Overexpression of Cytochrome P450 1A1 and Its Novel Spliced Variant in Ovarian Cancer Cells: Alternative Subcellular Enzyme Compartmentation May Contribute to Carcinogenesis

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Abstract

Epithelial ovarian cancer derived from the human ovarian surface epithelium (HOSE) is the leading cause of death from gynecologic malignancies among American women. Metabolic activation of endogenous and exogenous chemicals by cytochrome P450 (CYP) class I enzymes has been implicated in its etiology. In this study, we showed overexpression of CYP1A1 mRNA, but not CYP1B1 transcripts, in ovarian cancer cell lines when compared with primary cultures or immortalized HOSE cell lines. Importantly, we identified a novel, enzymatically active, spliced variant of CYP1A1 (CYP1A1v) formed by excision of an 84-bp cryptic intron in exon 2. CYP1A1v is overexpressed in ovarian cancer cell lines and exhibits a unique subcellular distribution restricted to the nucleus and mitochondria, contrary to the endoplasmic reticulum localization of the wild-type enzyme. In concordance, total CYP1A1 activity, as measured by the ethoxysorufin O-deethylase assay, was detected in mitochondrial, nuclear, and microsomal fractions of ovarian cancer cells but was notably absent in all subcellular fractions of HOSE cells. Immunocytochemistry studies in 30 clinical specimens revealed overexpression of CYP1A1 in various types of ovarian cancers compared with benign epithelia and frequent localization of the enzyme to cancer cell nuclei. Forced expression of CYP1A1wt or CYP1A1v in HOSE cells resulted in nuclear localization of the enzyme and acquisition of anchorage-independent growth, which was further exacerbated following exposure to benzo[a]pyrene or 17β-estradiol. Collectively, these data provided the first evidence that CYP1A1 overexpression and alternative splicing could contribute to ovarian cancer initiation and progression. (Cancer Res 2005; 65(9): 3726-34)

Introduction

In the United States, ovarian cancer is the leading cause of death from gynecologic neoplasms (1). About 23,000 cases are diagnosed each year and 14,000 women die from the disease. The majority (∼90%) of ovarian cancers are epithelial cancers arising from the human ovarian surface epithelium (HOSE), which is a simple squamous-to-cuboidal mesothelium covering the ovary.

Epidemiologic studies suggest dietary/environmental factors, such as high levels of meat consumption (2), caffeine intake (3), and cigarette smoking (4), are risk factors for ovarian cancer. In these instances, cytochrome P450 (CYP) 1A1 may play a major role in the metabolic activation of procarcinogens to carcinogens. For example, it is known that polycyclic aromatic hydrocarbons (PAH), by-products of incomplete combustion and pyrolysis of fossil fuels and organic matters, such as tobacco leaf, are direct carcinogens of HOSE cells (5). Other environmental pollutants, such as the halogenated aryl hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a by-product of some herbicides, are potent inducers of the enzyme (6) and may therefore exacerbate the metabolic activation of other procarcinogens to carcinogens. In a rat model, chronic treatment with TCDD promoted development of ovarian tumors (7). An industrial accident in Seveso, Italy, in 1976 contaminated the area with TCDD and raised ovarian cancer incidence among female residents (8). Similarly, endogenous factors, particularly estrogens, such as 17β-estradiol (E2) and estrone (E1), could be converted into catechol estrogens and other genotoxic metabolites via the action of CYP1A1 as well as CYP1B1 (9, 10). These reactive metabolites are known carcinogens of mammary cells (10) and may also be involved in the neoplastic transformation of HOSE cells, which are exposed to high levels of follicular estrogens associated with ovulation (11, 12). In this regard, women who carry polymorphic variants that confer higher CYP1A1 enzyme activity, such as the Ile/Val or Val/Val polymorphism in exon 7 (13), are at higher risk for ovarian cancer (14, 15). Additionally, positive interactions have been shown between tobacco smoking and CYP1A1 (Msp1) polymorphism (16) as well as between high caffeine consumption and Ile/Val polymorphism (14) in ovarian cancer patients. Based on findings from these studies, we hypothesized that CYP1A1 plays a key role in mediating the carcinogenicity of specific endogenous and exogenous chemicals on the ovarian surface epithelium.

Alternative splicing is an important mechanism for generating proteomic complexity and functional diversity (17). Removal of intron sequences from pre-mRNA is carried out by a ribonucleoprotein complex called a spliceosome. Most splicing events occur where the intron starts with GT and ends with AG, the so-called GT-AG rule. However, completion of the Human Genome Project has revealed other noncanonical splice pairs, including AT-AC (18), along with novel spliceosomes (19). New categories of alternative splicing events, including insertion of cryptic exons or excision of cryptic intron, production of exon/intron isoforms, and intron retention, have also been characterized (20, 21). However, few of these new splicing mechanisms have been directly implicated in the pathogenesis of cancers.

