Research Article

Single Nucleotide Polymorphisms in Selected Apoptotic Genes and BPDE-Induced Apoptotic Capacity in Apparently Normal Primary Lymphocytes: A Genotype-Phenotype Correlation Analysis

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Apoptotic capacity (AC) in primary lymphocytes may be a marker for cancer susceptibility, and functional single nucleotide polymorphisms (SNPs) in genes involved in apoptotic pathways may modulate cellular AC in response to DNA damage. To further examine the correlation between apoptotic genotypes and phenotype, we genotyped 14 published SNPs in 11 apoptosis-related genes (i.e., p53, Bcl-2, BAX, Bcl-2, DR4, Fas, Fasl, CASP8, CASP10, CASP3, and CASP7) and assessed the AC in response to benzo[a]pyrene-7,8,9,10-diol epoxide (BPDE) in cultured primary lymphocytes from 172 cancer-free subjects. We found that among these 14 SNPs, R72P, intron 3 16-bp del/ins, and intron 6 G>A in p53, −938C>A in Bcl-2, and I522L in CASP10 were significant predictors of the BPDE-induced lymphocytic AC in single-locus analysis. In the combined analysis of the three p53 variants, we found that the individuals with the diplotype carrying 0-1 copy of the common p53 R-del-G haplotype had higher AC values compared to other genotypes. Although the study size may not have the statistical power to detect the role of other SNPs in AC, our findings suggest that some SNPs in genes involved in the intrinsic apoptotic pathway may modulate lymphocytic AC in response to BPDE exposure in the general population. Larger studies are needed to validate these findings for further studying individual susceptibility to cancer and other apoptosis-related diseases.

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1. INTRODUCTION

Apoptosis, also known as the programmed cell death, is a biological process that regulates physiological cell death and plays an important role in the pathogenesis of a variety of human diseases, including cancer [1]. Resistance to apoptosis or reduced cellular apoptotic capacity (AC) provides a survival advantage of the cells that may develop into cancer cells, commonly seen in almost all types of malignant diseases, and mutations in the genes involved in apoptotic pathways are one of the molecular mechanisms underlying carcinogenesis [2, 3] and cancer therapy [3, 4].

Benzo[a]pyrene (B[a]P) is a classic DNA-damaging carcinogen found in both tobacco smoke and the environment as a result of fuel combustion [5]. Its bioactivated form, benzo[a]pyrene diol epoxide (BPDE), can cause irreversible damage to DNA by forming DNA adducts through covalent binding or oxidation [6, 7]. If these adducts are unrepaired, the cells will undergo apoptosis through activation of p53, caspase-9 (CASP9), and caspase-3 (CASP3) [8, 9]. As a pivotal regulator of cellular response to DNA damage, the transcription factor encoded by the p53 tumor suppressor gene has been clearly implicated in B[a]P-induced apoptosis, and the levels of p53 protein expression has been correlated with the levels of B[a]P-DNA adducts [8, 10]. Although details of the signaling pathways that trigger apoptosis in lymphocytes remain not fully understood, possible mechanisms include transcriptional activation of the Bcl-2 family members [11] and transcriptional upregulation of the death receptors (DRs) [12, 13]. These complex proteins participate in the activation of a sequential signaling that modulates two main apoptotic pathways [4]. One is the intrinsic or mitochondrial pathway, in which the stimuli of p53-Bcl-2 pathway lead to the activation of CASP9 and release
of cytochrome c from the mitochondria [14]. The other, referred to as the extrinsic cytoplasmic pathway, involves a group of proteins such as the DRs, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, caspase-8 (CASP8), and caspase-10 (CASP10) [15, 16]. Activation of these two pathways initiates a common downstream proteolytic cascade that involves CASP3 and caspase-7 (CASP7) [4].

