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Initiation of Breast Cancer by Endogenous Estrogens: Mechanism and Prevention

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Both endogenous and environmental factors are thought to contribute to cancer of the breast. Among these, estrogens have attracted intense interest for decades, but the mechanisms by which this class of hormone mediate normal and neoplastic growth have remained obscure. We provide in vitro data to support the hypothesis that 16α-hydroxyestrone (16α-OHE₁) is unique among estrogens with regard to its transforming properties in mammary cells. This suggests that the pathway leading to the formation of 16α-OHE₁ may be a determinant of mammary carcinogenesis, either because of genetic variation at this locus or because the locus is differentially regulated in healthy women and women with breast cancer.

We have identified Cyp2d-13 as a putative mediator of this pathway in mice. This represents a crucial advance in our efforts to adjudicate the hypothesis that estrogen metabolism is a critical factor in breast cancer. Expression of Cyp2d-13 is remarkably associated with the tumor phenotype in the mouse mammary gland. With regard to the human CYP2D6 locus, it is very intriguing that such a polymorphic locus might be linked to breast cancer. Ultimately, CYP2D6 genotyping might serve to identify those individuals who might most benefit from dietary or pharmacological strategies which induce the competing, 2-hydroxylase pathway.
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INTRODUCTION

Purpose of the research
Both endogenous and environmental factors are thought to contribute to cancer of the breast. Among these, estrogens have attracted intense interest for decades, but the mechanisms by which this class of hormone mediate normal and neoplastic growth have remained obscure. Epidemiological and biochemical data indicate that 16α-hydroxyestrone (16α-OHE1) is unique among estrogens with regard to its transforming properties in mammary cell lines and its association with breast cancer in women. This suggests that the pathway leading to the formation of 16α-OHE1 may be a determinant of mammary carcinogenesis, either because of genetic variation at this locus or because the locus is differentially regulated in healthy women and women with breast cancer. The purpose of our work has been to employ cellular and molecular approaches which elucidate the mechanisms by which 16α-OHE1 exerts its unique effects on estrogen receptor function. However, because the proximate cause of increased 16α-OHE1 in individuals is most likely due to increased levels of estrogen 16α-hydroxylase activity, we felt it very prudent to expand our approach. Thus, we have also sought to examine the role of estrogen metabolism in breast cancer by characterizing genes which 16α-hydroxylate estrogens.

Background

Estrogen metabolism and breast cancer
Estrogens are metabolized in a complex fashion (fig. 1), with different pathways having markedly divergent effects on the proliferative state of mammary epithelial cells (1). The clinical significance of these distinct metabolic pathways is suggested by the observation that a relative increase in the estrogen metabolite 16α-hydroxyestrone (16α-OHE1) is unique among estrogens in its association with breast cancer in women. Estrogen 16α-hydroxylase activity is selectively elevated both in post-menopausal women with breast cancer (2) and in women at risk for breast cancer (2) whereas the competing 2-hydroxylation and 17-ol oxidation pathways are not. Moreover, the degree of 16α-hydroxylation is influenced by certain dietary and environmental components, which correspondingly alter the relative risk for breast cancer in parallel fashion. Estrogen 16α-hydroxylase activity has also been found to be elevated in strains of mice with increased susceptibility to breast cancer (3).

These epidemiological observations have prompted a closer analysis of estrogen metabolism within the context of mammary carcinogenesis. Remarkably, these studies have consistently established a positive relationship between breast cancer and elevated 16α-hydroxylation in mammary ductal epithelium. For example, mammary explants from breast cancer-prone strains of mice are about 5 times more active than explants from low risk strains at 16α-hydroxylating estrogens (4). In liver cultures,
however, no such difference is seen. In nontumorous tissue from breast cancer patients, the increased activity has been specifically detected in the terminal duct lobular units (5), the presumed origin of ductal carcinoma which represents about 80% of all human breast cancer.

Cell culture models provide additional evidence to suggest a unique role for paracrine- or autocrine-derived 16α-OHE₁ in breast cancer. Cellular 16α-hydroxylase activity closely parallels the tumorigenic potential of mammary cell lines in nude mice (6). By the same token, exogenously added 16α-OHE₁ routinely stimulates cellular and molecular changes characteristic of transformation. For example, while 16α-OHE₁ and other estrogens demonstrate a comparable ability to accelerate the proliferation of mammary cells over time, only 16α-OHE₁-treated cells are able to maintain this growth rate upon withdrawal of the hormone (7). In addition, 16α-OHE₁ dramatically induces anchorage-independent growth and DNA repair in C57/MG mouse mammary epithelial cells (7, 8). The induction is comparable to that obtained with the mammary carcinogen DMBA, while estradiol and estriol are without effect. Several molecular changes occur in parallel with these events in 16α-OHE₁-treated cells: the formation of estrogen receptor and progesterone receptor is markedly diminished, while the levels of myc, fos, jun and neu are elevated (7) (our unpublished observations).

The affinity of 16α-OHE₁ for the estrogen receptor is relatively low when compared with estradiol. How, then, might this hormone achieve such a profound impact on the mammary epithelium? One clue may be that, among estrogens, 16α-OHE₁ has the unique ability to form covalent adducts with primary amines. This occurs nonenzymatically through a Schiff base formation and subsequent Heyns rearrangement (9). Such a mechanism could account for the observed propensity of 16α-OHE₁ to form DNA adducts (10) and irreversibly bind complexes with the estrogen receptor (11). Both of these events represent possible, yet unproven, explanations the unique growth-promoting effects of 16α-OHE₁. Given these properties, the formation of 16α-OHE₁ and its possible relationship to breast cancer is very reminiscent of the metabolism of xenobiotics to proximate carcinogens by certain CYP species.

