Combined Effect of CYP1B1 Codon 432 Polymorphism and N-Acetyltransferase 2 Slow Acetylator Phenotypes in Relation to Breast Cancer in the Turkish Population

YASEMIN K. ÖZBEK[1], TÜLIN ÖZTÜRK[2], BORA M. TÜZÜNER[1], ZERRIN CALAY[3], SENNUR ILVAN[3], FATIH M. SEYHAN[1], HALIL I. KISAKESEN[1], OĞUZ ÖZTÜRK[1] and TURGAY İSBIR[1]

[1]*Institute of Experimental Medical Research, Department of Molecular Medicine and*
[3]*Cerrahpasa Medical School, Department of Pathology, University of Istanbul, Instabul, Turkey;*
[2]*Department of Pathology, Bagcilar Education and Research Hospital, Istanbul, Turkey*

Abstract. *Background: Breast cancer (BC), is more prevalent in subjects who have had prolonged exposure to heterocyclic amines, aromatic amines and high levels of oestradiol. Cytochrome P450 1B1 (CYP1B1) and N-acetyltransferase2 (NAT2) have complementary role in metabolism of xenobiotics such as arylamines and heterocyclic amines, CYP1B1 also hyroxylates 17-β oestradiol. CYP1B1*3 polymorphism and seven missense and four silent polymorphisms of NAT2 were investigated. Patients and Methods: Sixty Turkish female BC patients and 103 healthy controls were phenotyped by polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP). Results and Conclusion: The distribution of NAT2 activity in the healthy control group was found to be correlated with that of healthy caucasians. Patients had slow acetylator phenotypes of NAT2, 1.8 times higher than controls but no statistical differences were found (p=0.07). In addition, the NAT2*5 alelle was more statistically correlated with breast cancer patients rather than the controls (p=0.02). Moreover, NAT2*5B was the most frequent haplotype of the NAT2*5 family (p=0.000). Breast cancer patients were detected to posses more CYP1B1*3 mutant alleles than the controls (p=0.043). The combined effect of CYP1B1*3 polymorphism and NAT2 slow acetylator genotype contributed to an increased risk for breast cancer in patients in this study (p=0.004).*

Differences in the distribution of gene polymorphisms in key discriminant genes involved in oestrogen and xenobiotic metabolism is considered to contribute to variations in breast cancer susceptibility among different racial/ethnic populations (1). Biotransformation of xenobiotics in the body includes activating hydroxylation reactions by Cytochrome P450 enzymes and detoxification by conjugating enzymes such as catechol-O-methyltransferase (COMT), glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs) (1). Polymorphisms in these enzymes may cause modifications in catalysing activity and stabilisation of the enzymes, thus increasing susceptibility to cancer due to prolonged exposure to oestradiol and xenobiotics (1). In this study, genetic polymorphisms of Cytochrome P450 1B1 (CYP1B1*3) and N-Acetyltransferase 2 (NAT2), and their contribution to breast cancer susceptibility was investigated among Turkish Women. Different populations have different polymorphism patterns, and the Turkish population is expected to display the genetic traits of the Caucasian population (1).

CYP1B1 is a haeme-thiolate monooxygenase that is involved in the NADPH-dependent monooxygenation of a considerable variety of carcinogens such as polyaromatic hydrocarbons (PAHs), heterocyclic aromatic amines (HAA), arylamines and nitrosamines formed in cooked food, tobacco products and found in industrial chemicals. CYP1B1 is located on chromosome 2p21 with three exons and two introns, encoding a 543 aminoacid length enzyme. CYP1B1 is mainly expressed in most cell types and secretory cells of breast tissue(2). CYP1B1 also mediates hydroxylation of 17-β oestradiol mainly to 4-hydroxyestradiol, the excess of which reacts further with DNA to form carcinogenic DNA adducts (3). Nearly, 80% of oestradiol is converted to 2-hydroxyestradiol (2-OHE2) by CYP1A1 in the liver and 20% of it to 4-hydroxyestradiol by CYP1B1 in breast tissue and uterus (3, 4). Various studies have established that increased blood oestrogen levels have the potential to induce breast and endometrial cancer, either by binding of oestrogen to its receptors, resulting in cell proliferation or, by conversion of cathechol form of oestrogens to reactive oxygen species

Correspondence to: Professor Dr. Turgay Isbir, Department of Molecular Medicine, Institute of Experimental Medicine (DETAE), Istanbul University, Vakıf Gureba Cad Sehremini, Istanbul, Turkey. Tel/Fax: +90 2126351959, e-mail: tisbir@superonline.com

Key Words: Breast cancer, oestrogen metabolism, N-acetyltransferase 2, cytochrome P450 1B1, heterocyclic arylamines.

