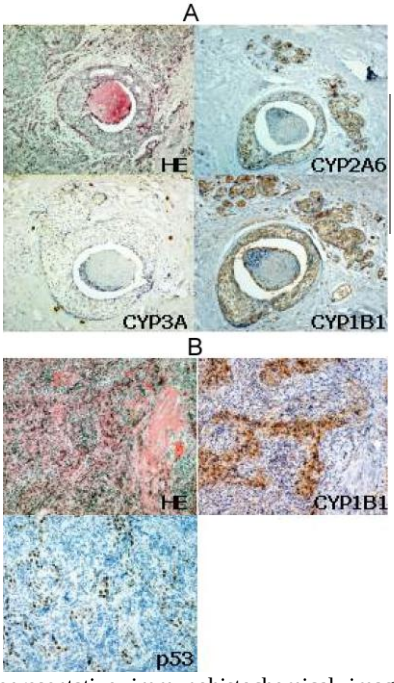

Figure 1. Representative immunohistochemical images of breast cancer. **A**, Hematoxylin-Eosin (HE) staining and immunohistochemistry (CYP2A6, CYP3A and CYP1B1) in invasive ductal carcinoma with a predominant intraductal component. CYP2A6 and CYP1B1 are strongly expressed in the same case although CYP3A is not expressed. **B**, Hematoxylin-Eosin (HE) staining and immunohistochemistry (CYP2A6, CYP1B1 and p53) in invasive ductal carcinoma. CYP1B1 and p53 are expressed in the same case.

more frequently in ER or PR positive cases than in ER or PR negative cases (table 1). These results are well consistent with the results from previous reports analyzed by RT-PCR (14), immunohistochemistry and immunoblotting (15, 16). As shown in Figure 1B, CYP1B1 was expressed in the cytoplasm of scchirous carcinoma and p53 was also expressed in the nuclei of the same invasive ductal carcinoma. The rate of p53 positive in CYP1B1 positive cases was 21.4% (6/28) and p53 positive rate in CYP1B1 negative cases 33.3% (2/6). The expression of CYP1B1 was not significantly related to the p53 mutations (Table 1) and was not effective on the survival of the patients with breast cancer (data not shown).

4.2. Expression of CYP3A

In humans, three isozymes are known in the CYP3A subfamily, CYP3A4 and CYP3A5 and the fetal form CYP3A7 (17, 18). Recently, enzymes of the CYP3A subfamily have been found to play roles not only in inactivation of the major anticancer drugs, such as taxol and vinca alkaloids but also in activation of the major anticancer prodrugs, such as cyclophosphamide and ifosphamide (19). Therefore, the local expression of CYP3A in breast cancer and surrounding tissues is of great interest for chemo-preventive treatment and management of breast cancer. CYP3A4 has been previously detected both in breast tumors and in normal tissues by immunoblot analysis (20). CYP3A was found in 22% of breast cancers by immunohistochemistry (21, 22) (Table 2). However, CYP3A was not detected in all 34 specimens from Japanese patients in this study (Table 1). This result indicates that breast tumor tissues from Japanese patients

express CYP3A at least less frequently than those from Caucasian patients.

4.3. Expression of CYP2A6

Breast cancer tissue from one patient was positively stained by anti-CYP2A6 (2.9%) in this study (Table 1) although the expression of CYP2A6 has not been previously shown in breast tumor tissues. This specimen was also positive for CYP1B1 and p53 **(Figure 1B)**. Several isoforms of CYP2A enzymes, such as CYP2A6, are involved in the metabolic activation of tobacco-specific nitrosamine (NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and *N*-nitrosodiethylamine associated with tobacco smoke. The active nitrosamine metabolites mediated by CYP2A6 are capable of forming DNA-adducts that may cause specific mutations in the p53 gene (23). Further, the homozygotes of a polymorphism CYP2A6*6 producing an inactive form of CYP2A6 have been found in 3.7% of 894 healthy Japanese subjects (24). Because the expression of CYP2A6 in breast cancer has been found in this study, the relationship among the CYP2A6, CYP2A6*6 polymorphism, p53 mutations, tobacco smoking, and breast cancer needs to be systematically investigated.

