Human Nasopharyngeal Epithelial CYP2E1 cDNA Cloning and Sequencing*

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Abstract **Objective** The cytochrome P4502E1 (CYP2E1) mRNA expression sequences of human embryonic nasopharyngeal epithelial (HENE) cell, in vitro malignant transformed HENE cell line 7429 and the nasopharyngeal carcinoma cell line HNE1 were compared to explore the characteristic roles of CYP2E1 in the nasopharyngeal carcinogenesis. **Methods** RT-PCR and DNA recombination techniques were applied to clone the CYP2E1 cDNA fragments in these three cell lines, and structural alterations were analyzed after sequencing. **Results** Compared with the CYP2E1 cDNA of HENE, the CYP2E1 of 7429 showed two mutations (A846T and A901G); and the CYP2E1 of HNE1 showed only an A901G mutation; while compared with CYP2E1 that derived from adult liver cells, the CYP 2E1 CDS of HNE1 was completely comparable, and that of HENE showed a G901A point mutation. But the mutation did not affect the corresponding amino acid sequence. **Conclusion** The results suggest that the CYP2E1 was highly preserved. Only minimal alteration during the carcinogentic process of nasopharyngeal carcinoma both in vitro and in vivo.

Key words Cytochrome P4502E1; cDNA; RT-PCR; Sequential analysis

There were reports which suggested that the nitrosamines (NAs) are related to the chemically induced carcinogenesis of nasopharyngeal carcinoma (NPC)[1,2]. As an indirect carcinogen, NAs must be metabolized by the cytochrome P4502E1 (CYP2E1) and then become carcinogenic active [3,4]. CYP2E1 is a member of the cytochrome P450 superfamily, which is distributed predominantly in the liver cells, and also in within the extrahepatic tissues such as the nasopharynx, esophagus, stomach, small and large intestine, lungs and kidneys, and is related with the development of malignant neoplasms in these sites[5]. Our laboratory has induced the HENE cells to develope a transformed cell line, the 7429 [6], that showed malignant phenotype in vitro, and reported that the CYP2E1 was expressed in both normal and malignant transformed nasopharyngeal tissues for the first time[7], which provided important evidence of the role of CYP2E1 in the carcinogenesis process of NPC. The present study was aimed to further explore the CYP2E1 mRNA primary structural alteration through a comparison of the CYP2E1 gene cDNA fragments during the malignant transformation process.

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MATERIALS AND METHODS

Reagents

Cell culture media RPMI 1640, RNA extract reagent TRIzol, and bovine serum were obtained from Gibco BRL. Advantage(r) cDNA PCR kit and polymerase Mix kit were provided by Clontech. AMV reverse transcription kit, T4 DNA ligase and pGEM-T vector were from Promega. The CYP2E1 cDNA cloning primers were designed according to sequence from the GenBank (No. XM_051310). The forward and reverse primers were 5′-GGCACCATGTCTGCCCTCGG-3′ and 5′-CCTCCACACACTCATGAGCG-3′, respectively, which were obtained through the primer 3 software designs and synthesized by Shanghai Boya Co. The product length was 1499bp, which included both the initial and terminal codes.

Cells and culture conditions

The HENE cells were obtained from fetal nasopharynx of the water balloon induced abortions of Xiangya Hospital and cultured with our established procedures [8]. Both the 7429 and HNE1 cell lines were established in our laboratory previously. These cell lines were cultured in RPM1 1640 media supplemented with 10% bovine serum and antibiotics

(penicillin G sodium, 100 units/ml; streptomycin sulfate 100μg/ml), in a humidified incubator at 37℃ with a 5% CO_2 atmosphere.

