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# **Association of CYP1B1 germline mutations with HNF1 $\alpha$ mutated hepatocellular adenoma**

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## Abstract

Biallelic somatic mutations of *TCF1* coding for hepatocyte nuclear factor 1 $\alpha$  (HNF1 $\alpha$ ) are found in 50% of the hepatocellular adenoma (HCA) cases usually associated to oral contraception. In rare cases, HNF1 $\alpha$  germline mutations could also predispose to familial adenomatosis. In order to identify new genetic factors predisposing to HNF1 $\alpha$  mutated HCA, we searched for mutations in genes involved in the estrogens metabolism. For 10 genes (*CYP1A1*, *CYP1A2*, *CYP3A4*, *CYP3A5*, *COMT*, *UGT2B7*, *NQO1*, *GSTM1*, *GSTP1*, and *GSTT1*), we did not find mutations nor differences in the allele distribution among 32 women presenting HNF1 $\alpha$  mutated adenomas compared to 58 controls. In contrast, we identified a CYP1B1 germline heterozygous mutation in 4 women among 32 presenting HNF1 $\alpha$  mutated adenomas compared to none in 58 controls. We confirmed these results with the identification of 4 additional CYP1B1 mutations in a second series of 26 cases. No mutations were found in the control group extended to 98 individuals and only a known rare genetic variant in two controls was observed ( $P=0.0003$ ). We performed an ethoxyresorufin O-deethylase (EROD) assay to evaluate the functional consequence of the CYP1B1 mutations. We found a reduced enzymatic activity of each CYP1B1 variant. In addition, an E229K CYP1B1 mutation was found in a woman with a germline HNF1 $\alpha$  mutation in a familial adenomatosis context. In this large family, all the three patients with adenomatosis bear both HNF1 and CYP1B1 germline mutations.

In conclusion, our data suggested that CYP1B1 germline inactivating mutations may increase the HCA incidence in women with HNF1 $\alpha$  mutation.

## Introduction

Use of oral contraception is an important risk factor to develop hepatocellular adenoma (HCA), a rare benign liver tumor (1, 2). HCA usually occur as a single tumor, but multiple nodules can be observed and the presence of more than 10 nodules in the liver defines liver adenomatosis (3). Most HCA usually remain stable but can increase in size after withdrawal of oral contraceptives while they rarely regress (4, 5). The search for genetic alterations in HCA led to the identification of mutations in two genes: (a) biallelic inactivating mutations of the *TCF1* gene (chromosomal locus: 12q24.31) coding for the transcription factor Hepatocyte Nuclear Factor 1 $\alpha$  (HNF1 $\alpha$ ) (6), and at a lower frequency (b) activating mutations in the *CTNNB1* gene coding for  $\beta$ -catenin (7, 8). Recently, mutation screening of these two genes in 96 HCA allowed us to identify strong genotype-phenotype correlations and we proposed a new classification of HCA tumors (8). Biallelic mutations of the gene coding for HNF1 $\alpha$  are found in half of the HCA cases. They define a homogeneous group of tumors representing the most usual form of adenoma characterized by a marked steatosis (8). In 84% of these cases both HNF1 $\alpha$  mutations in tumors are of somatic origin. In the remaining cases, one of the HNF1 $\alpha$  mutations was germline and patients developed adenomatosis. Familial analyses performed in 4 independent germline adenomatosis showed that all 11 relatives who developed adenomatosis displayed a germline HNF1 $\alpha$  mutation (9, 10). However, in these families, 16 individuals with a germline HNF1 $\alpha$  mutation did not develop any liver tumors. These observations suggested that germline HNF1 $\alpha$  mutations predisposed to liver adenomatosis with an incomplete penetrance and raised the possible existence of modifier genes.

HCA with somatic HNF1 $\alpha$  mutations appear mostly in women (97% of the cases) with 77% of these using oral contraceptive (8). Among them, we identified 4 women who developed

multiple HNF1 $\alpha$  mutated adenomas. These results raised the question of a potential role for a genetic predisposition to develop HNF1 $\alpha$  mutated adenomas, possibly related to estrogens metabolism.

In order to identify a genetic predisposition in women with somatic HNF1 $\alpha$  mutated HCA or to find a gene modifying the penetrance of adenomatosis in germline HNF1 $\alpha$  mutated patients, we search for alterations in candidate genes involved in estrogens metabolism. We screened a first group of 32 women with HNF1 $\alpha$  mutated HCA for genetic variants by direct sequencing of 11 genes (*CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A4*, *CYP3A5*, *COMT*, *UGT2B7*, *NQO1*, *GSTM1*, *GSTP1*, and *GSTT1*). The genotypes were compared to those of 58 control women with a HNF1 $\alpha$  non-mutated benign hepatocellular tumor. We validated the identified variants in a second group of 26 women presenting a HNF1 $\alpha$  mutated HCA and 40 control women.