**P450 enzymes in steroid metabolism**

Estrogen 16α-hydroxylase activity in mice and humans demonstrates apparently genetic and tissue-specific properties which could be very efficiently analyzed by a molecular biological approach. While the gene(s) responsible for this activity have remained uncharacterized, the very nature of this hydroxylation reaction implies that it is mediated by a cytochrome P450 species.

The superfamily of cytochrome P450 (CYP) genes encodes a vast number of structurally similar, broad-specificity enzymes that metabolize drugs, plant metabolites, environmental pollutants and endogenous steroids to forms which can then be further modified and excreted (12). Additionally, several P450 family members mediate highly specific reactions that participate in steroid hormone synthesis. The superfamily of CYP genes
is so ancient that it predates drugs and plant-animal interactions, implying that these so-called "drug-metabolizing" enzymes have an evolutionarily conserved role in regulating the levels of endogenous molecules that control basic homeostatic functions of the organism (13). More than 200 CYP genes have been isolated from 42 species (12). On the basis of sequence similarity and genomic structure, these have been divided into 36 families, 12 of which exist in all mammals examined to date. Some families have been further subdivided into subfamilies, which typically correspond to clusters of tightly linked genes. For example, the CYP2 family has been subdivided into CYP2A, CYP2B, CYP2C, CYP2D, etc. By convention, genes encoding human and rat P450 enzymes are prefaced in capital letters (CYP) while the P450-encoding genes in mice are indicated by an italicized prefix (Cyp).

Over the last decade, there has been a vast effort to characterize the catalytic functions of individual P450 enzymes, using either biochemically purified preparations or heterologous expression systems with cloned cDNAs. With regard to metabolism of endogenous steroids, the primary (if not sole) P450 families mediating these activities appear to be CYP 1, 2 and 3. Leaving aside the considerable xenobiotic-metabolizing functions of these families for the moment, the CYP 1 family members identified to date exclusively hydroxylate estrogens. CYP1A1 and CYP1A2 contain significant 2-hydroxylase activity (14-17), while CYP1B1 appears to be a major 4-hydroxylase (18). At the other end of the spectrum, CYP3 genes encode P450 enzymes with remarkably diverse steroid-metabolizing abilities. For example, human CYP3A4, which is the predominant hepatic P450 under normal circumstances, is an efficient metabolizer of estradiol, progesterone, testosterone, DHEA-sulfate and cortisol (19). CYP2 enzymes comprise an intermediate functional state; the family as a whole metabolizes most steroids, but some of the individual family members are relatively specific in their activity. For example, Cyp2d-9 exhibits a quite specific testosterone 16α-hydroxylase activity (20). Remarkably, none of the CYP genes cloned to date has been shown to encode an estrogen 16α-hydroxylase activity, although a partially purified CYP2D from rat liver can mediate this reaction (21).

We have utilized a PCR-based approach to search for CYP genes which mediate the 16α-hydroxylation of estrogens. Our evidence, described in detail in "The Body of Complied Research" below, provides strong evidence in support of the hypothesis that a novel, mammary tumor-associated P450, termed Cyp2d-13, may mediate this activity. Conclusive evidence of this hypothesis will ultimately permit us to test directly whether aberrant expression of this gene leads to mammary carcinogenesis.
BODY OF COMPLETED RESEARCH

This section has been divided into 7 parts. The first four of these summarize our continued efforts pertaining to the biochemical and cellular aspects of estrogen 16α-hydroxylation and its relationship to tumorigenesis. The last three parts describe our isolation and characterization of Cyp2d-13, along with its relationship to estrogen 16α-hydroxylation and the tumor phenotype.

**Altered E₂ metabolism in myc-transformed mouse mammary epithelial cells**

Our previous studies on immortalized, tumorigenic murine mammary epithelial cell lines have demonstrated that exposure to chemical carcinogens or transfection with oncogenes results in induction of hyperproliferation as evidenced by anchorage-independent growth (AIG) in *vitro* prior to mammary fat pad or subcutaneous transplantation (22). The experiment shown in table AT-1 was performed to examine whether the relative extent of AIG corresponds with the degree of transformation in the parental MMEC, oncogene-initiated MMEC/myc3 and oncogene-transformed MMEC/myc3-Pr1 cells. The AIG assay revealed a progressive increase in anchorage-independent colonies formed by MMEC/myc3 and MMEC/myc3-Pr1 cells and a positive correlation with tumorigenicity *in vivo*. The parental MMEC cells, however, were negative in the AIG and tumorigenicity assays. These results suggest that AIG may represent a valid cellular marker for preneoplastic and neoplastic transformation of mammary epithelial cells.

The experiment presented in table AT-2 was conducted to examine whether c-myc oncogene-induced tumorigenic transformation influences the cellular metabolism of E₂ and steroid receptor status. The relative extent of E₂ metabolism was determined by the radiometric assay measuring C2-hydroxylation and C16α-hydroxylation of E₂. The data is expressed as the ratio of C2/C16α-hydroxylation. The relative extent of estrogen receptor (ER) and progesterone receptor (PR) proteins was determined using the dextran-coated charcoal (DCC) assay. The C2/C16α ratio exhibited a progressive decrease in MMEC/myc3 and MMEC/myc3-Pr1 cells relative to that observed in MMEC cells. The observed decrease in the ratio was due to an increase in C16α-hydroxylation and concomitant decrease in C2-hydroxylation (data not shown). Consistent with the alteration in cellular metabolism of E₂, ER protein levels also decreased in MMEC/myc3 and MMEC/myc3-Pr1 cells relative to those in MMEC cells. By contrast, PR levels increased in MMEC/myc3 cells but not in MMEC/myc3-Pr1 cells. These results suggest that overexpression of c-myc may induce aberrant hyperproliferation in part by downregulating hormone responsiveness in mammary epithelial cells.
Estrogen responsiveness of myc-transformed mammary epithelial cells

The conventional determinants for estrogen response include: E$_2$-mediated initiation of replicative DNA synthesis, expression of early response genes, ER and PR upregulation, peptide growth factor expression and reversible inhibition of growth by antiestrogens. In addition, oxygenated metabolites of E$_2$ generated via P450 hydroxylases have been shown to exert direct effects on hormone responsive target tissue (7, 8). Our earlier observation that constitutive overexpression of c-myc confers aberrant hyperproliferation and partial abrogation of serum-derived growth factor requirement prompted us to examine the extent of E2 metabolism and degree of E$_2$ responsiveness in MMEC/myc3-Pr1 cells.