In this study, we identified a novel CYP1A1 variant (CYP1A1v) that is formed via excision of an 84-bp cryptic intron within exon 2 of the gene. Thus, the goals of this study were (a) to compare...
the expression levels of CYP1A1wt and CYP1A1v in normal HOSE cell lines to those in ovarian cancer cell lines, (b) to investigate the expression pattern of CYP1A1 in 30 cases of clinical specimens, (c) to determine whether CYP1A1v is enzymatically active and compare its subcellular distribution with that of CYP1A1wt, and (d) to assess whether these isoforms possess transforming potentials when expressed stably in HOSE cell lines in the absence or presence of procarcinogens, such as benzo[a]pyrene (BaP, a prototypic PAH) and estrogen (E2, an endogenous estrogen). Our findings showed that both CYP1A1 isoforms are overexpressed in ovarian cancer cell lines and clinical specimens. Importantly, CYP1A1v was found to be enzymatically active and exclusively localized to the mitochondria and nucleus. Forced expression of either isoforms induced anchorage-independent growth in HOSE cells, especially in the presence of BaP and E2. Collectively, these data provided the first evidence that CYP1A1 overexpression and alternative splicing could contribute to ovarian cancer initiation and progression.

Materials and Methods

Cell cultures and cell lines. Four normal primary HOSE cell cultures (HOSE 27, 20, 17, and 13), four immortalized normal HOSE cell lines (HOSE 301, 306, 636, and 646; ref. 22), and four ovarian cancer cell lines (OVCA 420, 429, 432, and 433; ref. 23) were used in this study. These cells were cultured and maintained as reported previously (22–24).

Clinical specimens. Human surgical ovarian tissues were collected by Z.J. at the Department of Pathology, University of Massachusetts Medical School (Worcester, MA) through routine procedures for histologic diagnosis. A total of 28 clinical specimens, including 24 ovarian neoplasms (18 serous adenocarcinomas, 3 mucinous adenocarcinomas, 2 borderline malignant adenocarcinomas, 3 mucinous adenocarcinomas, 2 borderline malignant tumors, and 1 benign serous cystadenoma), 3 normal ovaries, and 3 cases of benign serous cystadenomas, 3 normal ovaries, and 1 pancreatitis metastasis to ovary, were included in this study. Paraffin-embedded tissue sections were cut at 5 μm and mounted on SuperFrost Plus slides (Erie Scientific, Portsmouth, NH).

Immunohistochemistry. Epitope retrieval of 5 μm paraffin-embedded tissue sections was achieved through the use of antigen unmasking solution (Vector Laboratories, Burlingame, CA). A polyclonal rabbit-anti-human CYP1A1 antibody (Human Biologics International, Scottsdale, AZ) with GAPDH (GAPDH-F 5'-GAACGTA-3' and GAPDH-R: 5'-TCTAGACGGCAGGT-3') was used in all amplification reactions. The PCR program for CYP1A1wt and CYP1A1v (CYP1A1-F 5'-AAGCACGGTGAGGAGCTGATG-3' and CYP1A1-R 5'-ACATGGCCTTCTATCCACCTGCT-3', CYP1A1v (CYP1A1v-F 5'-GCCTAGTCAACCTGTGGATTTGGA-3' and CYP1A1v-R 5'-CGAAGGAAAGTGTGGCAGA-3') and CYP1B1 (CYP1B1-F 5'-GGCTGATGGTTTGA-GAACGTA-3' and CYP1B1-R: 5'-TCATACCTCTGCTGGTACG-3') was 30 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, except that glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GAPDH-F: 5'-CCACCCATGGGAAATTCCTGGA-3' and GAPDH-R: 5'-TCTAGACGAGGCT-CAGGTCAC-3') cDNA was amplified at 26 cycles. Signal intensities of CYP1A1, CYP1A1v, and CYP1B1 amplimers were normalized to those of GAPDH to prevent arbitrary units of relative abundance.

Direct DNA sequencing analysis. Reverse transcription-PCR (RT-PCR) products corresponding to CYP1A1 transcripts (wild-type and variant) were amplified using CYP1A1 primer sets listed above. The DNA bands of interest were excised and purified and subjected to radiolabeled primer cycle sequencing using the ThermoSequenase cycle sequencing kit (Amersham Biosciences Corp, Piscataway, NJ). Two separate rounds of sequencing were done to ensure reproducibility.

Western blot analysis. Nuclear, mitochondrial, microsomal, and cytosolic fractions were isolated according to the method of Le Ferre et al. (26). Protein concentrations of each fraction were measured by Bradford assay kit (Bio-Rad, Hercules, CA). Protein (30 μg) was separated on 10% SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Methods and reagents used in Western blot analyses were described previously (27).