## Materials and Methods

### Patients

A group of 16 French university hospitals participated to this study. Criteria for patient inclusion were women with confirmed HNF1 $\alpha$  mutated HCA and as control women with HNF1 $\alpha$  non mutated benign liver tumors surgically treated in the same hospitals and over the same period. A first group of patients was recruited between 1992 and 2003, and consisted in 32 women with HNF1 $\alpha$  mutated HCA and 58 women in the control group (25 HCA, 24 focal nodular hyperplasias and 9 telangiectatic focal nodular hyperplasias). A second group of patients was recruited from 2004 to 2006, and consisted in 26 women with HNF1 $\alpha$  mutated HCA and 40 women in the control group (23 adenomas, 11 focal nodular hyperplasias and 6 telangiectatic focal nodular hyperplasias). In the overall series, the mean age at diagnosis was 37 years old (ranging from 14 to 61) in HNF1 $\alpha$  mutated HCA cases and 38 years old (ranging from 12 to 62) in controls. Among the patients with a HNF1 $\alpha$  mutated tumor, 72% used an oral contraception (12 cases with missing data), in 6 cases one HNF1 $\alpha$  mutation was germline (data was unavailable in 3 cases). In the control group, 89% of the women had an oral contraception (11 cases with missing data). Among the 156 women, 38 cases of HNF1 $\alpha$  mutated adenomas and 31 cases of non-mutated tumors were previously described in Zucman-Rossi et al., and Bioulac-Sage et al., respectively (8, 11). All women but 4 were of European origin.

A family extensively described in Reznik et al., was also completely genotyped in the present work (10). Briefly, the proband, a 17 years old female, presented hepatomegaly. She developed early-onset diabetes at the age of 32 years of age. Liver nodules were discovered and liver adenomatosis was confirmed by pathological diagnosis, which led to a familial screening for

diabetes and adenomatosis. Germline HNF1 $\alpha$  mutations were found in 11 individuals and three of them were affected by adenomatosis defined by the presence of more than 10 lesions (Figure 1).

For all cases, a representative part of the HCA nodule, as well as of the non-tumor liver, was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use for molecular studies. All diagnosis were reviewed by a panel of liver pathologists and mutations were searched in the HNF1 $\alpha$  gene as previously described (8). In the present work gene variants were searched in non-tumor DNA and germline origin was validated by genotyping lymphocytic DNA. All patients were recruited in accordance with French law and institutional ethical guidelines. Ethical committee of the Saint-Louis hospital, Paris, France, approved the overall design of the study. Newly identified mutations were also tested in 92 unrelated women from the Centre d'Etude du Polymorphisme Humain (CEPH) families. The E229K CYP1B1 mutation was also tested in 125 independent and Caucasian women from the Human Genome Diversity Project (HGDP) (12).

### **Mutation screening**

Eight genes were screened for mutations or polymorphisms by direct sequencing of PCR products: CYP1A1 (exons 2 to 7, 3'UTR), CYP1A2 (polymorphism IVS1+734G>A and exons 2 to 7), CYP3A4 and CYP3A5 (exons 1 to 13), CYP1B1 (exons 1 to 3), COMT, NQO1, and UGT2B7 (exons 1 to 6). Sequencing was performed as previously described (6). All mutations were confirmed by DNA sequencing from 2 independent PCR amplifications. The screening for GSTM1, GSTP1 and GSTT1 variants was performed according to the protocol described by Cabelguenne et al., (13). Oligonucleotides sequences used for all PCR and programs are provided in the supplemental Table1.

The screen of the E229K CYP1B1 mutation in the HGDP population was performed by a PCR-RFLP assay. Briefly, a 517 pb fragment was generated with the PCR primers (forward) 5'-TGGCCAACGTCATGAGTGCC-3' and (reverse) 5'ACTCAGCATATTCTGTCTCTAC-3'. The generated fragment contains two EarI sites, the E229K CYP1B1 mutation removes one of the EarI sites.

### **Mutagenesis**

CYP1B1\*1 allele into the pDHFR mammalian expression vector (14) was a generous gift from T. Friedberg, University of Dundee, Dundee, UK. Wild type and mutated CYP1B1\*1, CYP1B1\*2 and CYP1B1\*3 alleles were generated using the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's conditions. Wild-type CYP1B1\*1, CYP1B1\*2 and CYP1B1\*3 alleles are described in <http://www.imm.ki.se/CYPalleles/cyp1b1.htm>. Expression of CYP1B1 alleles in mammalian COS-1 cells was performed according to Bandiera's conditions using lipofectAMINE reagent (Invitrogen) and the co-transfection of a pSV-β-galactosidase vector (15).