RT-PCR

Total cellular RNA was isolated from the three cell lines following the procedure provided by manufacturer. RNA concentration was determined by spectrophotometer, and the extraction efficacy was identified through electrophoresis. Reverse transcription (RT) was performed according to the manual provided by the AMV kit: 2μg of total RNA sample was add with 1μg Oligo$(dT)_{15}$ (0.5 μg/μl), denatured at 70℃ for 60 min, abruptly cold on ice. And then add with 2μl 10× reverse transcription buffer, 4μl 25 mmol/ml $MgCl_2$, 2μl 10 mmol/L dNTP, 20 U RNasin(r) inhibitor and 15 U AMV reverse transcriptase, supplement with RNase-free water to make the total volume reach 20μl; and then react at 42℃ for 60 min, inactivate at 95℃ for 5 min, and abruptly cold on ice. The PCR reaction mix containing 5μl cDNA product, 5μl 10× reaction buffer, 1μl 50× dNTP mixture (each containing 10 mmol/ml), 2μl CYP2E1 cDNA cloning primers mixture (each containing 10μmol/ml), 1 μl cDNA polymerase complex (50×), and was supplemented with PCR-grade water to reach a total volume of 50μl. PCR, performed on an Eppendorf PCR system, included an initial 1-min denaturation at 94℃, followed by 35 cycles of: 94℃ for 30 sec, 68℃ for 3 min, with a final 3-min extension at 68℃. The RT-PCR products were then identified by electrophoresis on 1% agarose gel.

Construction and identification of the 2E1-T recombinant vectors

10μl ligation reactions include 2μl PCR product, 1μl 2E1-T recombinant vector, 1μl 10× ligation buffer and 1μl T4 DNA ligase, and was supplemented with double distilled water. Mix the reaction by pipetting. Incubate the reactions at 14℃ for 16h. Fresh prepared JM109 competent cells were transformed with 2E1-T recombinant vector. Transformations using 5μl of each ligation were performed according to the protocol provided by manufacturer. And transformation cultures were plated onto LB/ampicillin/IPTG/X-Gal plate. Then proper white colonies were selected and amplified for identification. The positive bacteria strains were sent to Shanghai Boya Co. for sequence determination. The primers used for sequencing include the T7, SP6 sequencing primers and the WIF (5′-ATGGTGCTCCGGGTTGCTTC-3′) primer and the WIR (5′-ATGGACCTACCTGGAAGGAC-3′) primer that were synthesized by the Shanghai Boya Co. The sequencing data were compiled by the DNASTAR software and the comparative analysis between three cell lines, as well as with the nr database by using the BLAST program through Internet was conducted.

RESULTS

Identification of the total RNA extracts and the RT-PCR products

The OD260/OD280 values of total RNA from three cell lines were in the range of 1.8-2.0. Electrophoresis revealed that the 28s, 18s, and 5s bands were clearly seen (Fig.1); and the 28s/18s ratio was about 1.5/1. The results indicated that the RNA quality is excellent and showed no degradation. Electrophoresis of the RT-PCR products on the 1% agarose gel demonstrated a specific clear-cut band, which was located approximate the 1.5 kb site of DNA maker that is coordinated with the predicted size of the product (Fig.2).

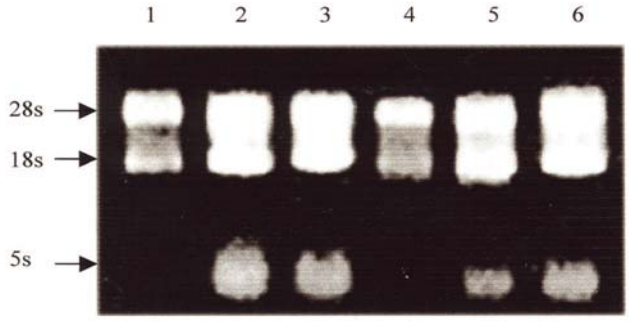

Fig.1 1% agarose gel electrophoresis of total cellular RNA.
Lane1-2: HENE; Lane3-4: 7429; Lane5-6: HNE1.