4.4. Detection of the p53 protein

The p53 protein was detected in 8 of 34 specimens (23.5%), suggesting that the 8 breast cancer tissues have mutations in the p53 gene. The frequency of p53 over-expression (Table 1) was consistent with previous reports (25, 26). There was no significant relationship of p53 mutations with the expression of CYP1B1.

4.5. Metabolism of estrogen and tamoxifen by CYPs expressed in breast cancer

In this study, 80% of specimens were estrogen-receptor positive (Table 1), which is comparable with approximately 70-80% of breast cancer is positive in previous reports. The estrogen-receptor positive breast cancer shows aberrant expression of aromatase in the cancer and surrounding adipose tissues, leading to the production of excess estrogen. This locally over-produced estrogen is associated with breast tumor growth and development. In breast tumor tissues, estrogen may be metabolized by CYPs expressed in tumor and surrounding tissues. As seen in Table 2, several CYPs have been reported to be expressed in normal and tumor breast tissues. Of them, CYP1A1 and CYP3A4 are favor to catalyze the conversion of estradiol to non-carcinogenic 2-hydroxyestradiol, which may protect cells against estrogen-induced carcinogenesis. In breast cancer, however, several groups have demonstrated the expressions of CYP1A1 and aryl hydrocarbon receptor that transcriptionally regulates CYP1A1 (3, 27). CYP1A1 detected in 40% of breast cancers (21, 22) has been reported that CYP1A1 expression was higher in breast tumors compared with normal breast tissues (3). Aryl hydrocarbon hydroxylase (AHH) activity reflecting the expression of CYP1A1 (28) was observed in 153 primary breast cancers, and the high AHH activity was associated with poor prognosis (29), while El-Rayes et al. (20) reported that CYP1A1 expression determined by immunohistochemistry was significantly lower in malignant breast tumor tissues as compared with normal tissues. On the other hand, CYP1B1 frequently expressed in breast cancer (14) plays the major role to convert estradiol to 4-hydroxyestradiol, a carcinogenic metabolite (30). The examination of estradiol hydroxylation activity in microsomes from human breast cancer showed significantly higher 4-hydroxy/2-hydroxy estradiol ratios in tumor tissues than in adjacent normal breast tissues (31), suggesting the importance of estrogen metabolism by CYP1B1 in breast tissues for development of breast cancer.

Tamoxifen, a selective estrogen receptor modulator, is used not only for all stages of estrogen receptor-positive breast cancer as an established hormonal treatment but also as for chemo-prevention in women at risk for developing the disease (32). Tamoxifen has been shown to be metabolized by human CYPs to N-desmethyl, 4-hydroxy, α-hydroxy, and 4-hydroxy-N-desmethyl metabolites. In the liver, tamoxifen is converted to trans-4-hydroxytamoxifen, the most potent anti-estrogenic metabolite, by CYP2D6 with some contribution of CYP2B6, CYP2C9, CYP3A. CYP3A also play the major role for the conversion of tamoxifen to N-desmethyltamoxifen that is a primary metabolite having anti-estrogenic activity similar to tamoxifen. On the other hand, CYP1B1 converts trans-4-hydroxytamoxifen to cis-4-hydroxytamoxifen, a less active anti-estrogenic metabolite. Because CYP2D6 has not been detected in breast cancer, the expression of CYP1B1 and CYP3A in breast cancer and surrounding tissue may alter the local activation or elimination of the anticancer drug. In this study, CYP3A was not detected in 32 breast cancer specimens from Japanese patients, suggesting the importance of in depth study of the ethnic dependence of relationship between the expression of CYPs and efficacy of anti-cancer agents.

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