It is likely that the efficiency of these apoptotic pathways is genetically determined. Therefore, we hypothesized that functional polymorphisms in genes involved in these apoptotic pathways may modulate the AC phenotype, thus contributing to individual variation in response to DNA damage. To test this hypothesis, we selected 14 potentially functional polymorphisms in 11 genes, that is, p53, Bcl-2, BAX, and CASP9 involved in the intrinsic pathway; DR4, Fas, Fasl, CASP8, and CASP10 involved in the extrinsic pathway; and CASP3 and CASP7, the effective CASPs. We genotyped these 14 polymorphisms and assessed in vitro AC with a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay using BPDE-treated primary lymphocytes from 172 subjects without cancer to evaluate associations between their polymorphic genotypes and the AC phenotype.

2. MATERIALS AND METHODS

2.1. Study population

Subjects in the current study were the control subjects in a molecular epidemiology study of lung cancer previously described [17]. Briefly, 172 subjects in this study were randomly selected from a pool of cancer-free control subjects recruited from the Kelsey-Seybold Clinics, a large multispecialty physician organization with several clinics throughout the Houston metropolitan area. Each subject was scheduled to be interviewed after a written informed consent was obtained. After the interview, a venous blood sample of about 20 mL was collected from each subject. The research protocol was approved by The University of Texas M. D. Anderson Cancer Center and the Kelsey-Seybold Foundation institutional review boards.

2.2. SNP selection

We used the National Center for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov), the National Institute of Environmental Health Sciences (NIEHS) Environment Genome Project SNP databases (http://egp.gs.washington.edu/directory.html and http://www.genome.utah.edu/genesnps/) and literature search to identify potentially functional variants in genes involved in both intrinsic and extrinsic apoptotic pathways. Polymorphisms with a minor allele frequency (MAF) of ≥0.05 were included, if they may theoretically result in amino acid changes (nonsynonymous SNP, nsSNP), located at regulating regions such as promoters, or are reportedly associated with known phenotypic effects. Three reported p53 SNPs were selected, including the well-known codon 72 SNP (R72P, G > C) and two intronic variants (a 16 bp-del/ins in intron 3 and a G-to-A transition in intron 6 because their haplotypes were found to be functional [18]. Two previously reported regulating SNPs in the promoters of the Bcl-2 family members, Bcl-2 (–938C > A) and BAX (–248G > A) [19, 20], were included. For the CASPs, we identified one nsSNP from each CASP8 (D302H, G > C), CASP10 (I522L, A > T) [21], and CASP7 (D255E, C > G, http://www.ncbi.nlm.nih.gov) and one of the two CASP9 nsSNPs in tight linkage disequilibrium (LD) (Q221R, G > A, http://egp.gs.washington.edu/directory.html and [22]. Because no nsSNP was found in the coding region of CASP3, we selected one common variant in its promoter region: −1337C > G (http://www.genome.utah.edu/genesnps/). For the death receptor genes, one nsSNP in DR4 and three promoter SNPs in Fas and Fasl were selected: T209R (C > G) in DR4, −1377G > A and −670A > G in Fas, and −844T > C in Fasl [23–26].

2.3. Genotyping

The genotyping methods used to distinguish the 14 selected polymorphisms in 11 apoptosis-related genes are presented in Table 1. Genotyping methods for seven of the polymorphisms were previously described: p53 R72P [27]), p53 intron 3 16 bp-del/ins and intron 6 G > A [18], DR4 T209R [28], Fas −1377G > A and −670A > G [29], and Fasl −844T > C [30]. The remaining seven polymorphisms (i.e., Bcl-2 (–938C > A), BAX (–248G > A), CASP9 Q221R, CASP8 D302H, CASP10 I522L, CASP3 (−1337C > G, and CASP7 D255E) were detected by using a primer-introduced restriction analysis (PIRA)—polymerase chain reaction (PCR) assay [31] and summarized in Table 1. Genotyping was performed without knowledge of the subjects’ phenotype; more than 10% of the samples were randomly selected for confirmation, and the results were 100% concordant. For the seven self-designed genotyping assays, PCR products containing each target genotype were purified and the sequences were confirmed by direct sequencing.