### **EROD assay on whole cells**

EROD assay was performed according to Bandiera's conditions and as briefly described below. After transfection (48 h), cells were incubated with a 400 nM solution of 7-ethoxyresorufin (Sigma-Aldrich) in PBS 1X at 37°C/5% CO<sub>2</sub>. The fluorescent product resorufin is released from EROD by CYP1B1. Fluorescence was measured in duplicate aliquots of supernatant at time = 0 min, 5 min and 10 min (excitation = 544 nm; emission = 590 nm). The amount of resorufin formed was calculated by comparison with a standard curve ranging from 30 μM to 3.75 nM (16).

## **Quantification of Protein Expression**

After resorufin analysis, cells were lysed (lysis reagent as supplied for the  $\beta$ -Gal Reporter Gene Assay chemiluminescent kit; Roche Molecular Biochemicals, Mannheim, Germany) and concentration protein was determined using a BCA protein assay (BCA Protein Assay Kit; Pierce). Lysates were subjected to immunoblot analysis employing a polyclonal anti-human CYP1B1 primary antibody (BD Gentest) according to the manufacturer's instructions. EROD assay was normalized to the transfection efficiency determined with the  $\beta$ -Gal Reporter Gene Assay chemiluminescent kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

## **Statistical analysis**

Statistical analyses were performed using the GraphPad prism software version 4 (GraphPad Software Inc., San Diego, CA). Frequencies of CYP1B1 mutations in patients with HNF1 $\alpha$  mutations and in control populations were compared in contingency tables using a Fisher's exact test. The different activities of the mutated and non-mutated alleles of CYP1B1 were compared with a two-tailed, unpaired *t* test with 95% confidence intervals.

## Results

### 1- Genotype analysis

We scanned for genetic variants in the *CYP1A1*, *CYP1A2*, *CYP3A4*, *CYP3A5*, *COMT*, *NQO1*, and *UGT2B7* genes in the first series of patients including 32 women with HNF1 $\alpha$  mutated tumors and 58 women presenting non HNF1 $\alpha$  mutated benign liver tumor. Allele frequencies of all identified coding single nucleotide polymorphisms (SNPs) in the two patient groups are provided in Table 1. We did not observe significant differences between the 2 groups and these frequencies were similar to those from in the SNP500 database. Moreover, we did not identify new variants in these genes when compared to available genetic databases.

### 2- Identification of CYP1B1 mutations in patients with HNF1 $\alpha$ mutated adenoma.

Among the first series of 32 women with a HNF1 $\alpha$  mutated adenoma, we identified an heterozygous CYP1B1 germline mutation in 4 individuals. No mutations or rare variant were observed in the 58 women presenting non HNF1 $\alpha$  mutated benign liver tumor. Sequencing a second series of 26 cases with a HNF1 $\alpha$  mutated HCA, we identified 4 additional women with a CYP1B1 germline mutation. None of the CYP1B1 identified mutations were found in SNP available databases (NIEHS, SNP500, dbSNP, Panther, Ensembl). No mutations were found in 40 supplementary women presenting non HNF1 $\alpha$  mutated benign liver tumor (Table 2). However, among this second control group, we identified two Caucasian individuals with a rare CYP1B1 heterozygous variant leading to an amino acid substitution at codon 81 (Y81N) never found in women presenting HNF1 $\alpha$  mutated HCA (Table 2). The Y81N polymorphism is characterized as a rare variant in the SNP500 database with a 2% allelic frequency in Caucasian population. Taken together we found a significant association between CYP1B1 germline

mutation and the occurrence of HNF1 $\alpha$  mutated adenoma ( $P = 0.0003$ , Fisher exact test). The 8 identified mutated individuals corresponded to 4 different nucleotide mutations. An amino acid substitution at codon 229 (E229K) was observed in 5 cases. Two other amino acid substitutions (P52L and G329S) were found. The last mutation was a small deletion of 13 nucleotides leading to a frameshift (R355fs). We have tested a third control group of 92 independent women from CEPH collection for these mutations and none was found. Because the E229K CYP1B1 mutation was observed in five cases, we genotyped 125 additional Caucasian women from the HGDP collection. No E229K CYP1B1 mutation was observed in this population and we concluded that the E229K substitution was a mutation.

Among the women with HNF1 $\alpha$  mutated HCA, there were no significant differences in clinical characteristics (oral contraceptive use, age, number of nodules) when comparing CYP1B1 mutated and non-mutated women. One of the E229K CYP1B1 mutations was found in a woman with a germline HNF1 $\alpha$  mutation in a familial adenomatosis context. We genotyped 16 members of her family and identified the mutation in 8 relatives (Figure 1). We observed that the 3 cases with an adenomatosis (II-2, II-6, III-11) had both CYP1B1 and HNF1 $\alpha$  mutations and only a 31 years old man (III-6) without tumors presented both CYP1B1 and HNF1 $\alpha$  mutations.