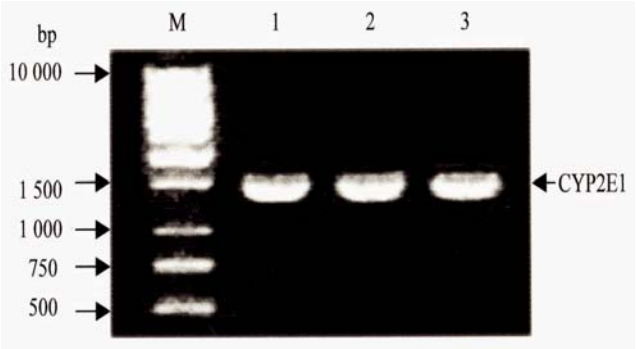

Fig.2 1% agarose gel electrophoresis of RT-PCR product of CYP2E1.
M: 1kb DNA Ladder Marker; Lane1: 7429-2E1; Lane2: HNE1-2E1; Lane3: HENE-2E1.

Construction and Identification of the 2E1–T recombination vectors

The white colonies on the LB/ampicillin/IPTG/X–Gal plates were randomly selected and cultured to amplify. The plasmids were extracted and digested with EcoR I (see Fig.3). The results show that they had been divided into two bands. One is the pGEM–T vector (about 3.0 kb); the other is the CYP2E1 cDNA (about 1.5 kb). The result was also confirmed by sequencing. The recombination plasmids transformed bacteria strains were sent to the Shanghai Boya Co. to performing sequencing in four reactions each sample. The DNASTAR software compiled the data. And then the target CYP2E1 cDNA sequences (1 499 bp) were obtained.

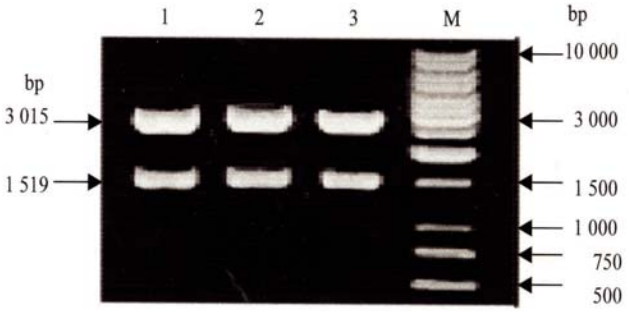

Fig.3 Identification of the recombination plasmids by digesting with EcoR I.
M: 1kb DNA Ladder Marker;
Lane1: 7429–2E1–T digested with EcoR I;
Lane2: HENE–2E1–T digested with EcoR I;
Lane3: HNE1–2E1–T digested with EcoR I.

Comparison of the CYP2E1 cDNA fragments from the three cell lines

The DNASTAR software Seqman program comparison demonstrated that as compared with the CYP 2E1 cDNA of HENE, the CYP2E1 of 7429 showed two point mutations, which are, A846T and A901G, and the CYP2E1 of HNE1 showed only an A901G point mutation (Tab.1).

Table.1 Comparison results of the CYP2E1 cDNA fragments from the three cell lines

	HENE	7429	HNE1
The base at 846 locus	A	T	A
The base at 901 locus	A	G	G

Comparison of the CYP2E1 from HNE1 and HENE with the human adult hepatocellular CYP 2E1

The CYP2E1 derived from adult human liver cells has the GenBank No. J02843. And the BLAST program through the INTERNET performed comparison. It was revealed that the CYP2E1 CDS sequence of HNE1 was completely comparable; the CYP2E1 CDS of HENE showed a G901A point mutation (Fig.4). The mutation was located on the 6th exon, and the corresponding amino acid sequence not affected.

DISCUSSION

As a chemical inducer, mechanism of the activation of DNP did not clarify yet. Since our laboratory successfully obtained a nasopharyngeal cell line with malignant phenotype after human embryonic nasopharyngeal epithelium cells (HENE) were induced in vitro alone by using the ring nitrosamine compound N, N–dinitropiperazine (DNP) [8]. We had proposed the hypothesis that a metabolic enzyme, which activates the DNP, might be presented. And this hypothesis had been demonstrated recently[7]. Our study demonstrated the expression of human cytochrome p4502E1 (hCYP2E1), which can specifically metabolize nitosamine chemicals do present in both the normal nasopharyngeal epithelial cells, the malignant transformed nasopharyngeal cell line cells, and the nasopharyngeal carcinoma cell line cells and tissues to certain extent. In addition, DNP as a metabolic substrate can enhances the transcription level of CYP2E1. These data strongly