As summarized in table AT-3, MMEC/myc3-Pr1 cells were incubated with 10 nM E$_2$ for 96 hours and the culture medium was used for product isolation and identification by gas chromatography and mass spectrometry (GC/MS). These data clearly demonstrate that the cells were capable of converting E$_2$ into estrone (E$_1$), 16α-OHE$_1$, 2-OHE$_1$, and estriol. These results, when combined with data obtained by radiometric analysis, strongly support the contention that mammary epithelial cells in culture retain the ability to metabolize E$_2$.

The persistence of E$_2$ responsiveness in MMEC/myc3-Pr1 cells was examined by determining whether E$_2$ can upregulate growth and increase ER content in these cells. Table AT-4 indicates that a continuos 5-day exposure to E$_2$ resulted in a dose-dependent increase in cell number and levels of ER protein. These effects occurred at physiological levels (1-10 nM) of hormone.

Modulation of E$_2$ metabolism and proliferation in MCF-7 cells

Our earlier studies on the murine mammary epithelial cell culture systems have shown that prototypic initiators of tumorigenesis (chemical carcinogens and oncogenes) results in decreased ratio of C2/C16α-hydroxylation of E$_2$ and increased aberrant hyperproliferation (8). These biochemical and cellular perturbations are reversed by the presence of known inhibitors of mammary carcinogenesis (23). The experiment shown in table AT-5 was designed to validate E$_2$ metabolism and hyperproliferation as markers for human mammary carcinogenesis. The extent of alteration in 2-OHE$_1$ formation as an endpoint for E$_2$ metabolism, and in AIG as an endpoint for aberrant hyperproliferation was measured in MCF 7 cells treated with the tumor inhibitors indole-3-carbinol (I3C), tamoxifen (TAM) and 4-hydroxytamoxifen (4-OHTAM).

Treatment with either I3C or 4OHTAM stimulated the formation of 2-OHE$_1$, inhibiting the extent of AIG. Since direct addition of 2-OHE$_1$ also inhibited AIG, this suggests that 4-OHTAM may act in part by inducing 2-OHE$_1$. TAM also inhibited AIG (to a lesser extent than 4-OHTAM), but had no effect on 2-hydroxylation. We therefore propose that the tamoxifen metabolite 4-OHTAM functions both by acting as a receptor antagonist and by altering estrogen metabolism, and that both mechanisms are required for its full antiproliferative effect.
In vitro models examining the role of estrogens in mammary carcinogenesis

In vitro models derived from mammary explant and cell culture systems provide an innovative approach to examining the effects of exogenous agents on the process of mammary carcinogenesis and its modulation directly at the level of the target tissue (22,23). Experiments conducted on murine mammary explant culture systems have shown that treatment of the non-involved mammary tissue to chemical carcinogens results in increased carcinogen-DNA binding, enhanced C16α-hydroxylation of E2 and high incidence of mammary hyperplasia in vitro, prior to tumorigenicity in vivo. Independent studies using the epithelial cell culture model have also shown that treatment with carcinogens or transfection with oncogenes results in upregulation of some biochemical and cellular endpoints (8). Thus, both of these in vitro models provide strong evidence that perturbed biochemical and cellular events occurring prior to tumor formation represent reasonable markers for preneoplastic transformation.

We therefore used murine and human explant cultures to examine the relationship between estrogen 16α-hydroxylation and risk of breast cancer (Table AT-6). Remarkably, E2 C16α-hydroxylation was 3- to 4-fold higher in explants from the high risk groups when compared to the corresponding low risk groups. In addition, explants from the high-risk groups exhibited a greater response to carcinogen-induced perturbation of E2 C16α-hydroxylation (data not shown). These data indicate that estrogen 16α-hydroxylation represents a biochemical marker for endocrine responsiveness whose alteration may predict the susceptibility of the target tissue to carcinogenesis.

We next examined whether C16α-hydroxylated metabolites of E2 may act as initiators of mammary carcinogenesis (Table AT-7). Various metabolites were tested for their ability to influence unscheduled DNA synthesis (a marker for genotoxicity) and anchorage-independent growth (a marker for aberrant proliferation). Among the metabolites tested, 16α-OHE1 exhibited a clearly superior ability to induce these markers. Indeed, the effects of 16α-OHE1 rivaled those of the classical mammary carcinogen DMBA.

Identification of a potential estrogen 16α-hydroxylase selectively elevated in mammary tumors

The conserved nature of selected regions in CYP families permitted the use of a PCR-based approach to search for CYP genes encoding enzymes with estrogen 16α-hydroxylase activity. We used degenerate primers specific for CYP families 1, 2, or 3 because most steroid-metabolizing P450 enzymes fall within one of these families (see above). All primer sets were designed to span at least one intron in order to avoid potential confusion from contaminating genomic DNA. As template for PCR, we used cDNA
derived from mouse T<sub>1</sub>Pr<sub>1</sub> and human MCF-10F cells, which contain high levels of estrogen 16α-hydroxylase activity (unpublished observations).