Table I. *Distribution of CYP1B1 polymorphisms in control and breast cancer patients.*

Genotype		Control N (%)	Breast cancer N (%)
CYP 1B1 Leu432Val	Leu/Leu	50 (56.2)	23 (38.3)
	Leu/Val	31 (34.8)	24 (40.0)
	Val/Val	8 (9)	12 (20.0)

(ROS) and related quinone and semiquinone products *via* redox cycling (5).

N-acetyltransferase-accompanied detoxification is a basic biotransformation pathway for the metabolism of drugs and environmental xenobiotics, specifically hydrazine drugs, homo- and hetero-cyclic amines and arylamines (6). The NAT2 detoxifying enzyme represents the N- and O-acetylation process in phase 2 metabolisation of biomolecules forming unstable N-acetylated compounds (7-8). If not sufficiently excreted from the organism, these N-acetoxy metabolites may be converted to aryl nitrenium ions that have the potential to react with DNA (6-9).

NAT2 is located at chromosome 8p21.3-23.1 and polymorphisms in this region cause individual and interethnic variation, thus affecting the acetylation capacity of the individual. Human populations can be divided into subgroups according to their acetylation capacities. To date 25 polymorphisms have been identified, of which 7 single nucleotide polymorphisms (SNPs) are the most abundant in various human populations, and are all found within the 870 bp coding region of the NAT2. The wild type allele is denoted as NAT2*4. NAT2*12 and NAT2*13 represent other fast phenotypes. NAT2*5, NAT2*6 and NAT2*7 haplotypes are documented as the slowest acetylating phenotypes (7). Individuals possessing both slow haplotypes are classified as slow acetylators. In this regard, individuals possessing both fast alleles, NAT2*4, NAT2*12 and NAT2*13 are classified as the fastest acetylators. Individuals possessing one fast and one slow allele are classified as intermediate acetylators (6-8).

This population based case-control study conducted among Turkish women, investigates the genetic frequencies of CYP1B1*3 and NAT2, and their possible individual or cooperative involvement in susceptibility to breast cancer.

Patients and Methods

Patient selection. A total of 163 unrelated individuals were included in this study: 60 female breast cancer patients, (median age: 53.7±11.31, range 31-72 years) and 103 healthy controls, (median age: 41.87±14.33, range 22-86 years). Surgical samples from the 60 breast cancer patients were obtained by the Department of Pathology, Istanbul University Cerrahpasa Medicine School, Istanbul. The healthy control group consisted of female healthy blood donors with no prior cancer history or any other vital disease history. Both postmenopausal and premenopausal women were included in the study. Twenty six (43.3%) patients and 70 (68.0%) controls were premenopausal and 34 (56.7%) patients and 33 (32.2%) controls were postmenopausal.

Table II. *Allele frequencies of NAT2 haplotypes.*

Haplotype	Controls		Breast cancer	
	N	%	N	%
4	36	17.8	12	10
5A	25	12.4	7	5.85
5B	16	7.9	26	21.65
5C	11	5.45	10	8.35
5D	5	2.5	2	1.65
5E	1	0.5	-	-
6A	21	10.4	17	14.15
6B	6	3	4	3.35
6C	7	3.5	-	-
6J	16	7.9	12	17.5
7A	27	12.5	18.3	15
7B	8	3.95	3	2.5
12A	8	3.95	-	-
12B	3	1.5	-	-
12C	3	1.5	1	0.85
13	9	4.45	8	6.65
Total	202	100	120	100

DNA extraction. Patient DNA was extracted from paraffin-embedded tissue using the method of Wright and Manos (10). Blood samples from the control group were collected in tubes containing EDTA, and DNA was prepared from leukocyte pellets by SDS lysis, ammonium acetate extraction and ethanol precipitation (11).