Immunofluorescence staining. All four OVCA cell lines were grown on coverslips to 80% confluence and then stained with 2 μmol/L MitoTracker Red CM-HXXROS (Molecular Probes, Eugene, OR) in culture medium for 30 minutes. Methods of fixation, permeabilization, and antibody staining were followed as reported previously (28) with following modifications. Goat polyclonal human CYP1A1 antibody (Gentest, Woburn, MA) and chick anti-goat antibody tagged with Alexa Fluor 488 (Molecular Probes) were diluted to 1:100 and 1:1,000, respectively. The control experiment used human CYP1A1 supersomes (Gentest) preabsorbed primary antibody.

Ethoxyresorufin O-deethylase activity assay. Ethoxyresorufin O-deethylase (EROD) activity in different subcellular fractions from various OVCA cell lines was assessed according to the method of Burke et al. (29). Reaction mixtures contained 25 μg protein and the NADPH regenerating system were employed. To determine maximal CYP1A1 activity, each sample was assayed concurrently with and without the addition of 1 unit CYP reductase (Sigma).

Generation of stable transfected human ovarian surface epithelium cell lines. Full-length CYP1A1wt and CYP1A1v cDNAs were amplified using Platinum Taq High Fidelity System (Invitrogen, Carlsbad, CA) and a specific pair of primers (forward primer tgtgatAGTTTTTTTTGAATC- CAT and reverse primer atttttgcccctTAAGAGGGGCGCTGCAT). To facilitate directional cloning of the PCR products, two restriction sites, Nhel1 and NotI, were engineered to the 5' end of forward and reverse primers, respectively (as shown in small letters). PCR reactions were initiated at 94°C for 3 minutes and followed by 40 cycles of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 68°C and continued to incubate at 68°C for 10 minutes at the end of cycle. PCR products were gel purified, digested, and cloned into pcDNA3.1 vector (Invitrogen) to generate pcDNA-1A1wt and pcDNA-1A1v vectors that express full-length transcripts of CYP1A1wt and CYP1A1v, respectively. Identity and reading frame of pcDNA-1A1wt and pcDNA-1A1v were confirmed by DNA sequencing (Macrogen, Inc., Seoul, Korea). Each of the immortalized HOSE cell lines (HOSE 301, 306, 636, and 642) was stably transfected with pcDNA-1A1wt and pcDNA-1A1v, separately, using LipofectAMINE Plus reagent (Invitrogen). Empty vector was also stably transfected into HOSE cell lines to serve as a control. Stable transfectants of HOSE cell lines carrying the empty vector, pcDNA-1A1wt, and pcDNA-1A1v were named as CTL, WT, and V, respectively. For each of the four HOSE lines, we generated their respective CTL, WT, and V derivatives.

Soft agar assay. Stable transfected HOSE cells were trypsinized, counted, and mixed with 0.4% Noble agar in complete medium with 5% charcoal-stripped serum (labeled as UT), charcoal-stripped medium with 1 μmol/L estrogen (labeled as E2), or charcoal-stripped medium with 1 μmol/L BaP at a concentration of 1.250 cells/250 μL and then transferred into a 0.8% Noble agar precoated 24-well plates. Plates were incubated in 2% medium for up to 4 weeks. The number of colonies formed in soft agar for each of the four HOSE cell lines transfected with the same vector was averaged and SDs were calculated.

In situ analyses. Extensive sequence analyses were carried out using nucleotide and protein BLAST programs provided by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). DNA sequences comparison was done using Clustalw from European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Possible signal sequences and the hydrophobic/hydrophobicity of CYP1A1 protein were analyzed using PSORTII (http://psort.lims.u-tokyo.ac.jp/form2.html) and SwissProt from Swiss Institute of Bioinformatics (http://us.expasy.org/).
Statistical analyses. One-way ANOVA was used to detect significant
differences among the various group means. A multiple range test using the
Tukey-B procedure was employed to compare the individual group means.
Significance was set at $P < 0.05$ or 0.01.

Results
Identification of a novel CYP1A1v in OVCA cells. In our initial
experiments, when RT-PCR analyses were conducted using a
primer set designed for CYP1A1, two PCR products of different
sizes were detected in cDNA samples prepared from OVCA cells
(Fig. 1A): a 387-bp product expected to be amplified from wild-type
CYP1A1 transcripts and a smaller (303 bp) product. To minimize the
chances of PCR artifacts, a more stringent annealing
temperature (65°C) was used for all subsequent PCRs. The
smaller-sized fragment was consistently amplified from all
reactions under these conditions. DNA from the smaller band
was isolated and sequenced, revealing that it could be derived from
transcripts of a splicing variant. When compared with the wild-type
transcript, the splicing variant has an 84-bp deletion in exon 2
(Fig. 2A). To our knowledge, its sequence had never been reported.
We therefore concluded that it is a novel splicing variant, which we
here called CYP1A1v. This sequence has since been deposited in
Genbank with the assigned accession no. AY310359. A new set of
specific primers designed to amplify CYP1A1v covering the variant-
specific exon junction was used for subsequent semiquantification
of the variant transcripts.