Two sets of degenerate primers (specific for families 1 or 2) successfully amplified fragments of the correct size, using T<sub>1</sub>Pr<sub>1</sub> cDNA as template. Because these fragments may be heterogeneous, we subcloned them into the vector pGEM-T (Promega) and sequenced numerous clones. For family 1, all clones analyzed thus far correspond to CYP1A2, which is a stronger estrogen 2-hydroxylase than CYP1A1. This is somewhat surprising, because previous reports had indicated that CYP1A1, but not CYP1A2, is expressed in human MCF-7 cells (24). Our primers amplify CYP1A1, CYP1A2 and CYP1B1 with similar efficiency; hence we currently have no explanation for the distinct expression of CYP1A species. In any event, since CYP1A2 does not 16-hydroxylate estrogens, and no other CYP1 PCR fragments have been detected in our analysis to date, we have adopted the working hypothesis that the 16α-hydroxylase is not a CYP1 family member.

Using the family 2 primers and T<sub>1</sub>Pr<sub>1</sub> cDNA as template, we amplified a particularly strong DNA fragment of the correct size. Sequencing data from 16 subclones and restriction enzyme analysis of the uncloned PCR fragment indicates that the amplified product is homogeneous and corresponds to a novel mouse Cyp2d family member (provisionally termed Cyp2d-13). This PCR product has been used to obtain full-length cDNAs corresponding to Cyp2d-13 (fig. 2). Cyp2d-13 exhibits several features which suggest that it is indeed a 16α-hydroxylase. The sequence of Cyp2d-13 is most similar to Cyp2d-9, which 16α-hydroxylates testosterone, and rat CYP2D4, which is not a 16α-hydroxylase but has been shown to cross-react with antisera directed against a purified P450 preparation with estrogen 16α-hydroxylase activity (fig 3). This observation alone is rather provocative.

A comparison of the predicted amino acid sequence of Cyp2d-13 and other Cyp2d family members suggests that their functional differences might primarily be in relation to substrate specificity (fig. 4). Substrate specificity in the CYP2D family appears to be conferred at least in part by 6 small, dispersed regions, termed substrate recognition sites (SRS). SRSs in the CYP2 family have been determined by aligning CYP2 sequences with CYP101A (P450cam) (25), whose substrate recognition sites (SRSs) have been identified conclusively by X-ray crystallography. Mutagenesis and domain-swapping experiments with a number of CYP2 genes have been entirely consistent with the proposed SRS regions. It is important to emphasize here that SRSs appear to determine substrate specificity rather than enzymatic activity. For example, a single amino acid substitution in SRS-5 of rat CYP2D1 (Ile380→Phe) results in decreased catalytic activity toward bufuralol but not debrisoquine (26), suggesting that the enzymatic function of the protein has remained intact. In the partial alignment shown in figure 4, note that the predicted amino acid sequence of Cyp2d-13 diverges from other Cyp2d subfamily members primarily in two small regions that correspond to SRS-5 and SRS-6.

To obtain further evidence that Cyp2d-13 might represent an estrogen 16α-hydroxylase, we used a quantitative RT-PCR approach (27) to examine Cyp2d-13 expression in different mammary cell lines with more than 20-
fold variation in their enzymatic activity. Unlike Northern or RNase Protection analyses, which would likely suffer from significant cross-hybridization with other Cyp2d members, here we were able to use primers completely specific for Cyp2d-13. SP1 primers were used as an internal control. We found that Cyp2d-13 RNA levels are very high in tumor-derived RIII/Pr1 cells, but barely detectable in MMEC and RIII/MG cells (derived from normal mouse mammary epithelium) (fig. 5). This is in excellent agreement with the relative ability of these cells to 16α-hydroxylate estrogens. We also found that the mammary carcinogen dimethylbenz[a]anthracene (DMBA) induces Cyp2d-13 by roughly 2-fold in T1Pr1 cells and several other mouse mammary cell lines (not shown). This correlates well with previously observed effects of DMBA on estrogen 16α-hydroxylase activity (28).

Cyp2d-13 expression correlates with both C16α-hydroxylation and the tumorigenicity of the above cell types. Interestingly, this association is also true in primary mouse mammary tumors, where Cyp2d-13 RNA levels are much higher than in the normal mammary gland (fig. 5). We also examined the expression pattern of Cyp2d-13 in a panel of normal mouse tissues. Only liver contained appreciable levels of this P450 species, but most tissues did express Cyp2d-13 at very low levels (fig. 6). These observations are consistent with previously published data showing that estrogen 16α-hydroxylase activity is normally mainly in the liver but strongly increased in mammary tumor tissue (4).

**Development of Cyp2d-13-specific antisera**

The foregoing observations would be strengthened by a corresponding analysis of P450 2d-13 protein levels. Moreover, the availability of antisera which inhibit P450 2d-13 function would be a valuable tool for testing whether this enzyme mediates estrogen 16α-hydroxylase activity in liver and mammary tumor samples. Because of the high degree of homology between Cyp2d-13 and other Cyp2d species, we chose a peptide-based strategy to generate specific antisera. We synthesized two peptides corresponding to SRS-1 (SQGIVLARYGPAC) and SRS-5 (LPLGVPHTSKRHDIEC) because these regions of P450 2d-13: (1) should be on the surface of the protein based on alignment with bacterial P450 enzymes whose structures have been resolved 0, (2) correspond to presumptive substrate-binding regions and therefore may generate antisera with immuno-inhibitory properties toward enzymatic function, and (3) contain some residues not conserved in other P45 2d family members (9/12 residues match SRS-1 in P450 2d-9 and 7/14 residues match SRS-5 in P450 2d-9). A cysteine residue was added at the C-terminus of each peptide to permit coupling to a carrier protein (KLH) for immunization in rabbits.