*Genotyping method for CYP1B1*3 and NAT2*. For genotyping, DNA was extracted from the blood of the controls and samples embedded in paraffin from non-tumoural neighbouring breast tissue. The CYP1B1*3 genotype was determined following Polymerase Chain Reaction (PCR) according to the method of Zheng *et al*. (12). Genotyping for NAT2 polymorphism, including 11 SNPs and 7 missense mutations, was conducted according to the method of Deitz *et al*. (13). The NAT2 haplotype nomenclature used in this study was based on the study of Sabbagh *et al*. (7, 8).

Statistical analysis. Statistical analyses were performed using SPSS version 7.5 (SPSS Inc. Chicago, USA) including the Chi-square (χ^2) test, Fisher's exact test and the Pearson correlation test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated.

Results

The allelic variant frequencies of CYP1B1*3 polymorphism are shown on Table I. 20.3% of the patients were homozygous for mutant allele (Val allele), while 40.7% of the patients were heterozygous mutant and 39% were homozygous for wild allele (Leu) (p=0.043, χ^2: 3.911, OR: 2.58, 95% CI: 0.99-6.75).

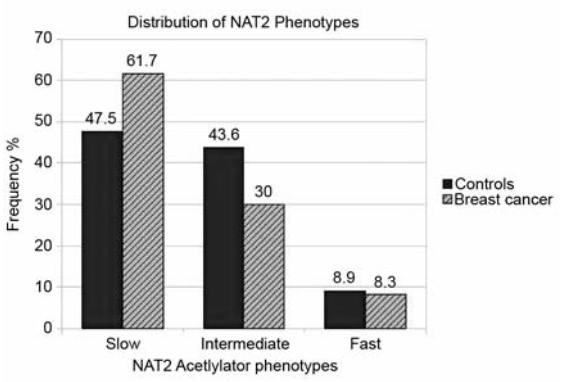

Figure 1. *Distribution of NAT2 acetylator phenotypes in patients and controls.*

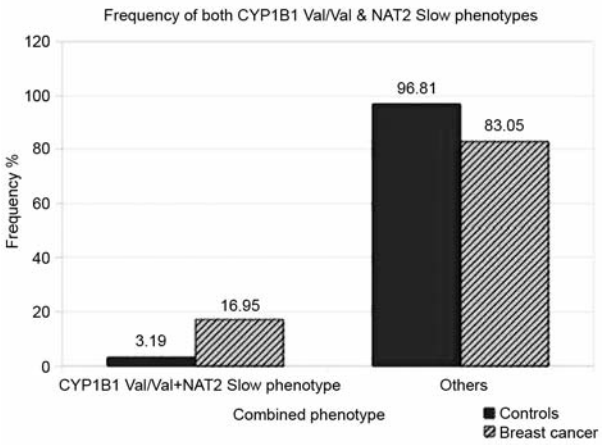

Figure 2. *Distribution of carriers of both risky phenotypes (homzygous mutant for CYP1B1*3 and carrying two slow acetylator haplotype of NAT2).*

The NAT2 gene was analysed for 11 polymorphisms: T111C, G191A, C282T, T341C, A434C, C481T, G590A, C759T, A803G, A845C, and G857A. None of the polymorphisms at nucleotides 111, 191, 434, 759 and 845 were observed in either control or patient samples.. NAT2 allelic frequencies using the nomenclature of Sabbagh *et al*. (7, 8) are shown on Table II.

N-Acetyltransferase 2 genotyping in patients and controls was identified through the combinations of all polymorphisms. When these combined genotypes were evaluated with their phenotypic activities, fast acetylators and intermediate acetylators were found to be less frequent in the patient group (Figure 1). Slow acetylator phenotype in the patient group was observed to be 1.8 times higher than in the control group. Nevertheless, the results did not reach statistical significance (p=0.07, χ^2: 3.24, OR: 1.82, 95% CI: 0.94-3.50).

NAT2*4/4 'Fast Acetylator' phenotype, as the wild type, was observed in 3% of the controls, while the haplotype was not present in any of the patients. Total NAT2*5 haplotype frequency was statistically more abundant in patients than in controls. 56% of controls and 75% of patients carried NAT2*5 (p=0.02, χ^2: 5.04, OR: 2.22, 95% CI: 1.10-4.50). Within these NAT2*5 haplotypes, NAT2*5B was the most frequent (p=0.000, χ^2: 14.75, OR: 4.06, 95% CI: 1.94-8.51).