Overexpression of CYP1A1wt and the novel CYP1A1v in
OVCA cells compared with human ovarian surface epithelium
cells. Semiquantitative RT-PCR analyses showed that primary
cultures of normal HOSE cells and immortalized HOSE cell lines
expressed minimal levels of CYP1A1wt mRNA and CYP1A1v
mRNA (Fig. 1A and B). In contrast, the four OVCA cell lines
consistently showed overexpression of the two CYP1A1 transcripts
compared with levels found in HOSE cell cultures. About a 5-fold
increase in the CYP1A1wt mRNA and a 10-fold increase in
CYP1A1v mRNA were observed in the OVCA cell lines on average
(Fig. 1B). In contrast, there was no observable difference in
CYP1B1 mRNA expression levels among the various HOSE and
OVCA cell lines.

Overexpression of CYP1A1 protein and atypical nuclear
staining in human ovarian cancer specimens. A total of 27
clinical specimens, including 24 ovarian neoplasms (18 serous
adenocarcinomas, 3 mucinous adenocarcinomas, 2 borderline
malignant mucinous tumors, and 1 benign serous cystadenoma)
and 3 normal ovaries were immunostained with a polyclonal
rabbit anti-human CYP1A1 antibody to assess their expression of
CYP1A1 proteins (Fig. 3). Strong cytoplasmic (nonnuclear) staining
was restricted to the cancer cells of serous and mucinous
adenocarcinomas but was more widespread in stromal cells (Fig.
3A-C). Some cancer cells in these specimens showed both
cytoplasmic and nuclear staining (Fig. 3D). CYP1A1 protein was
also immunolocalized in the epithelial cells of borderline
mucinous lesions (Fig. 3A). The majority of serous and mucinous
carcinoma cells showed immunopositivity in both nuclei and
cytoplasmic staining (Table 1). Expression of cytoplasmic and
nuclear CYP1A1 seems to decline concomitantly in types of
carcinoma with lower malignancy. CYP1A1 immunonegativity was
detected in benign serous cystadenoma (Fig. 3D) and normal
human ovary. The one human tissue specimen of pancreatic
metastasis to ovary in this study showed no detectable CYP1A1
protein expression (data not shown).

Overexpression of CYP1A1wt protein and CYP1A1v protein
in ovarian cancer cell lines and exclusive localization of
CYP1A1v in cell nucleus and mitochondria. Western blot
analyses revealed that wild-type and variant CYP1A1 protein were
expressed at very low levels in HOSE cells but were abundant in
OVCA cell lines (Fig. 4A). Subcellular fractionation of OVCA cells
revealed that the wild-type and variant proteins are localized in
different cellular compartments. CYP1A1wt was principally associ-
ated with the microsomal fraction, whereas CYP1A1v resided
predominantly in the nuclear and mitochondrial compartments
of OVCA cells (Fig. 4A). The unique subcellular distribution of
CYP1A1v is specific, because no wild-type enzyme was colocalized
to these fractions. Interestingly, neither protein was detected at any
significant level in the cytosolic fraction. The unusual localization
of CYP1A1v in the nucleus and mitochondria was also confirmed by
immunofluorescence microscopy (Fig. 4B). The fluorescence signals
of CYP1A1v immunoreactivity (Fig. 4B, 2) completely coincided with
signals from nuclear (blue; Fig. 4B, 1) and mitochondrial (red;
Fig. 4B, 3) trackers in contrast to the results obtained with an

Figure 1. A, relative abundance of CYP1A1wt, CYP1A1v, CYP1B1, and
GAPDH transcripts in different normal, immunortalized normal, and ovarian
cancer cell lines. RT-PCRs were done on 1 μg RNA using primer sets for
corresponding genes. Representative fluorographs of the RT-PCR products.
Bottom bands appearing exclusively in cancer cells were purified and
sequenced. B, relative abundance of CYP1A1wt (white), CYP1A1v (gray), and
CYP1B1 (black) transcripts in HOSE and OVCA cells. Columns, mean relative
abundance of gene transcripts from all primary cultures of normal and
immortalized HOSE cells and those from the four OVCA cell lines (n = 12
(HOSE); n = 12 (immortalized HOSE); and n = 12 (OVCA)); bars, SD. One-way
ANOVA was applied to calculate the statistical significance of CYP1A1wt and
CYP1A1v mRNA expression among HOSE, immortalized HOSE, and OVCA
samples ($P < 0.01$).
Figure 2. Schematic diagram showing CYP1A1v protein and mRNA. A, location of 28–amino acid residues (deletion) and NLS. B, Clustalw alignment of CYP1A1v against CYP1A1wt. The deleted cDNA region (84 bp) is highlighted (bold) in the CYP1A1wt sequence. Right, cDNA positions of both wild-type and variant transcripts. C, eight possible modes of processing CYP1A1 pre-mRNA. Various donor and acceptor splice sites are highlighted.