2.4. Apoptosis assay

The apoptosis phenotype (i.e., apoptotic capacity [AC]) was detected with the TUNEL assay previously described [32]. Briefly, two parallel short-term cultures from each blood sample were incubated at 37°C without CO2 for 67 hours before BPDE treatment. At the end of the incubation, one of the two parallel cultures was treated with BPDE (98% pure; Midwest Research Institute, Kansas City, Mo, USA) at a final concentration of 4 μM. After an additional 5-hour incubation, all cells were pelleted by centrifugation, resuspended with lysis buffer (Human Erythrocyte Lysing Kit, R&D Systems, Minneapolis, Minn, USA), fixed for 1 hour, rinsed with phosphate-buffered saline, and finally stored in 70% ethanol at −20°C until used for the TUNEL assay.

For the TUNEL assay, we used the APO-BRDU kit (Phoenix Flow Systems, San Diego, Calif, USA) and followed the manufacturer’s recommended protocol. The ratio of the
difference in the percentages of apoptotic cells in a subject’s BPDE-treated and untreated cultures to the percentage of apoptotic cells in the untreated culture was recorded as the AC (AC% = [AC_{treated} - AC_{baseline}] / AC_{baseline} × 100) [32].

### 2.5. Statistical analysis

DNA quality or quantity was insufficient for genotyping in 2 subjects; thus, the final analysis included 170 persons.
Differences of the continuous AC measurements between genotypes/diplotypes of apoptotic genes were evaluated by using Student’s t-test. Trend test was performed by using the general linear regression model with adjustment for age and sex. We dichotomized the continuous phenotype measurements by using the median (150%) as the cutoff value to obtain an almost equal low-AC subgroup (84 subjects) and high-AC subgroup (86 subjects). Logistic regression analyses were used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) between combined genetic variants and dichotomized AC phenotype with adjustment for age and sex. Alleles/haplotypes associated with the lower AC phenotype in individual polymorphism analysis were termed as “at-risk” alleles hereinafter. We used the PHASE 2.0 program [33] to infer haplotype frequencies based on the observed genotypes for each gene. Diplotype was the most probable haplotype pair for each individual. The potential effects of the continuous AC measurements between the subgroups according to age and sex (data not shown). Table 2 shows the continuous AC measurements by genotypes of the selected apoptotic genes. The observed genotype frequencies were all consistent with those expected from the Hardy-Weinberg equilibrium (data not shown). For SNPs in genes involved in the intrinsic apoptotic pathway, variant homozygotes of p53 intron 3 16-bpdel/ins, p53 intron 6 AA, and Bcl-2 −938AA all had significantly higher AC than their corresponding wild-type homozygotes (496.07 ± 121.26 versus 204.22 ± 183.21 for p53 intron 3 16-bpdel/ins, P = 0.027; 496.07 ± 121.26 versus 199.44 ± 179.10 for p53 intron 6 G>A, P = 0.021, and 247.62 ± 225.67 versus 164.06 ± 154.89 for Bcl-2 −938C>A, P = 0.046). However, the significant P values for the trend of higher AC with increasing number of the variant alleles were observed only for p53 R72P (P = 0.016) and Bcl-2 −938C>A (P = 0.057) as assessed in the general linear regression model with adjustment for age and sex (Table 2). In contrast, only the variant homozygotes of CASP10 1522L out of all SNPs in genes involved in the extrinsic apoptotic pathway had significantly lower AC (159.49 ± 171.44) than the II homozygote (239.07 ± 205.18, P = 0.046) as well as a significant trend of lower AC with increasing number of the variant alleles (P = 0.046).