To evaluate the specificity of the antisera, we used a transient expression assay in human embryonic kidney 293T cells. These cells transflect at very high efficiency and permit potent expression of cDNA clones controlled by the cytomegalovirus (CMV) promoter (A.M.C. Brown, personal communication). We generated CMV-driven expression plasmids
containing Cyp2d-13, Cyp2d-9, or human CYP1A1, each with the coding sequence for a unique "FLAG" epitope (29) at the C-terminus of the indicated P450. A monoclonal antibody highly specific for this epitope is commercially available (Eastman Kodak); this can be used to demonstrate that transfection of each expression plasmid results in similar levels of protein expression. Transfection of 293T cells with a CMV-Cyp2d-13/FLAG expression plasmid and subsequent immunostaining with either the monoclonal FLAG antibody or an SRS-1 antiserum (termed 422) results in intense staining of a subpopulation of cells (see figure 7A, 7B), while cells transfected with the "empty" CMV expression plasmid are not stained by either antiserum (figure 7C, 7D). Preimmune 422 serum did not stain transfected cells (not shown). An SRS-5 antiserum (399) also caused intense staining of CMV-Cyp2d-13/FLAG-transfected cells (not shown).

Transfected 293T cells seemed to express P450 2d-13 at such high levels that the cells became "overloaded". There was not a clear microsomal localization as would be expected, but rather an enormous inclusion body-like appearance. The stained cells also possessed an irregular morphology when compared to their nontransfected neighbors. Therefore, we used another cell line, termed COS-1, which has been used extensively as a vehicle for analyzing the functional activities of cloned CYP genes. CMV-Cyp2d-13/FLAG-transfected COS-1 cells give a typical microsomal staining pattern (figure 7E). Remarkably, transfection of COS-1 cells with the Cyp2d-9 or CYP1A1 containing expression plasmids did not result in significant staining with the 422 antiserum (not shown), indicating that this antiserum is highly specific for P450 2d-13.

Cyp2d-13 is expressed at much higher levels in RIII/Pr1 cells than in MMEC cells. Thus, to determine whether our antisera could detect endogenously expressed P450 2d-13, we stained these cells with the 422 antiserum (figure 8). The perinuclear microsomal region of RIII/Pr1 cells were intensely stained with this antiserum (figure 8A, 8B), while MMEC cells gave no staining whatsoever (figure 8C).

All of the above data has also been corroborated by immunoblot analysis (not shown). In sum, our data strongly suggest that the SRS-1- and SRS-5-directed antisera should be excellent tools for examining Cyp2d-13 expression and may very well be appropriate for future immuno-inhibition studies.

A novel CYP2D6 haplotype in C16α-hydroxylating human mammary cells

Collectively, the above observations give considerable weight to the hypothesis that Cyp2d-13 may be a bona fide estrogen 16α-hydroxylating enzyme in mouse mammary tissue. This hypothesis is further supported by our PCR analysis in human MCF-10F cells, which has also selectively detected CYP2D expression. As in mice and rats, the human CYP2D locus on chromosome 22 has multiple tightly linked members, but only CYP2D6 appears to be expressed (30,31). The CYP2D6 locus is highly polymorphic, resulting in marked variations in the metabolism of certain commonly prescribed drugs (32). CYP2D6 polymorphisms have therefore been of
intense pharmacological interest. There are four characterized variant CYP2D6 alleles which collectively account for the poor ability of 5-10% of Caucasians to metabolize debrisoquine and numerous related plant alkaloids (33, 34). Additionally, at least two distinct haplotypes are associated with the "extensive metabolizer" phenotype (fig. 9) (35). One of these, termed CYP2D6L, is occasionally amplified in some families, resulting in their ability to metabolize certain drugs at an "ultra-rapid" rate (36). Enzymatic reactions mediated by CYP2D6 are therefore prone to wide inter-individual variation.

Sequence analysis of the CYP2-derived PCR fragment in MCF-10F cells indicates the presence of a previously undescribed CYP2D6 haplotype (which we have termed CYP2D6M). This haplotype contains a cluster of variant bases, resulting in the deletion of Val386 and two nearby, unconserved amino acid changes (fig. 9). Similar to the above observations with Cyp2d-13., these changes occur in the region corresponding to SRS-5, which helps determine substrate specificity in other CYP2 family members. Indeed, the substrate specificity of SRS-5 has been demonstrated experimentally using rat CYP2D1 (26), which is orthologous to CYP2D6. MCF-7 cells, which possess little C16α-hydroxylase activity, do not contain the CYP2D6M haplotype. We are currently characterizing this haplotype in full, after which we will engage in a comparative analysis of estrogen-metabolizing ability amongst the various CYP2D6 polymorphisms. Should this approach prove fruitful, it will ultimately be of great interest to examine the association of these polymorphisms with breast cancer.
CONCLUSIONS

Consistent with previous studies, myc oncogene transformation of mammary epithelial cells leads to induction of C16α-hydroxylation, anchorage-independent growth and tumorigenesis. Agents which specifically reduce C16α-hydroxylation (e.g. indole-3-carbinol) inhibit hyperproliferation such as that observed here; moreover, ER antagonists such as tamoxifen only achieve their full inhibitory effect when converted to metabolites (4-OHTAM) that also reduce C16α-hydroxylation. These data support our hypothesis that this pathway of estrogen metabolism leads to the formation of estrogens that promote mammary tumorigenesis. Indeed, we observed that 16α-OHE₁ was unique among estrogens in its ability to stimulate genotoxic damage and anchorage-independent growth in C57/MG cells.

Our efforts to identify precisely the molecular changes which occur when mammary cells are chronically exposed to 16α-OHE₁ have been severely hampered by difficulties in growing MCF-7 cells long-term in culture in steroid-depleted medium and the prolonged process of moving the laboratory to a new location. However, we have made extensive progress in the characterization of mouse and human genes that may mediate the formation of 16α-OHE₁. We soon expect to have confirmation of this by heterologous expression of the cloned cDNAs, in which case we will be able to utilize less problematic molecular approaches (stable transfection, transgenics) to approach these experiments.