antibody preparation preabsorbed with the antigen (Fig. 4B, 6). The low levels of CYP1A1 signal observed in the nonmitochondrial compartment of the cytoplasm (Fig. 4B, 4 and 8-10) could be due to association of CYP1A1wt with the microsomes.

Computer-aided analysis of the CYP1A1v sequence. Analysis of the full-length sequence revealed that the 84-bp deletion is in-frame and would not cause premature termination (Fig. 2B), implying that the variant protein will have 28 fewer amino acid residues (amino acids 224 and 228 in helix F were deleted from the variant; C, hotspot [amino acid residue 462] of CYP1A1 polymorphism. B, Clustalw alignment of CYP1A1v against CYP1A1wt. The deleted cDNA region (84 bp) is highlighted (bold) in the CYP1A1wt sequence. Right, cDNA positions of both wild-type and variant transcripts. C, eight possible modes of processing CYP1A1 pre-mRNA. Various donor and acceptor splice sites are highlighted.

By comparing the sequence of CYP1A1v mRNA to that of the wild-type transcript, eight possible splice junction pairs were recognized (i.e., the dinucleotides upstream and downstream of the splice site could be CC-TC, CT-CC, TG-CC, GA-CT, AA-TG, AT-GA, TA-AA, or AA-AT; Fig. 2C). However, none of these obeyed the GT-AG rule (30), suggesting that a non-canonical splice site is responsible for alternative splicing of the CYP1A1 pre-mRNA. Moreover, among the eight possible sites, because only one splice junction pair, AA-AT, has been suggested to exist by statistical analysis of all annotated genes in the mammalian genome (18), it may be the pair involved in splicing of this variant.

To investigate why only the CYP1A1v (not the wild-type protein) is targeted to the nucleus and mitochondria, an extensive in silico search of possible signaling peptides was done. A putative bipartite basic nuclear localization signal (NLS) was identified in the CYP1A1 amino acid sequence from residues 341 to 357 (RKIQEELDT-VIGRSRRP; Fig. 2A), which matches the consensus motif (K/R)2X10-12(K/R)3 of nucleoplasm (31). However, these searches failed to identify a mitochondrial matrix-targeting signal, most commonly composed of positive charge and hydroxylated residues (32), in the CYP1A1v. Of interest, hydrophobicity analysis revealed a decrease in the overall hydrophobicity in the NH2-terminal region of CYP1A1, most markedly around amino acids 230 to 260, which encompasses the deleted region (33). Thus, perturbation in this general area may result in the formation of a functional mitochondrial targeting signal.

According to a recent molecular modeling study of human CYP1A1 (34), both resorufins and BaP are primarily stabilized by hydrophobic interactions in the substrate binding pocket. Within this substrate binding pocket, two key amino acid residues in helix F responsible for substrate stabilization (amino acids 224 and 228; Fig. 2A) were found to be deleted from the CYP1A1v. Loss of these amino acids may result in alterations in substrate specificity and/or binding affinity in the variant protein. CYP1A1v in both nucleus and mitochondria is active in ethoxyresorufin O-deethylase assay. To determine whether the mitochondria- and nucleus-localized CYP1A1 protein, presumably derived from the CYP1A1v, is catalytically active, the EROD assay was done after subcellular fraction of ovarian cancer cell lines (OVCA 429, 432, and 433) in the presence and absence of exogenous CYP oxidoreductase. The enzyme activity found in subcellular fractions of each cell lines were determined and averaged (Fig. 5). In the absence of exogenous CYP oxidoreductase, the highest EROD activity was detected in the nuclear fraction followed by the mitochondrial and microsomal fractions, whereas the cytosolic fraction was devoid of EROD activity. In the presence of extra CYP oxidoreductase, significantly higher levels (3- to 4-fold increases) of EROD activities were found in all fractions. The greatest increase was observed in the microsomal fraction followed by the mitochondrial and nuclear fractions. Under this condition, a low level of cytosolic EROD activity was also detected. We noticed that NAPDH oxidoreductase plays a greater role in enhancing the enzyme activity of CYP1A1wt and CYP1A1v in ovarian cancer cell lines. It seems that the activities of both CYP1A1wt and CYP1A1v are limited by low levels of oxidoreductase activity in ovarian cancer cells when compared with TCDD-stimulated MCF-7 cells (data not shown). Because we have shown exclusive localization of the CYP1A1v in the mitochondria and nucleus by Western blotting, together with previously reported association of CYP1A1wt with the microsomes (35), the EROD data now strongly support the notion that the CYP1A1v is catalytically as active as CYP1A1wt.