Linkage disequilibrium (LD) analysis showed that the three loci in p53 were in LD (r² = 0.14, D’ = 0.64 for R72P and intron 3 16-bpdel/ins; r² = 0.24, D’ = 0.85 for R72P and intron 6 G>A; and r² = 0.60, D’ = 0.79 for intron 3 16-bpdel/ins and intron 6 G>A). Therefore, we performed haplotype/diplotype inference using the PHASE 2.0 program based on the observed genotypes. Overall, three common haplotypes were derived (Table 3). The diplotype carrying zero copy of the p53 R-del-G haplotype and the diplotype carrying two copies of the p53 P-Ins-A haplotype all had significantly higher AC (termed as “protective” hereinafter) and the effect of the R-del-G haplotype was in a dose-response manner (P for trend: 0.016; Table 3).

4. DISCUSSION

In the genotype-phenotype analysis, we examined the role of potentially functional variants in selected apoptotic genes in the AC phenotype induced by BPDE treatment in primary lymphocytes. We found that R72P, intron 3 16-bpdel/ins, intron 6 G>A in p53, −938C>A in Bcl-2, and 1522L in CASP10 may be predictors of AC, but the effects of the p53 variants might also be modulated by its downstream genes involved in the intrinsic pathway. To the best of our knowledge, this is the first multigene genotype-phenotype correlation analysis in relation to the apoptotic pathways in primary lymphocytes at a population level.

Because there is tissue specificity in response to carcinogen exposure, it would be ideal to compare the BPDE-induced AC measurements among different tissues of the same person. However, few reported studies have addressed this tissue specificity, nor did our study have such an opportunity. It was reported that B[a]P-induced apoptosis of murine Hepa1c1c7 cells was through CASP-9 activation related with p53 accumulation and activation [8] and that a decrease in the expression of Bcl-2 to Bax ratio was another hallmark of the process [8, 34]. Although obtained from different cell types, these findings are consistent with our current observations that genetic variants in the genes involved in the intrinsic apoptotic pathway may play an important role in the prediction of AC phenotype. The Bcl-2 family is a group of evolutionarily conserved pro- and anti-apoptotic proteins that play a pivotal role in the regulation of the mitochondrial-mediated (intrinsic) apoptotic pathway [35]. Bcl-2 inhibits apoptosis through heterodimerization with proapoptotic members of the Bcl-2 family, such as Bax and also through formation of channels that stabilize the mitochondrial membrane [36]. Bcl-2 expression has also been implicated in the pathogenesis of cancers [37, 38], and the expression of Bcl-2 to Bax ratio seems to be important in determining both in vitro and in vivo response to chemotherapeutic drugs [39]. Recently, variant allele of Bcl-2 −938C>A was found to be associated with reduced prostate cancer risk in Caucasians in a small case-control study, possibly due to the elimination of an Sp1 binding site, a downregulation of Bcl-2 mRNA transcript levels, and unregulated programmed cell death [40], which is consistent with what we found in the current study (the variant A allele carriers were associated with high-AC phenotype that may help eliminating possible malignant cells).

Our results on the p53 polymorphisms were not consistent with published data. For example, the wild-type 72R
Table 2: Comparisons of mean BPDE-induced apoptosis capacity in apparently normal primary lymphocytes by the genotypes of selected apoptotic genes.