The identification of Cyp2d-13 as a putative estrogen 16α-hydroxylase is a crucial advance in our efforts to adjudicate the hypothesis that estrogen metabolism is a critical factor in breast cancer. Expression of Cyp2d-13 is remarkably associated with the tumor phenotype in the mouse mammary gland; moreover, the gene possesses many of the features expected of a bona fide estrogen 16α-hydroxylase. With regard to the human CYP2D6 locus, it is very intriguing that such a polymorphic locus might be linked to breast cancer. Ultimately, CYP2D6 genotyping might serve to identify those individuals who might most benefit from dietary or pharmacological strategies which induce the competing, 2-hydroxylase pathway.
REFERENCES


11. Swaneck GE, Fishman J 1988 Covalent binding of endogenous estrogen 16α-hydroxyestrone to estradiol receptor in human breast cancer
cells: characterization and intranuclear localization. Proc Natl Acad Sci USA 85:7831-7835


29. Roeder RG and Chiang C-M 1993 Expression and purification of general transcription factors by FLAG epitope-tagging and peptide elution Peptide Res 6:2


31. Kimura S, Umeno M, Skoda RC, Meyer UA, Gonzalez FJ 1989 The human debrisoquine 4-hydroxylase (CYP2D) locus: sequence and
identification of the polymorphic CYP2D6 gene, a related gene, and a pseudogene. Am J Hum Genet 45:889-904


APPENDIX

Grant Number DAMD17-94-J-4441
Induction of Aberrant Hyperproliferation and Tumorigenic Transformation by C-myc Oncogene in Mouse Mammary Epithelial Cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Status of Transformation</th>
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<tbody>
<tr>
<td></td>
<td>in vitro</td>
</tr>
<tr>
<td></td>
<td>Anchorage-independent</td>
</tr>
<tr>
<td></td>
<td>growth (%CFE)(^a)</td>
</tr>
<tr>
<td>MMEC</td>
<td>ND</td>
</tr>
<tr>
<td>MMEC/myc(_3)</td>
<td>12.8 ± 3.8</td>
</tr>
<tr>
<td>MMEC/myc(_3)-Pr(_1)</td>
<td>26.8 ± 3.8</td>
</tr>
</tbody>
</table>

\(^a\) Number of colonies ÷ Initial seeding density \times 100

\(^b\) Number of tumors ÷ Number of transplant sites
AT-2

C-myc Oncogene-Mediated Alteration in Estradiol Metabolism and in Hormone Receptor Status

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>E2 Metabolism(^a) (C2/C16α-hydroxylation)</th>
<th>Receptor Protein Content(^b) (fmoles per 1.0 x 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ERP</td>
</tr>
<tr>
<td>MMEC</td>
<td>5.4 ± 0.4</td>
<td>11.9 ± 0.5</td>
</tr>
<tr>
<td>MMEC/(\text{myc}_3)</td>
<td>2.7 ± 0.4</td>
<td>8.8 ± 1.5</td>
</tr>
<tr>
<td>MMEC/(\text{myc}^3)-(\text{Pr}_1)</td>
<td>1.6 ± 0.6</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) determined by \(^3\)H\(_2\)O formation from [C2-\(^3\)HJE\(_2\) and [C16α-\(^3\)HJE\(_2\)

\(^b\) determined by dextran coated charcoal absorption (DCC) assay
AT-3

Metabolism of 17β-Estradiol (E₂) in MMEC/myc₉-Pr₁ Cells

<table>
<thead>
<tr>
<th>E₂ Metabolites</th>
<th>Relative Abundanceᵃᵇ (Normalized per 1.0 x 10⁷ cells)</th>
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<tbody>
<tr>
<td>E₁</td>
<td>1.465 ± 304</td>
</tr>
<tr>
<td>16α-OHE₁</td>
<td>206 ± 20</td>
</tr>
<tr>
<td>2-OHE₁</td>
<td>799 ± 178</td>
</tr>
<tr>
<td>E₃</td>
<td>190 ± 14</td>
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</tbody>
</table>

ᵃ determined by the RP-HPLC and GC-M5 analysis of the culture medium

ᵇ MMEC/myc₉-Pr₁ cells incubated with 1.0 x 10⁻⁹M E₂ for 96 hours
Ligand-mediated Upregulation of Proliferation and Hormone Receptor Status in MMEC/myc3-Pr1 Cells

<table>
<thead>
<tr>
<th>E2 Treatment (nM)</th>
<th>Increase in cell number&lt;sup&gt;a&lt;/sup&gt; (x10&lt;sup&gt;5&lt;/sup&gt;)</th>
<th>Receptor Protein Content&lt;sup&gt;b&lt;/sup&gt; (fmoles/1.0 x 10&lt;sup&gt;6&lt;/sup&gt; cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ERP</td>
</tr>
<tr>
<td>0.0</td>
<td>2.3 ± 0.6</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>2.8 ± 0.4</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>4.2 ± 0.5</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>10.0</td>
<td>4.4 ± 0.5</td>
<td>13.7 ± 3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> cells maintained in serum-free medium for 120 hours

<sup>b</sup> determined by dextran-coated charcoal (DCC) absorption assay
AT-5

Modulation of Estradiol 2-Hydroxylation and Aberrant Hyperproliferation in Human Mammary Carcinoma MCF-7 Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>2-hydroxylation of E₂(^a) (% per 1.0 x 10^6 cells)</th>
<th>Anchorage-Independent Growth (% CFE)(^b)</th>
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<tbody>
<tr>
<td>EtOH</td>
<td></td>
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<tr>
<td>(solvent control)</td>
<td>2.4 ± 0.4</td>
<td>15.3 ± 1.4</td>
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<tr>
<td>I3C</td>
<td>6.2 ± 0.3</td>
<td>5.0 ± 1.1</td>
</tr>
<tr>
<td>TAM</td>
<td>1.6 ± 0.1</td>
<td>3.7 ± 0.7</td>
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<tr>
<td>4-OH TAM</td>
<td>3.6 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>2-OHE(^1)</td>
<td>ND</td>
<td>4.9 ± 2.9</td>
</tr>
<tr>
<td>16α-OHE(^1)</td>
<td>ND</td>
<td>25.9 ± 2.0</td>
</tr>
</tbody>
</table>