In a combined analysis of CYP1B1*3 and NAT2 phenotypes, the frequency of individuals carrying both the CYP1B1 Val/Val and NAT2 slow haplotypes was 6.2 times higher in the patient group than in the control group (p=0.004, $\kappa\chi^2$: 8.82, OR: 6.70, 95% CI: 1.63-23.55).

Discussion

This study investigated the risk of breast cancer in association with metabolic transformations of chemical carcinogens and analysed genetic modifications in order to determine risk assessment. Cytochrome P450 1B1 activation of chemicals and N-Acetyltransferase 2 detoxifying mechanisms may indicate either an increased or decreased cancer risk (12, 14).

N-Acetyltransferase 2 is one of the mostly differentiated gene family whose pharmacokinetic characteristics have already been studied (14, 15). Individual genetic variations lead to differences in NAT2 activation and susceptibility to diseases (14, 15). Smoking, consuming well-cooked meat and exposure to industrial chemicals are all risk inducing factors. The NAT2*5 family, including the T341C polymorphism, has been determined to be the most dominant subtype that causes declination in N-, O-acetylation functions. NAT2*14 including G191A, NAT2*6 including G590A and NAT2*7 including G857A are also the other slow acetylator subtypes in the order of increasing activity (7,8). Moreover, it has been demonstrated that T341C, A434C and G590A polymorphisms reduce protein expression and G191A, A845C and G857A cause decreases in protein stabilisation (7, 8). These studies show that a slow acetylator phenotype is not homogenous but is composed of phenotypes related on the heritage of certain SNPs and alleles.

The association between NAT2 acetylation and breast cancer has been evaluated by a number of studies. Among them are studies that obtained positive correlations while there has been research with no consistent associations (15). Alberg *et al*. established fast acetylation as a risk factor in women in all menopausal status (16). In contrast, Sillanpaa *et al*. and Huang *et al*. stated that slow acetylation increases cancer risk and the risk reaches the highest levels in postmenopausal women (17, 18).

In the present study, NAT2*4/4 homozygous wild type variant was present in 3% of the controls. NAT2*4 allele frequency was 18% in controls and 10% in patients.

Combinations of fast variants with other variant types were 8.9 % of controls and 8.3 % of patients. In conclusion, the frequency of fast variants in Turkish population seems to be in accordance with that of the Caucasian population and to be smaller than in Asian populations according to Sabbagh *et al*. (8).

Phenotypic schematisation of patients and controls confers 1.8 times elevated but not significant risks of breast cancer for slow acetylators. Statistically, NAT2*5 allele is harboured mostly by patients (p=0.02). Meanwhile, NAT2*5B allele is determined to be the most abundant phenotype among the NAT2 family with a frequency of 43 % in patients and 15.8 % in controls. In support of this observation, this risk associated phenotype (NAT2*5B) is also the most common phenotype among Turkish population according to Sabbagh *et al*. (8).

Studies combining acetylator phenotype and smoking status in breast cancer does not provide consistent associations. Huang *et al*., Hunter *et al*. and Millikan *et al*. have shown association with different acetylator haplotypes (17, 19, 20). Turkish women over fifty years old have low smoking rates and smoking rates of participants in our study were found to be quite low, only 9 of the patients and 23 of the cases were smoking 3.66±10.66 and 3.91±9.11 packs/year respectively. Therefore the smoking status was not included in the statistical analysis.

CYP1B1*3 induces risk with respect to activation of xenobiotics and oestrogen catabolism. Expression of CYP1B1 takes place mainly in the liver and is also detected in normal tissues (21). However, CYP1B1 expression level, determined in tumor tissues in immunohistochemical studies and enzyme activity experiments, reaches a negligible level in pretumoral progression and neoplastic tissues (21). Cancer risk due to conversion of 4-OH oestradiol to quinone and semiquinone metabolites and formation of DNA adducts has been investigated by Cavalieri *et al*. (22). Hanna *et al*. examined functional differences in oestrogen hydroxylating activities and concluded that CYP1B1*3 had a nearly 4-fold 4-hydroxylase function (23). CYP1B1*3 polymorphism localised in the haeme-binding region of the enzyme, leads to changes in functional activities with polymorphic modifications. As a consequence, excessive functioning of the enzyme may increase oestrogen catabolism and formation of reactive oestrogen catabolites capable of binding DNA (24, 25). In accordance with the results presented here, breast cancer patients carry the mutant genotype in approximately 2.5 fold higher frequencies when compared to controls. However, age average of homozygous mutant phenotype among the rest of the patients is similar in this study.