CYP1A1wt and CYP1A1v are both involved in benzo(a)pyrene- or 17β-estradiol-induced cellular transformation. This experiment sought to determine (a) whether overexpression of CYP1A1wt and CYP1A1v in HOSE cells also results in their protein products localized to different cellular compartment, (b)
whether overexpression of CYP1A1wt or CYP1A1v induces neoplastic transformation per se in HOSE cells, and (c) whether overexpression of the enzyme mediates cellular transformation caused by an endogenous/exogenous procarcinogen. The soft agar colony formation assay was used to measure acquisition of anchorage-independent growth potential in the four HOSE cell lines (HOSE 301, 306, 636, and 646), each stably transfected with empty pcDNA3.1 (HOSE-CTL), pcDNA-1A1wt (HOSE-WT), or pcDNA-1A1v (HOSE-V). BaP, a major PAH produced during cigarette smoking (36), was used as a prototype exogenous environmental procarcinogen. E2, the major circulating and follicular estrogen, was chosen for our study because ovarian surface epithelial (OSE) cells are often exposed to high levels of this steroid around ovulation or when trapped in the stroma as inclusion cysts (11). Both BaP and E2 are the known substrates for CYP1A1, and their metabolites are carcinogenic (9, 10, 37).

Table 1. Expression of CYP1A1 in ovarian carcinoma

<table>
<thead>
<tr>
<th>Type of tumors</th>
<th>No. cases</th>
<th>Cytoplasmic staining</th>
<th>Nuclear staining</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;75%</td>
<td>30-75%</td>
</tr>
<tr>
<td>Serous cancer</td>
<td>20</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Mucinous cancer</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Serous, low malignant potential</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>(of borderline malignancy)</td>
<td></td>
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<tr>
<td>Mucinous, low malignant potential</td>
<td>2</td>
<td>0</td>
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<tr>
<td>(of borderline malignancy)</td>
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<td></td>
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</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>17 (57%)</td>
<td>13 (43%)</td>
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*Nonnuclear region includes mitochondria, microsomes, and cytosol.

All four HOSE-CTL cell lines expressed no detectable CYP1A1wt or CYP1A1v mRNA as shown by semiquantification of the transcripts by RT-PCR (Fig. 6A). As expected, HOSE-V cell lines expressed high levels of CYP1A1v transcript and no detectable CYP1A1wt mRNA (Fig. 6A, top). Surprisingly, HOSE-WT cell lines, in addition to high levels of CYP1A1wt mRNA, also expressed detectable levels of CYP1A1v transcripts, likely products of alternative splicing of the wild-type transcript (Fig. 6A, middle). Exposure of HOSE cell lines (CTL, WT, or V) to BaP and E2 did not alter expression levels of CYP1A1wt, CYP1A1v, or CYP1B1.

Two approaches were used to determine whether HOSE-WT and HOSE-V cell lines overexpressed the CYP1A1s that exhibited extra-endoplasmic reticulum (ER) localization. EROD assays were done on subcellular fractions of three types of stable transfected HOSE cell lines (HOSE-CTL, HOSE-WT and HOSE-V) and revealed the following: (a) the enzyme activities of CYP1A1 were elevated in...
both HOSE-WT and HOSE-V cell lines when compared with HOSE-CTL cells, (b) the enzyme activities were predominantly detected in the nuclear fractions (Fig. 6B), and (c) little to no enzymatic activities were present in the mitochondrial fraction. Results from immunofluorescence assays (data not shown) confirmed this observation. Of interest to note, the quantity of mitochondria in those HOSE cell lines was extremely low when compared with primary cultures of normal HOSE cells and four immortalized HOSE cell lines, which express little or no CYP1A1 transcripts. Immunocytochemistry revealed overexpression of the enzyme in various types of ovarian cancers with both cytosolic and nuclear localization. Comparable levels of CYP1A1 activity, as measured by the EROD assay, were found in nuclear, mitochondrial, and microsomal fractions of OVCA cells but not in HOSE cells. Enzyme activity in all three compartments depended on NADPH regeneration. Further investigation showed acquisition of anchorage-independent growth in HOSE cells engineered to stably overexpress exogenous CYP1A1wt or CYP1A1v. On exposure of these cells to BaP or E2 (especially to the steroid, E2), further enhancement of anchorage-independent growth was observed. Taken together, these findings provide strong support for an essential role of CYP1A1 in neoplastic transformation of HOSE cells likely via metabolic activation of exogenous/endogenous procarcinogens and utilization of a unique alternative splicing mechanism to broaden the distribution of the enzyme to critical organelles. The overall hypothesis of this study has been summarized in Fig. 7.