\(^a\) determined from \(^3\)H₂0 formation after a 48 hour incubation with [C₂\(^3\)H] E₂

\(^b\) Number of colonies

\[
\frac{\text{Number of colonies}}{\text{Initial seeding density}} \times 100
\]
Breast Cancer Risk-Dependent Enhancement of Estradiol 16α-Hydroxylation in Murine and Human Mammary Explant Cultures

<table>
<thead>
<tr>
<th>Origin</th>
<th>Cancer Risk</th>
<th>Tissue Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Estradiol C16α-hydroxylation (% per mg tissue)&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Murine</td>
<td>Low</td>
<td>NFS-MDE</td>
<td>0.16 ± 0.05</td>
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<tr>
<td></td>
<td>High</td>
<td>C3H-MDE</td>
<td>0.59 ± 0.13</td>
</tr>
<tr>
<td>Human</td>
<td>Low</td>
<td>TDLU-LR</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>TDLU-HR</td>
<td>0.31 ± 0.05</td>
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</tbody>
</table>

<sup>a</sup> Mammary ductal epithelium from low risk MFS strain, mammary ductal epithelium from high risk C3H strain, terminal duct lobular units from reduction mammoplasty, terminal duct lobular units from cancer mastectomy

<sup>b</sup> determined from <sup>3</sup>H<sub>2</sub>O formation after a 48 hour incubation with [C16α-<sup>3</sup>H] E₂
Induction of Unscheduled DNA Synthesis and Aberrant Hyperproliferation by Estradiol Metabolites in Mouse Mammary Epithelial C57/MG Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Unscheduled DNA Synthesis (HU-insensitive $^3$H-TdR uptake, cpm x $10^6$/mg DNA)</th>
<th>Anchorage-Independent Growth (% CFE)$^a$</th>
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<tbody>
<tr>
<td>DMSO</td>
<td>12.5 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>(solvent control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBA</td>
<td>21.4 ± 2.2</td>
<td>24.0 ± 2.4</td>
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<tr>
<td>(positive control)</td>
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<td></td>
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<tr>
<td>E$_2$</td>
<td>12.5 ± 1.0</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>16$\alpha$-OHE$_1$</td>
<td>22.0 ± 3.5</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>2-OHE$_1$</td>
<td>13.0 ± 0.9</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ Number of colonies

\[
\frac{\text{Number of colonies}}{\text{initial seeding density}} \times 100
\]
Figure 1

Sites of oxidative metabolism in estradiol
Figure 2

Cyp2d-13 sequence

gaucgtttccaaagacacccccaaagacccagacccagcggcgcgcc
ATG AGA CAT CGG ACC GCC GCT GTA GCA TGG GCC ATG
M R L F T G A E L W P I 12

GCC ATA TTC ACG GTC ATC TTC TTG ATT CTG TCA AGG ACC TGG GCC CAG CGG TGG ACT GCC
A I F T V I F L I L V H W R Q W T A 34

CAC TAC CCT CCA GCC CCT ATG CGG TCT CTA GGG AAC CGG CTC CTG CAC ATG GAC TTC CAG ANT
H Y P P G P M P W P V L G N L L H M D F Q N 56

ATG CCA GCA GCC TTC CAA AAG CTG CCG GGT CCT TAT GGG CAC CTG TTG CAC AGC CTA GAG CGC TCT
M P A G F Q K L R G R Y G D L F S L Q L A S 78

GAG TCA GTG GGT GTA CTA AAT GGG CTG ACG GCC CTC TGA AAA GCC TGA CTG GCA AGC GAG GAC
E S V V V L N G L T L R E A L V K H S E D 100

ACT GCT GAC CGG CCA CGG CTG CAT TTC AAT GAC CTG CCG TTC TGG GTA CCA CGC TCT CAA GTG ATA
T A D R F P F L H E F N D L L G F G P R S Q G I 122

GTC CTA GCA CGG TAT GGG CCT GCC TGG CTT CAG CAG CGG TTC TCT GTC TCT ACC ATG CAC CAC
V L A R Y G P A W R Q Q R R F S V T S M H H 144

TTT GCC CTG GCC AAC AAG TCA CTG GAG CAG TGG GTG ACT GAG GCC AAG AGA GCC TCA TTC GTG GCC
F C L G K S K L E Q W V T E E R C L C A A 166

TTC CTA GAC CAT ACT GGA CAT CCT TTC ACC ACC CTA TTG GAG AAA GCA GTG TTC AAG GCT
F A D H T G H P F S P N T L L D K A V C N V 188

ATC GCC TTC CTC CTT CAT GCC TGG CGC TTC GAG TAC GAT GAC CCA CGC TTC ATC AGG CTA CTG GCC
I A S L L Y C R F E Y D D P F P R I R L L G 210

TTG TTG AAG GAA ACT CTT AAG GAG GAA ACT GGA TTC CTA CCA GEN TTC AGG TCC ATG
L L K E T K E F N D F L E F L N V F P F M 232

CTG CTG CGC ATC CGG CGG CTG GGT GCC AAG GTC TTC GCC GTC GGG AAA AAG GCC TTT GGT ACC ATG
L L R I P G L V G K V F P G K R A F V I T N L 254

GAT GAG CTA CTG ACT GCC TCA ACG ACC CTA TGG GCC CAC CCT ACC CAG CCA CCC CGA GAT TTG ACT GAT
D E L L A E H K T T W D P T Q P P R D L T 276