Finally, no differences in the frequency of common CYP1B1 polymorphisms and alleles were observed between the 58 patients with HNF1 $\alpha$  mutated HCA and the 98 control women presenting a non-mutated benign liver tumor (supplemental Table 2).

### **3- Identified mutations decrease CYP1B1 enzymatic activity.**

The three different identified CYP1B1 mutations change evolutionary conserved amino acids in mammals (Figure 2) and the modified amino acids are all located either the hinge region or in some putative substrate recognition sites (SRSs) (17). We therefore tested for the functional

consequences of the different mutations using the 7-ethoxyresorufin *O*-deethylation (EROD) assay (15). The allelic context of the mutations P52L, E229K, G329S and R355fs were determined in the patients by cloning and sequencing CYP1B1 cDNA from non-tumor tissues of the corresponding patient. Interestingly, E229K were located in the same allele \*2 in all 5 mutated patients. Each mutation was introduced in its respective allelic context by site directed mutagenesis in a pDHFR plasmid. Wild type and mutated constructs were transfected in COS-1 cells, which does not express CYP1B1 (15). CYP1B1 expression was assayed by immunoblotting 48 h after transfection. A distinct and comparable signal was obtained by western-blot in cells transfected with all the CYP1B1 wild type and mutated alleles. CYP1B1\*1-R355fs mutation was undetectable with the antibody since the variant encodes a protein in which the C-terminal that contains the epitope is modified by the frameshift mutation (Figure 3). We tested by quantitative RT-PCR the cells transfected with CYP1B1\*1-R355fs and observed that CYP1B1 was expressed at a similar mRNA level when compared to wild type allele \*1 (data not shown). The EROD activity of all normal and mutated alleles was determined after normalization to the transfection efficiency as previously described (15). Because CYP1B1\*3 was the most frequently found allele in our tested populations (39% allelic frequency in all patients), we arbitrarily normalized our EROD assay results to the activity of this allele. We did not find any significant differences of activity between the 3 wild type alleles, CYP1B1\*1, \*2 and \*3. In contrast, all mutated alleles exhibited a significant reduced activity when compared to their corresponding wild type allele (Figure 3).

## Discussion

Our study showed the identification of CYP1B1 germline heterozygous mutations in 14% of the women presenting a HNF1 $\alpha$  mutated HCA. All identified CYP1B1 mutations led to a decreased enzymatic activity as tested using the EROD assay. We also identified a rare natural polymorphism (Y81N) in two women of the control group. Curiously, the Y81N CYP1B1 variant exhibited a reduced enzymatic activity as shown using the EROD test (supplemental figure 1). These results suggest that *in vivo* susceptibility to develop HNF1 $\alpha$  mutated HCA is not only dependent on the *in vitro* EROD activity test and probably should be addressed with specific substrates. In fact, the EROD assay estimates the CYP1B1 activity by measuring the rate of cleaved resorufin, but it may reflect only partially the hydroxylation of endogenous substrates. We can hypothesize that, *in vivo*, the effects of the E229K and P52L CYP1B1 mutations would have a more important impact than the Y81N polymorphism towards endogenous substrates such as estrogens. We propose that CYP1B1 variants affecting specific domains such the hinge region or SRSs may act as potent genetic factors predisposing to the development of HNF1 $\alpha$  mutated HCA in sporadic and familial cases.

CYP1B1 mutations were first identified by Stoilov and collaborators in primary congenital glaucoma (PCG, OMIM#231300) (18). PCG is an autosomal recessive disorder that is associated with developmental defects of the anterior chamber (19) with an increase of the intraocular pressure leading to optic nerve damage and blindness. In the families presented by Stoilov et al., the CYP1B1 mutations were homozygous and co-segregated in the affected but normal members of the families. As shown in the present work using the EROD assay, the CYP1B1 mutations observed in PCG are expected to inactivate the cytochrome P450 activity (18). However, the precise mechanism by which CYP1B1 inactivation can lead to PCG remains

unknown. Two CYP1B1 mutations observed in women with HNF1 $\alpha$  mutated HCA (E229K and R355fs) have already been reported in PCG. Another CYP1B1 mutation (P52L) observed in a woman with HNF1 $\alpha$  HCA has recently been identified by Lopez-Garrido et al., in one individual of its control group (20). In contrast, the remaining mutation (G329S) observed in HCA had never been described before.