| Variable         | No. (%) | AC (mean ± SD) | P value(a) | P value(b) |
|------------------|---------|----------------|------------|------------|
| **Intrinsic pathway** |         |                |            |            |
| p53 R72P         |         |                |            |            |
| RR               | 91 (53.5) | 180.47 ± 164.13 | Ref.       |            |
| RP               | 65 (38.2) | 223.07 ± 196.22 | .143       |            |
| PP               | 14 (8.2)  | 313.40 ± 243.25 | .067       | .016       |
| p53 intron 3     |         |                |            |            |
| 16-bpdel/del     | 134 (78.8) | 204.22 ± 183.21 | Ref.       |            |
| 16-bpdel/ins     | 34 (20.0)  | 204.51 ± 194.62 | .993       |            |
| 16-bpins/ins     | 2 (1.2)    | 496.07 ± 121.26 | .027       | .274       |
| p53 intron 6     |         |                |            |            |
| GG               | 135 (79.4) | 199.44 ± 179.10 | Ref.       |            |
| GA               | 33 (19.4)  | 224.07 ± 209.17 | .495       |            |
| AA               | 2 (1.2)    | 496.07 ± 121.26 | .021       | .099       |
| Bcl-2 –938C>A    |         |                |            |            |
| CC               | 53 (31.2)  | 164.06 ± 154.89 | Ref.       |            |
| CA               | 76 (44.7)  | 216.62 ± 180.38 | .087       |            |
| AA               | 41 (24.1)  | 247.62 ± 225.67 | .046       | .037       |
| BAX –248G>A      |         |                |            |            |
| GG               | 144 (84.7) | 205.39 ± 189.73 | Ref.       |            |
| GA               | 25 (14.7)  | 225.99 ± 173.85 | .613       |            |
| AA               | 1 (0.6)    | 84.85           | —          |            |
| CASP9 Q221R      |         |                |            |            |
| QQ               | 53 (31.2)  | 194.75 ± 169.29 | Ref.       |            |
| QR               | 75 (44.1)  | 213.91 ± 199.95 | .571       |            |
| RR               | 42 (24.7)  | 213.00 ± 187.11 | .620       | .704       |
| **Extrinsic pathway** |         |                |            |            |
| DR4 T209R        |         |                |            |            |
| TT               | 52 (30.6)  | 179.61 ± 179.86 | Ref.       |            |
| TR               | 75 (44.1)  | 229.67 ± 206.45 | .160       | .485       |
| RR               | 43 (25.3)  | 203.39 ± 155.49 | .497       |            |
| Fas –1377G>A     |         |                |            |            |
| GG               | 126 (74.1) | 218.66 ± 195.82 | Ref.       |            |
| GA               | 42 (24.7)  | 179.99 ± 158.13 | .248       |            |
| AA               | 2 (1.2)    | 100.14 ± 92.38  | .396       | .182       |
| Fas –670A>G      |         |                |            |            |
| AA               | 43 (25.3)  | 210.82 ± 173.91 | Ref.       |            |
| GA               | 86 (50.6)  | 236.97 ± 203.18 | .472       |            |
| GG               | 41 (24.1)  | 143.07 ± 147.70 | .058       | .085       |
| FasL –844T>C     |         |                |            |            |
| CC               | 79 (46.5)  | 225.68 ± 195.41 | Ref.       |            |
| CT               | 74 (43.5)  | 190.11 ± 180.80 | .245       |            |
| TT               | 17 (10.0)  | 200.82 ± 173.81 | .629       | .464       |
| CASP8 D302H      |         |                |            |            |
| DD               | 127 (74.7) | 202.30 ± 184.45 | Ref.       |            |
| DH               | 38 (22.4)  | 210.97 ± 195.10 | .802       |            |
| HH               | 5 (2.9)    | 320.41 ± 183.38 | .162       | .389       |
| CASP10 I522L     |         |                |            |            |
| II               | 54 (31.8)  | 239.07 ± 203.18 | Ref.       |            |
| IL               | 74 (43.5)  | 212.19 ± 177.84 | .430       |            |
| LL               | 42 (24.7)  | 159.49 ± 171.44 | .046       | .046       |
lymphocytes may be relevant to other types of tissue as well. However, our findings of this intrinsic apoptotic pathway in only borderline significant due to a limited study power. The interaction between polymorphisms of p53 to be an important role in the B[a]P-induced apoptosis [8]. In the genes involved in the intrinsic pathway, which may play an important role in regulating the apoptotic response to carcinogen exposure, at least in primary lymphocytes, although there is tissue specificity in response to exposure to carcinogens. Such a modification of host carcinogen-induced AC in primary lymphocytes may contribute to variation in individual susceptibility to cancer in the general population. Although this study may be limited due to small sample size, multiple tests, and lack of repeated AC measurements for the same individuals, the findings, if validated in more rigorously designed and larger studies, should facilitate the design of future studies aimed at identifying subpopulations at risk of cancer and other apoptosis-related diseases.