GCC TTC CTG GCT GAG GTG GAG AAG GCC AAG GGG AAT CCT GAG ACC AGC TTC AAT GAT GAG AAC CTG
A F L A E V E K L G P K G T T L I T N L S S A A L K D 298

GCC ACG GTA GTG GCT GAC CTG TTC TCT QCA GGG AGG ATG GTG ACC ACC ACC TCA ACC ACA CTG TCC TGG GCC
R T V G D L F S A G M W T V T S T T L L S W A 320

CTG ATG ATC ATC ATG ATC CTG CAT CCA GAT GAG CGA CGC GCA GTA CAA CAG GAG ATC GAT GAA GTC ATA
L M L M L I H P D V Q R R V Q V Q E I D E V I 342

GGG CAG GTG CAG TCG GCC GAA ATG GCC GAC GCT CCG ATG GCC CAC TAC ACC ACC AAT GCT GTC ATT CAT
G Q V Q V Q C P E H M A Q D A R M P Y T N A V I H 364

GAG GTG CAG CCC TTT GCA GAC ATT CTC CCT CCT GTG GTA CCT CAC AAG ACT CTG CCT GAC ATT GAA
E V Q R F D I L D I L G V P H K T S R D I E 386

CTA CAC GCC TTC CTT ATC CCT AAG GGG ACC TGC ACG ACC TGC TAC TCC CGG CTA AAA GAT
L Q G F I P K G T T L I T N L S S A A L K D 408

GAG ACT GTG GAG AAG CCC CTC TGC TTC CAT CCT GAA CAC CTG GAT GCC CAG GCC CAC TTT
E T V W E K L C F T H P E H F L D A Q G H F 430

GTG AAG CCT GAG GCC TTC ATG CCA TTC TCA GCC GCC GCC AAG TCA TCG CTG GGG GAG CCC CTG GCC
V K P R A F M G S A R G R S C L G E P L A 452

GCC ATG GAG CTC TTC TTC TTC TTC TCC ACC TTC ACC CTG GAG CAG TAT TGG AAC GCT TCA GCA GCC GCC
R M E L L F L F P T C L L Q R F S I S V P D G 474

CAG CCC CAG ACC GAC CAT GCC GTG TTT AGG GCT CTG ACA ACC CCA TGC CCC TAC CAG CTC TGT
Q P Q P S D R H A L T T P C Y Q L C 496

GCT TGG CCC CCC TAA gaggagatgtatatcatctccaaactcactctgtgtggttgggtctctaattgtgcataaagccatattttac
A L P R A * 500
**Figure 3**

Partial alignment of Cyp2-d13 with rat CYP2D4 (P450-CMF3) and mouse Cyp2-d9 (testosterone 16α-hydroxylase)

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<tr>
<td>CYP2D4</td>
<td>TCCCTGTCATC CATAGGGTGC AGCGCTTTTCG AGACATTCTC CCTCTTGGTG TgCCTCACAA</td>
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<tr>
<td>Cyp2-d9</td>
<td>TCCCTGTCATT CATAGGGTGC AGCGCTTTTGc gGACATTGgg CcGgTgaaTt TgCcCaCgCAt</td>
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<td>CYP2D4</td>
<td>GACTTCTCGT GACATGGAag TgCAGGGCCTT CCTTATCCCT AAGGGACaAC CTCATCACCC</td>
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<td>Cyp2-d9</td>
<td>gACaagTCaT GACATGGAag TgCAGGaCTT CCTcATCCCc AAGGGACGAt CTCcTCcCC</td>
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<td>Cyp2-d13</td>
<td>AACCTGTCCT CGCGCTAAGA AGATGAGACT GTCTGGGAGA AGCCCTCT</td>
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<tr>
<td>CYP2D4</td>
<td>AACCTGTCCT GAGTGCTGAGA gGATGAGAcC GTCTGGGAGA AGCC-Ct</td>
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<tr>
<td>Cyp2-d9</td>
<td>AACaTGTCCT CCaTGCTGAA AGATGAGCTG GTCTGGGAGA AGCC-Ct</td>
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30
## Figure 4

Partial Amino acid alignment of the Cyp2d subfamily

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<tr>
<th></th>
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<tr>
<td>2d-13</td>
<td>AVVHEVQRFA DILPLCVPHK TSRDIELQGF LIPKGTTLIT NLSSALKDET VWEKPLCFHP</td>
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<td>2d-13</td>
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<tr>
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<td>h a n</td>
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<tr>
<td>2d-11</td>
<td>yr haipva f</td>
<td></td>
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</table>
Figure 5

*Cyp2d-13* expression is elevated in mammary tumors and tumor-derived cell lines

A: MMEC  
B: RIII/MG  
C: RIII/Pr1  
D: RIII normal mammary gland  
E: RIII primary mammary tumor
Figure 6

*Cyp2d-13* is primarily confined to the liver in normal tissues

M: Mammary Gland
O: Ovary
L: Liver
K: Kidney
I: Intestine (Duodenum)
A: Adrenal
H: Heart
U: Uterus
B: Brain
S: Spleen
Figure 7

Immunocytochemical detection of P450 2d-13 in transfected 293T and COS-1 cells

Antiserum

FLAG

422 (α-SRS-1)

CMV-2d-13 (293T)

A

B

CMV (293T)

C

D

CMV-2d-13 (COS-1)

E
Figure 8
Immunocytochemical detection of P450 2d-13 in tumor-derived RIII/Pr1 cells

RIII/Pr1 (α-SRS-1)

MMEC (α-SRS-1)
CYP2D6 Polymorphisms

(2D6-A)  (2D6-B)  (2D6-C)

ΔAGA

ΔA

Splice mutation

S→T

R→C

R→H

M→V

L→M

H→R

P→S

1 kb