**Discussion**

In this study, we identified a novel CYP1A1v produced by a splicing event that involves an excision of an 84-bp cryptic intron. CYP1A1v, which is enzymatically active and is uniquely and exclusively located in the nucleus and the mitochondria instead of in the ER, where the wild-type enzyme resides. Importantly, when HOSE cells were engineered to overexpress CYP1A1wt, this alternative splicing mechanism generated CYP1A1v from the mature wild-type transcript. Hence, an overall increase in CYP1A1 gene expression would give rise to both transcripts and proteins and extend cellular localization of the enzyme to new cellular compartments. Of significance, we detected higher levels of CYP1A1wt and CYP1A1v mRNA in four ovarian cancer cell lines when compared with primary cultures of normal HOSE cells and four immortalized HOSE cell lines, which express little or no CYP1A1 transcripts. Immunocytochemistry revealed overexpression of the enzyme in various types of ovarian cancers with both cytosolic and nuclear localization. Comparable levels of CYP1A1 activity, as measured by the EROD assay, were found in nuclear, mitochondrial, and microsomal fractions of OVCA cells but not in HOSE cells. Enzyme activity in all three compartments depended on NADPH regeneration. Further investigation showed acquisition of anchorage-independent growth in HOSE cells engineered to stably overexpress exogenous CYP1A1wt or CYP1A1v. On exposure of these cells to BaP or E2 (especially to the steroid, E2), further enhancement of anchorage-independent growth was observed. Taken together, these findings provide strong support for an essential role of CYP1A1 in neoplastic transformation of HOSE cells likely via metabolic activation of exogenous/endogenous procarcinogens and utilization of a unique alternative splicing mechanism to broaden the distribution of the enzyme to critical organelles. The overall hypothesis of this study has been summarized in Fig. 7.
Splicing events have been characterized and divided into four different categories (20, 21): insertion of cryptic exons or excision of cryptic introns, production of exon/intron isoforms, and intron retention. In our study, an 84-bp cryptic intron is spliced from exon 2 of CYP1A1wt, possibly via an AA-AT splice junction pair, as suggested by our in silico analysis. This is a noncanonical splice pair, because >96% of pre-mRNA splicing use the conservative GT-AG pair (18). The involvement of noncanonical splice junction pairs may be due to poorly conserved 5’ and 3’ splice sites and/or many cryptic splice sites (38). Recent findings suggest that introns flanked by another noncanonical AT-AC pair are excised by a spliceosome composed of nonclassic types of small nuclear ribonucleoproteins (19). Interestingly, when HOSE cells are stably transfected with a CYP1A1wt expression vector, we found that CYP1A1v coexisted with the wild-type transcript. This finding indicates that the mature transcripts of CYP1A1wt are susceptible to this splicing event. Thus, induction of CYP1A1wt overexpression in normal OSE cells by xenobiotic/endobiotics or physiologic stresses could yield both transcripts along with their protein products and might extend the coverage of enzyme to new cellular organelles, where they may contribute to an imbalance in activation/detoxification of procarcinogens. This splicing event may exist in tissues other than the OSE because we also observed expression of CYP1A1v mRNA in breast and prostate cancer cells.5

Although a few early reports detected CYP1A1-like enzyme activity in cell nucleus (39) and mitochondria (40) in rodent models, the enzyme identities remain unclear. CYP1A1v is the first human CYP1A1v to be identified and characterized at both molecular and biological levels. Thus, alternative splicing of CYP1A1 could be considered a mechanism for targeting the enzyme to extra-ER locales. Computational analyses identified a putative bipartite basic NLS, which is masked in the CYP1A1wt. One can speculate that removal of the cryptic intron results in the activation of this NLS and nuclear targeting of CYP1A1v. However, we cannot yet explain the acquisition of the mitochondrial matrix targeting signal via deletion of the 84-bp region, except to hypothesize that the deletion induces an increase in hydrophobicity in the NH2 terminus of the molecule and creates a mitochondrial targeting signal (32) for the variant enzyme. Most of the CYP enzymes residing in the ER have been shown to depend on NADPH-oxidoreductase for electron transfer (41), and their activities are enhanced in the presence of an NADPH regenerating system. Here, we found that the EROD activity in the mitochondrial, nuclear, and microsomal fractions of ovarian cancer cells were all elevated in the presence of exogenous NADPH-oxidoreductase, suggesting that comparable cofactor requirements exist between the wild-type and the variant enzyme.