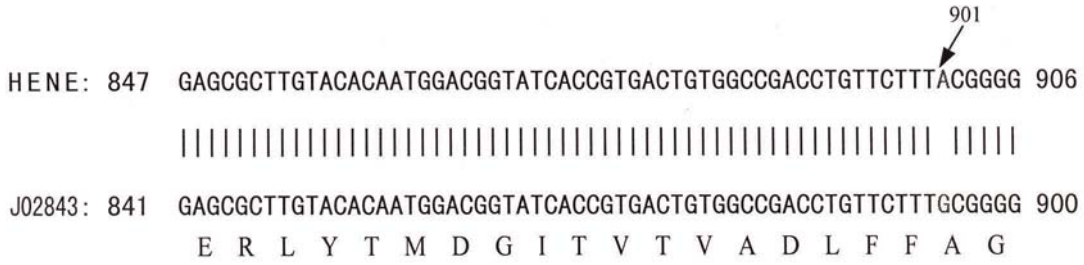

Fig.4. In comparison with Human (adult, ethanol–inducible) liver CYP 2E1 gene, the CYP 2E1 CDS of HENE showed a G901A point mutation.

suggest that the CYP2E1 plays a critical role in the chemically induced carcinogenesis of NPC. Whether the structure and function of CYP2E1 was altered during the carcinogenesis process? It is important to understand the factors that modulate the CYP2E1 activity. The present study is aimed to further explore the features and rules that the CYP2E1 follows during the chemical carcinogenesis process so as to disclose the mechanisms of chemically induced carcinogenesis more comprehensively.

The CYP2E1 gene included 9 exons, and its full length CDS was 1482 bp, which had its mRNA expression in the extrahepatic tissues at very low levels. In order to obtain the real amplified sequence from the low expressed nasopharyngeal epithelial tissue, we used the Advantage(r) cDNA kit and Polymerase Mix kit provided by Clontech. The Advantage cDNA polymerase complex has the features of highy efficacy, highy authentic, and can amplify the long fragment DNA effectively. In addition, we obtained the 1499 bp length clones of CYP2E1 fragments with the application of specific denaturing, annealing, and extending approaches provided that the RNA quality is ensured. The approach is useful in direct clone experiments of the tissue-cell low expression level mRNA.

There are reports indicate that the metabolic activity of CYP2E1 is closely related with gene polymorphism[9], and further related to the susceptibility of the corresponding carcinomas [10,11]. The data of the human cytochrome P450 nomenclature committee indicated that the CYP2E1 gene had 7 allelic gene-types and 12 sub-allelic gene-types (http://www.imm.ki.se/CYPalleles). The majority of the polymorphism loci were distributed at the 5′-franking regulatory region and some polymorphism loci that presented within the coding region were mostly synonymous mutations, and even if being mutations in the open reading frame which cause amino acid exchanges, they do neither alter nor affect its enzymic activity[12, 13] . The three cell line cells´ CYP2E1 gene coding regions cloned in the present study do exist mutations (see Table 1), but all are synonymous mutations, which is coordinated with the literatures. The results suggest that during the HENE cell in vitro malignant transformation into the 7429 cell line cells process or the in vivo malignant transformation into the HNE1 cell line cells process there was not any significant coding region mutation of the CYP2E1 gene, which showed highly preserved characteristics. Interestingly, the HNE1 derived CYP2E1 CDS was completely consistent with the adult liver derived CYP2E1; but the normal human embryonic nasopharyngeal cell (HENE) derived CYP2E1 showed a G901A synonymous mutation (Fig.4). The former suggests that the mature somatic cell CYP2E1 coding region structure is stable, while the later might be a phenomenon during maturation of the somatic cells. The mechanisms require further investigation, however.

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