CYP1B1 is also responsible for hormone metabolism and the hydroxylation of both endogenous and exogenous molecules that can lead to the formation of toxic metabolites (see for review Sissung TM, 2006) (21). The up-regulation of CYP1B1 is mediated by some sex hormones and their metabolites in addition to environmental carcinogens, such as polyaromatic hydrocarbons. Once up-regulated, CYP1B1 catalyzes the conversion of steroid hormones and exogenous substrates into hydroxylated metabolites. Depending on the hydroxylated molecule, this step of hydroxylation may increase the genotoxic and oxidative load on the cell and modulate cell signaling. This could further explain the role of CYP1B1 in neoplastic progression. CYP1B1 is responsible for the hydroxylation of estradiol (E2) or estrone, and may form chemically reactive catechol estrogens (CE). Among the different CE, CYP1B1 catalyzes mainly the formation of 4-OH-estradiol (22) that may directly bind to DNA. The catechol estrogens (CE) are in majority inactivated by COMT which catalyses an O-methylation. If this inactivation is incomplete, the CE may be oxidized to reactive quinone capable of direct formation of DNA adducts. This step of oxidation also generates free radical formation.

Several studies showed that CYP1B1 was implicated in the occurrence of tumors. First of all, Liehr et al. observed a significant and higher level of 4-hydroxylation of E2 in breast cancer microsomes compared with a very low level of 4-hydroxylation in normal breast tissue (23).

Moreover, immunohistochemical studies of CYP1B1 showed enhanced CYP1B1 in several types of human cancer including breast cancer (24, 25).

Several polymorphisms within the CYP1B1 gene have also been implicated in different cancer risk. To date, the association between CYP1B1 polymorphisms and estrogens metabolism is unclear (see for review Sissung TM, 2006) (21). To our knowledge, this study is the first to associate a decreased activity of CYP1B1 with tumor occurrence in human. Since CYP1B1 is poorly expressed in the liver (24, 26, 27), we suspect that CYP1B1 reduced activity observed in our patients may be related to a peripheral effect of the perturbed estrogens metabolism. In this scenario, a decreased CYP1B1 activity in extrahepatic organs would lead to a saturation of hepatic cytochromes P450 and potentially disturb the estrogens metabolism in the liver. We propose a mechanism through an estrogen metabolites genotoxic effect leading to HNF1 $\alpha$  mutations to be at the origin of HCA. This hypothesis is consistent with the high rate of point mutations and more precisely of nucleotide tranversions observed in the spectrum of somatic HNF1 $\alpha$  mutations identified in HCA (8).

In conclusion, CYP1B1 germline inactivating mutations appears to predispose to the development of HNF1 $\alpha$  mutated HCA in women. In addition, it seems to modify the penetrance of the liver adenomatosis phenotype in HNF1 $\alpha$  germline mutated patients. However, the mechanism by which CYP1B1 inactivation predispose to the development of HNF1 $\alpha$  mutated benign liver tumors remains to be elucidated.

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## References

1. Baum JK, Bookstein JJ, Holtz F, and Klein EW. Possible association between benign hepatomas and oral contraceptives. *Lancet* 1973;2:926-9.
2. Edmondson HA, Henderson B, and Benton B. Liver-cell adenomas associated with use of oral contraceptives. *N Engl J Med* 1976;294:470-2.
3. Flejou JF, Barge J, Menu Y, et al. Liver adenomatosis. An entity distinct from liver adenoma? *Gastroenterology* 1985;89:1132-8.
4. Buhler H, Pirovino M, Akobiantz A, et al. Regression of liver cell adenoma. A follow-up study of three consecutive patients after discontinuation of oral contraceptive use. *Gastroenterology* 1982;82:775-82.
5. Edmondson HA, Reynolds TB, Henderson B, and Benton B. Regression of liver cell adenomas associated with oral contraceptives. *Ann Intern Med* 1977;86:180-2.
6. Bluteau O, Jeannot E, Bioulac-Sage P, et al. Bi-allelic inactivation of TCF1 in hepatic adenomas. *Nat Genet* 2002;32:312-5.
7. Chen YW, Jeng YM, Yeh SH, and Chen PJ. P53 gene and Wnt signaling in benign neoplasms: beta-catenin mutations in hepatic adenoma but not in focal nodular hyperplasia. *Hepatology* 2002;36:927-35.
8. Zucman-Rossi J, Jeannot E, Nhieu JT, et al. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* 2006;43:515-24.
9. Bacq Y, Jacquemin E, Balabaud C, et al. Familial liver adenomatosis associated with hepatocyte nuclear factor 1alpha inactivation. *Gastroenterology* 2003;125:1470-5.