In line with previous studies, risk association seems more intense from the side of xenobiotic/oestrogen activation and acetylation mechanisms in the present study. To the authors' knowledge, only one previous study evaluated CYP1B1*3 and NAT2 slow acetylator phenotypes with significant results, however, only in light smokers (26). With respect to the present results, the coexistence of CYP1B1*3 and slow acetylator phenotype both demonstrate a positive relationship with breast cancer in patients and pose a statistically significant risk of breast cancer compared to women carrying the other variations (p=0.004, χ^2: 8.82, OR: 6.70, 95% CI: 1.63-23.55). Due to the relatively small number of individuals included in this study, statistically significant findings were limited. It is therefore necessary to perform this study with larger groups and various populations. Furthermore both individual and cooperative effects of CYP1B1*3 and NAT2 polymorphisms should be investigated.

References

1 Gadducci A, Biglia N, Sismondi P and Genazzani AR: Breast Cancer and sex steroids: Critical review of epidemiological, experimental and clinical investigations on etiopathogenesis, chemoprevention and endocrine treatment of breast cancer. Gyn End *20*: 343-360, 2005.

2 Kumaraguruparan R, Mohan KVPC and Nagini S: Xenobiotic-metabolising enzymes in patients with adenocarcinoma of the breast: Correlation with clinical stage and menopausal status. The Breast *15*: 58-68, 2006.

3 Sissung TM, Price DK, Sparreboom A and Figg WD: Pharmacogenetics and Regulation of Human Cytochrome P450 1B1: Implications in Hormone-Mediated Tumor Metabolism and a Novel Target for Therapeutic Intervention. Mol Cancer Res *4*: 135-150, 2006.

4 Perez CB, Valdiglesias V, Pásaro E, Méndez J and Laffon B: Genetic Polymorphism in Cytochrome 1B1 in a Spanish Population. Basic & Clin Pharmacology & Toxicology *101*: 70-72, 2007.

5 Cavalieri EL, Stack DE, Devanesan PD , Todorovıc R, Dwıvedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML , Gooden JK, Ramanathan R, Cerny RL and Rogan EG: Molecular Origin Of Cancer: Catechol Estrogen-3,4-Quinones As Endogenous Tumor initiators. Proc Natl Acad Sci USA *94*: 10937-10942, 1997.

6 Meyer UA: Polymorphism of Human Acetyltransferases. Env Health Pers *102*: 213-216 1994.

7 Sabbagh A and Darlu P: Inferring haplotypes at the NAT2 locus: the computational approach. BMC Genetics *6*: 30, 2005.

8 Sabbagh A, Langaney A, Darlu P, Gerard N, Krishnamoorthy R and Poloni ES: Worldwide distribution of NAT 2 diversity: Implications for NAT 2 evolutionary history. BMC Genetics *9*: 21, 2008.

9 Ozturk O, Kagnici OF, Ozturk T, Durak H, Tuzuner BM, Kisakesen HI, Cakalir C and Isbir T: 192R Allele of Paraoxanase 1 (PON1) Gene as a New Marker for Susceptibility to Bladder Cancer. Anticancer Research *29*: 4041-4046, 2009.

10 Wright DK and Manos MM: Sample preparation from paraffin-embedded tissues. *In*: PCR Protocols, A Guide to Methods and Applications. U.K, Inis MA, Gelfand DH, Sninsky JJ, White TJ (eds.). Academic Press, pp. 153-158, 1990.