Because this kind of induced apoptosis in lymphocytes may be inheritable [44]. Thus these results, although preliminary, need to be substantiated in larger studies.

Previous finding on the CASP10 I522L polymorphism from a large breast cancer study showed that the variant LL genotype was associated with a borderline significant 1.30-fold increased cancer risk compared with the wild-type II homozygote [21], which is consistent with the notion that the LL homozygote contributes to a diminished AC. However, the role of the extrinsic pathway (or only CASP10) in B[a]P-induced apoptosis was not obvious in the present study, which needs further evaluation.

In conclusion, this proof-of-principle study of genotype-phenotype correlation provides evidence that potentially functional polymorphisms in the core genes of the apoptotic pathways may have a role in regulating the apoptotic response to carcinogen exposure, at least in primary lymphocytes, although there is tissue specificity in response to exposure to carcinogens. Such a modification of host carcinogen-induced AC in primary lymphocytes may contribute to variation in individual susceptibility to cancer in the general population. Although this study may be limited due to small sample size, multiple tests, and lack of repeated AC measurements for the same individuals, the findings, if validated in more rigorously designed and larger studies, should facilitate the design of future studies aimed at identifying subpopulations at risk of cancer and other apoptosis-related diseases.

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| Table 2: Continued. |
|----------------------|
| Variable              | No. (%) | AC (mean ± SD) | P value(a) | P value(b) |
| Effective CASPs       |         |                |            |            |
| CASP3–1337C>G         |         |                |            |            |
| CC                   | 107 (62.9) | 210.98 ± 202.46 | Ref. |            |
| CG                   | 51 (30.0)  | 212.23 ± 166.75 | .970 |            |
| GG                   | 12 (7.1)   | 159.34 ± 109.53 | .388 | .516 |
| CASP7 D255E          |         |                |            |            |
| DD                   | 95 (55.9)  | 214.49 ± 190.29 | Ref. |            |
| DE                   | 68 (40.0)  | 195.79 ± 166.92 | .516 |            |
| EE                   | 7 (4.1)    | 231.51 ± 318.48 | .829 | .801 |

(a) Two-sided Student t-test.
(b) Trend test obtained from general linear regress model with adjustment for age and sex.

| Table 3: Comparisons of mean BPDE-induced apoptosis capacity in apparently normal primary lymphocytes by p53 genotypes. |
|---------------------------------------------------------------|
| p53 genotypes No. (%) | AC (mean ± SD) | P value(a) | P value(b) |
| R-del-G              |                |            |            |
| 2 copies             | 81 (47.6)      | 177.57 ± 159.94 | Ref. |            |
| 1 copy               | 74 (43.5)      | 220.39 ± 197.21 | .138 |            |
| 0 copy               | 15 (8.8)       | 307.90 ± 235.37 | .009 | .016 |
| R-ins-G              |                |            |            |
| 0 copy               | 116 (68.2)     | 190.05 ± 174.57 | Ref. |            |
| 1 copy               | 49 (28.8)      | 238.99 ± 197.24 | .116 |            |
| 2 copies             | 5 (2.9)        | 310.86 ± 311.47 | .146 | .094 |
| P-ins-A              |                |            |            |
| 0 copy               | 143 (84.1)     | 202.33 ± 179.92 | Ref. |            |
| 1 copy               | 25 (14.7)      | 215.43 ± 215.45 | .745 |            |
| 2 copies             | 2 (1.2)        | 496.07 ± 121.26 | .023 | .154 |

(a) Two-sided Student t-test.
(b) Trend test obtained from general linear regress model with adjustment for age and sex.
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