10. Reznik Y, Dao T, Coutant R, et al. Hepatocyte nuclear factor-1 alpha gene inactivation: cosegregation between liver adenomatosis and diabetes phenotypes in two maturity-onset diabetes of the young (MODY)3 families. *J Clin Endocrinol Metab* 2004;89:1476-80.
11. Bioulac-Sage P, Rebouissou S, Sa Cunha A, et al. Clinical, morphologic, and molecular features defining so-called telangiectatic focal nodular hyperplasias of the liver. *Gastroenterology* 2005;128:1211-8.
12. Cann HM, de Toma C, Cazes L, et al. A human genome diversity cell line panel. *Science* 2002;296:261-2.
13. Cabelguenne A, Lorient MA, Stucker I, et al. Glutathione-associated enzymes in head and neck squamous cell carcinoma and response to cisplatin-based neoadjuvant chemotherapy. *Int J Cancer* 2001;93:725-30.
14. Ding S, Yao D, Deeni YY, et al. Human NADPH-P450 oxidoreductase modulates the level of cytochrome P450 CYP2D6 holoprotein via haem oxygenase-dependent and -independent pathways. *Biochem J* 2001;356:613-9.
15. Bandiera S, Weidlich S, Harth V, et al. Proteasomal degradation of human CYP1B1: effect of the Asn453Ser polymorphism on the post-translational regulation of CYP1B1 expression. *Mol Pharmacol* 2005;67:435-43.
16. Li DN, Seidel A, Pritchard MP, Wolf CR, and Friedberg T. Polymorphisms in P450 CYP1B1 affect the conversion of estradiol to the potentially carcinogenic metabolite 4-hydroxyestradiol. *Pharmacogenetics* 2000;10:343-53.
17. Watanabe J, Shimada T, Gillam EM, et al. Association of CYP1B1 genetic polymorphism with incidence to breast and lung cancer. *Pharmacogenetics* 2000;10:25-33.
18. Stoilov I, Akarsu AN, and Sarfarazi M. Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital

- glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum Mol Genet* 1997;6:641-7.
19. Sarfarazi M, Akarsu AN, Hossain A, et al. Assignment of a locus (GLC3A) for primary congenital glaucoma (Buphthalmos) to 2p21 and evidence for genetic heterogeneity. *Genomics* 1995;30:171-7.
  20. Lopez-Garrido MP, Sanchez-Sanchez F, Lopez-Martinez F, et al. Heterozygous CYP1B1 gene mutations in Spanish patients with primary open-angle glaucoma. *Mol Vis* 2006;12:748-55.
  21. Sissung TM, Price DK, Sparreboom A, and Figg WD. Pharmacogenetics and regulation of human cytochrome P450 1B1: implications in hormone-mediated tumor metabolism and a novel target for therapeutic intervention. *Mol Cancer Res* 2006;4:135-50.
  22. Hayes CL, Spink DC, Spink BC, et al. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc Natl Acad Sci U S A* 1996;93:9776-81.
  23. Liehr JG and Ricci MJ. 4-Hydroxylation of estrogens as marker of human mammary tumors. *Proc Natl Acad Sci U S A* 1996;93:3294-6.
  24. McFadyen MC, Breeman S, Payne S, et al. Immunohistochemical localization of cytochrome P450 CYP1B1 in breast cancer with monoclonal antibodies specific for CYP1B1. *J Histochem Cytochem* 1999;47:1457-64.
  25. Murray GI, Taylor MC, McFadyen MC, et al. Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res* 1997;57:3026-31.
  26. Muskhelishvili L, Thompson PA, Kusewitt DF, Wang C, and Kadlubar FF. In situ hybridization and immunohistochemical analysis of cytochrome P450 1B1 expression in human normal tissues. *J Histochem Cytochem* 2001;49:229-36.

27. Tang YM, Chen GF, Thompson PA, et al. Development of an antipeptide antibody that binds to the C-terminal region of human CYP1B1. *Drug Metab Dispos* 1999;27:274-80.
28. van Schaik RH, van der Heiden IP, van den Anker JN, and Lindemans J. CYP3A5 variant allele frequencies in Dutch Caucasians. *Clin Chem* 2002;48:1668-71.
29. Mammen JS, Pittman GS, Li Y, et al. Single amino acid mutations, but not common polymorphisms, decrease the activity of CYP1B1 against (-)benzo[a]pyrene-7R-trans-7,8-dihydrodiol. *Carcinogenesis* 2003;24:1247-55.
30. Gotoh O. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J Biol Chem* 1992;267:83-90.

Table 1: Allele frequencies of all tested SNPs in the two groups of patients.