11 Miller SA, Dykes DD and Polesky HS: Simple salting-out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res *16*: 1215, 1988.
12 Zheng W, Xie D, Jin F, Cheng J, Dai Q, Wen W, Shu X and Gao Y: Genetic Polymorphism of Cytochrome P450-1B1 and Risk of Breast Cancer. Cancer Epid Biom & Prev *9*: 147-150, 2000.
13 Deitz AC, Zheng W, Leff MA, Gross M, Wen W, Doll MA, Xiao GH, Folsom AR and Hein DW: N-Acetyltransferase-2 Genetic Polymorphism, Well-done Meat Intake, and Breast Cancer Risk among Postmenopausal Women. Cancer Epid Biom & Prev *9*: 905-910, 2000.
14 Hein DW, Doll MA, Fretland AJ, Leff MA, Webb SJ, Xiao GH, Devanaboyina U, Nangju NA and Feng Y: Molecular Genetics and Epidemiology of the NAT 1 and NAT 2 Acetylation Polymorphisms. Cancer Epid Biom & Prev *9*: 29-42, 2000.
15 Ochs-Balcom HM, Wiesner G and Elston RC: A Meta-Analysis of the Association of N-Acetyltransferase 2 Gene (NAT2) Variants with Breast Cancer. Am J Epidemiol *166*: 246-254, 2007.
16 Alberg AJ, Daudt A, Huang HY, Hoffman SC, Comstock GW, Helzlsouer KJ, Strickland PT and Bell DA: N-acetyltransferase 2 (NAT2) genotypes,cigarette smoking, and the risk of breast cancer. Cancer Detect Prev *28*: 187-197, 2004.
17 Huang CS, Chern HD, Shen CY, Hsu SM and Chang KJ: Association between Nacetyltransferase 2 (NAT2) genetic polymorphism and development of breast cancer in post-menopausal Chinese women in Taiwan, an area of great increase in breast cancer incidence. Int J Cancer *82*: 175-179, 1999.
18 Sillanpaa P, Hirvonen A, Kataja V, Kataja V, Eskelinen M, Kosma VM, Uusitupa M, Vainio H and Mitrunen K: NAT2 slow acetylator genotype as an important modifier of breast cancer risk. Int J Cancer *114*: 579-584, 2005.
19 Hunter DJ, Hankinson SE, Hough H, Gertig DM, Garcia-Closas M, Spiegelman D, Manson JE, Colditz GA, Willett WC, Speizer FE and Kelsey K: A prospective study of NAT2 acetylation genotype, cigarette smoking, and risk of breast cancer. Carcinogenesis *18*: 2127-2132, 1997.
20 Millikan RC, Pittman GS, Newman B, Tse CK, Selmin O, Rockhill B, Savitz D, Moorman PG and Bell DA: Cigarette smoking, N acetyltransferases 1 and 2, and breast cancer risk. Cancer Epidemiol Biomarkers Prev *7*: 371-378, 1998.
21 McFadyen MC, Rooney PH, Melvin WT and Murray G: Quantitative analysis of the Ah receptor/cytochrome P450 CYP1B1/CYP1A1 signalling pathway. Biochem Pharmacol *65*: 1663-1674, 2003.
22 Cavalieri E, Frenkel K, Liehr JG, Rogan E and Roy D: Estrogens as endogenous genotoxic agents-DNA adducts and mutations. J Natl Cancer Inst Monogr *27*: 75-93, 2000.
23 Hanna IH, Dawling S, Roodi N, Guengerich FP and Parl FF: Cytochrome P450 1B1 (CYP1B1) Pharmacogenetics: Association of Polymorphisms with Functional Differences in Estrogen Hydroxylation Activity. Cancer Research *60*: 3440-3444, 2000.
24 Liehr JG and Roy D: Free radical generation by redox cycling of estrogens. Free Radical Biol Med *8*: 415-423, 1990.
25 Huang Z, Fasco MJ, Figge HL, Keyomarsi K and Kaminsky LS: Expression of cytochromes P450 in human breast tissue and tumors. Drug Metab Dispos *24*: 899-905, 1996.
26 Sillanpaa P, Heikinhemo L, Kataja V, Eskelinen M, Komsa V, Uusitupa M, Vainio H, Metsola K and Hirvonen A: CYP 1A1 and CYP 1B1 genetic polymorphisms, smoking and breast cancer in a Finnish Caucasian population. Breast Cancer Res Treat *104*: 287-297, 2007.

Received December 24, 2009
Revised March 29, 2010
Accepted March 29, 2010