Our results strongly support a role for aberrant expression of CYP1A1 in ovarian carcinogenesis. Exposure of HOSE cells to BaP or E2 per se did not induce increased expression of the enzyme. In the absence of CYP1A1 expression, these two procarcinogens were ineffective in inducing an anchorage-independent growth phenotype in these cells. In marked contrast, overexpression of CYP1A1wt or CYP1A1v in HOSE cell lines significantly increased anchorage-independent growth potentials of HOSE cells even in the absence of BaP or E2. Elevated levels of CYP1A1wt and/or CYP1A1v expression probably had caused neoplastic transformation via activation of procarcinogens existing in the culture medium per se. Exposing the

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5 Unpublished data.
Figure 7. Schematic diagram summarizing the hypothesis of this study. In normal HOSE cells, the expression level of CYP1A1wt (diamond) is low or undetectable. On exogenous (environmental factors) or endogenous (hormone factors) stimuli, CYP1A1wt can be significantly induced in HOSE cells. Some of the CYP1A1wt transcripts can be partially converted into CYP1A1v (broken diamond) by alternative splicing via cryptic intron excision. The protein products of CYP1A1v mRNAs are targeted to nucleus and mitochondria instead of microsomes (ER) where CYP1A1wt resides. Both CYP1A1wt and CYP1A1v can metabolize various kinds of procarcinogens into highly reactive carcinogens, which in turn attack protein and DNA within their compartments. As a result of DNA adduct formation, DNA mutations accumulate and eventually impair genome stability. HOSE can be finally transformed with more cancer phenotypes. With the perpetual stimulation of activated carcinogens, transformed cells may progress to more advanced stage.

stable transfectants to BaP or E2 increased soft agar colony formation in both HOSE-WT and HOSE-V cell lines. Of interest is the marked elevation of soft agar colony formation in HOSE-WT cell lines compared with HOSE-V cell lines. In contrast, BaP elicited roughly the same response in both types of stable transfectants. Because the overexpression of CYP1A1 in HOSE-WT and HOSE-V cell lines were localized primarily in nuclear compartment possibly due to low abundance of mitochondria in these cell lines, we suggest that nuclear localization of CYP1A1 is sufficient to mediate BaP-induced neoplastic transformation, whereas the E2-induced transformation may involve the presence of the enzyme in multiple subcellular organelles. Indeed, BaP carcinogenicity is believed to be mediated by the translocation of the procarcinogen to the cell nucleus followed by formation of the mutagenic metabolite BaP-7,8-diol 9,10-epoxide and DNA adducts, which in turn initiate mutagenesis (42, 43).

On the contrary to BaP, estrogen carcinogenicity is much more complex and involves multiple phase I and II enzymes with different subcellular locales. Estrogens are converted to catechol-estrogens (2-OH and 4-OH estrogens) and estrogen quinones, which are genotoxic, via CYP1A1 and CYP1B1 metabolic activation (9, 10). Catechol-O-methyltransferase catalyzes the methylation of catechol estrogens to methoxyestrogens, which may reduce the potential for genomic damage and suppress cell proliferation via enhancement of 2-MeOE2 formation (44, 45). Aberrant or altered expression of these estrogen metabolic enzymes is implicated in the initiation of human breast and endometrial tumors (9, 10, 46, 47). Analogous information has been scarce for ovarian carcinogenesis. The human ovarian epithelium is routinely exposed to high levels of estrogens such as those produced during the follicular phase of each female cycle. Alternatively, the ovarian epithelium is exposed to abnormal levels of stromal estrogens with the formation of inclusion cysts, which in fact have been proposed as the origin of epithelial ovarian cancer (48). In general, CYP1A1 produces mostly 2-OH estrogens, but a significant amount of 4-OH estrogens is generated simultaneously (49). It has been shown that 4-OH estrogens are more carcinogenic than 2-OH estrogens in a mammary epithelial cell model (10). Whether a similar scenario exists for OSE remains to be investigated in future experiment.

In summary, we here provide the first evidence that CYP1A1 is overexpressed in ovarian cancer cell lines and ovarian cancers. We also report the identification of a novel variant of CYP1A1 that is enzymatically active and resides exclusively in cell nucleus and mitochondria. Our data suggest that a noncanonical splice group is responsible for the removal of a cryptic intron from the CYP1A1 transcript. These findings raise the possibility that alternative splicing underlies increased functional diversity of CYP1A1 by increasing its subcellular coverage. They also suggest that chronic or repeated induction of CYP1A1 expression in HOSE cells would result in expression of the enzyme in multiple cellular organelles, leading to an imbalanced detoxification-activation homeostasis of xenobiots/endobiots, a mechanism long suspected to induce neoplastic transformation.

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Overexpression of *Cytochrome P450 1A1* and Its Novel Spliced Variant in Ovarian Cancer Cells: Alternative Subcellular Enzyme Compartmentation May Contribute to Carcinogenesis


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