Chromosomal loci	Genotypes	Allelic frequencies			Fisher exact test *	Allelic frequencies
			32 women with a mutated HNF1a adenoma	58 women with a non mutated benign tumor	P value	SNP500 database
22q11.2	COMT V158M	Val	41%	49%	0.27	53%
		Met	59%	51%		47%
15q22	CYP1A1 T461N	Thr	97%	99%	0.28	93%
		Asn	3%	1%		7%
15q22	CYP1A1 I462V	Ile	98%	92%	0.09	89%
		Val	2%	8%		11%
15q22	CYP1A2	C	33%	34%	0.87	34%
	IVS1-163 C>A	A	67%	66%		66%
2p21	CYP1B1 R48G	Arg	77%	65%	0.12	75%
		Gly	23%	35%		25%
2p21	CYP1B1 A119S	Ala	72%	65%	0.4	77%
		Ser	28%	35%		23%
2p21	CYP1B1 L432V	Leu	56%	60%	0.63	55%
		Val	44%	40%		45%
2p21	CYP1B1 N453I	Asn	75%	82%	0.33	81%
		Ile	25%	18%		19%
7q22.1	CYP3A5	G	91%	ND	ND	92% †
	IVS3-1387 G>A	A	9%	ND		8% †
1p13.3	GSTM1	null	44%	59%	0.19	47% ††
		non-null	56%	41%		53% ††
11q13	GSTP1 I105V	Ile	67%	66%	1	69%
		Val	33%	34%		31%
22q11.2	GSTT1	null	41%	40%	1	29%
		non-null	59%	60%		71%
16q22.1	NQO1 P187S	Pro	82%	84%	0.83	82%
		Ser	18%	16%		18%

4q13	UGT2B7 H268Y	His	47%	52%	0.63	ND
		Tyr	53%	48%		ND

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\* Comparison between the women with a mutated HNF1 $\alpha$  adenoma and the women with a non mutated benign tumor. ND, Not Determined, † data from van Schaik et al. (28); †† data from Cabelguenne et al. (13).

Table 2: CYP1B1 germline mutations identified in tumors.

CYP1B1 mutations		Patients with mutated HNF1 $\alpha$ adenoma	Population control
Nucleotide change	Amino acid change	N = 58	N = 98
155 C>T	P52L	1	0
684 G>A	E229K	5	0
984 G>A	G329S	1	0
1064_1076del	R355fsX	1	0
Total		8	0
<i>P</i> value (Fisher exact test)		0.0003	

## Figure Legends

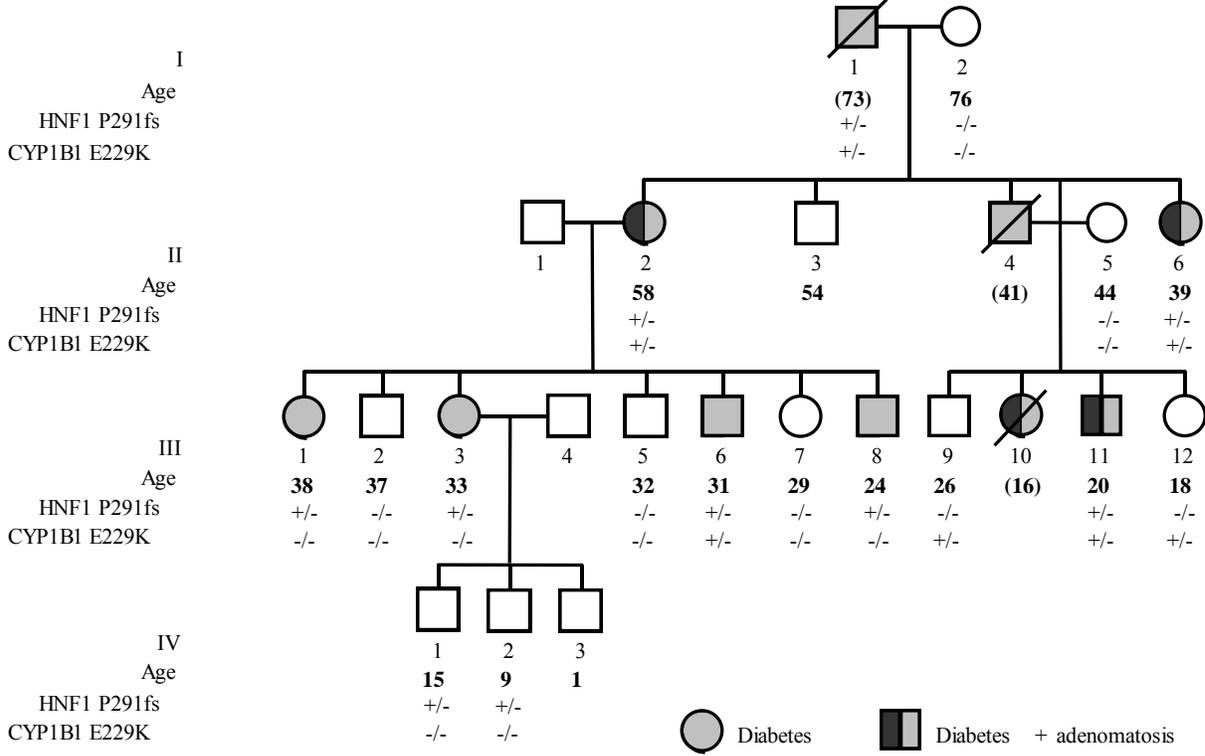
Figure 1: Cosegregation of liver adenomatosis and CYP1B1 mutations. Patients are identified by generation numbers (roman numerals on the left) and individual number within each generation below the symbol. The second line under the symbols corresponds to the age of patients. Age in brackets corresponds to the age of death. The HNF1 $\alpha$  and CYP1B1 genotypes of each tested patient are indicated on the third and fourth line, respectively (+/-, patients carrying either mutation; -/-, patients not carrying mutations).

Figure 2: Protein diagram showing the locations of the mutations. The transmembrane segment, the hinge region, the meander region, the heme binding domain and the conserved helices I, J, K and L were located according to Mammen et al. (29). The putative CYP1B1 SRSs, originally determined by Gotoh, were located according Watanabe et al. (17, 30). The alignments of amino acids of Human, Rat, Mouse, Opossum and Drosophila CYP1B1 gene were represented below each substitution.

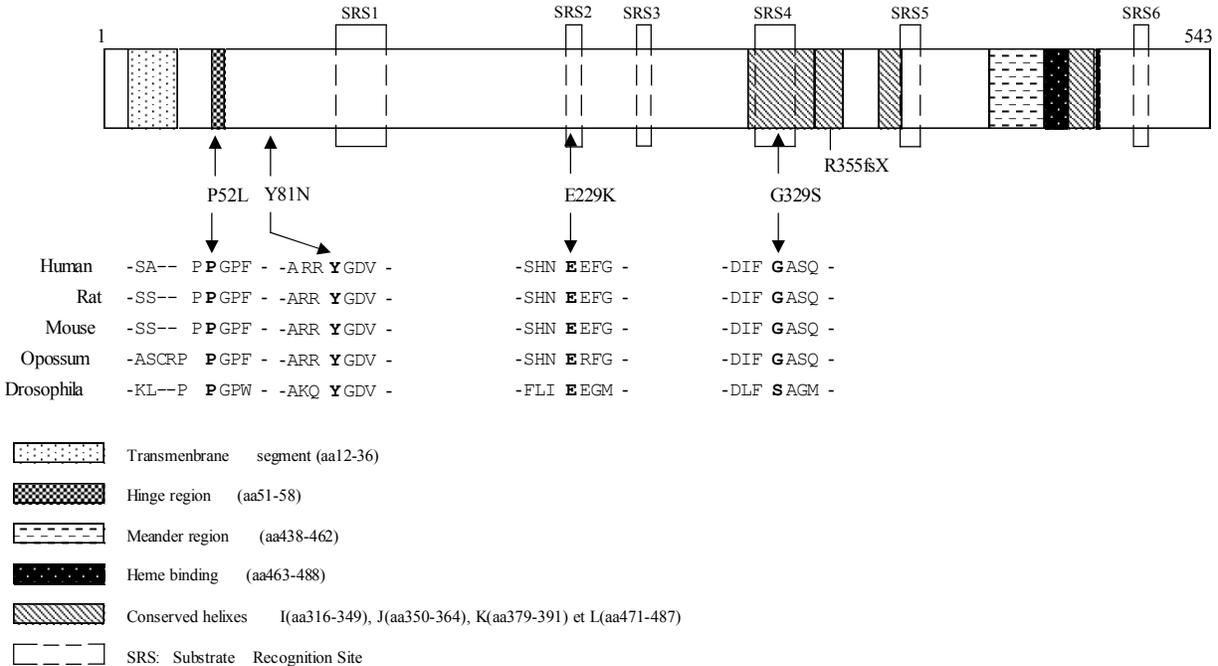
Figure 3: Effect of E229K, G329S, P52L and R355fs mutations on CYP1B1 catalytic activity. \*1, \*2 and \*3 are the different non-mutated alleles of CYP1B1, defined by 3 polymorphisms. Each mutation was replaced in its haplotypic context, E229K and G329S in \*2 allele, P52L in \*3 allele and R355fs in \*1 allele (*see Materials and Methods*). The EROD activity is expressed as picomoles of resorufin per milligram of protein per minute and normalized to the total  $\beta$ -galactosidase activity representing transfection efficiency. Values are means +/- S.D. (n = 4 replicates). EROD activity of the CYP1B1\*3 allele was used as reference and fixed to 1.

Corresponding immunoblot analysis using an anti-CYP1B1 primary antibody are indicated by an arrow.

Jeannot Figure 1



Jeannot Figure 2



Jeannot Figure 3

